CURRENT ASPECTS IN CHEMOPREVENTIVE STRATEGIES, VOLUME II

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CURRENT ASPECTS IN CHEMOPREVENTIVE STRATEGIES, VOLUME II

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EDITORIAL
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Editorial: Current Aspects in Chemopreventive Strategies, Volume II

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Keywords: anti-cancer agents, apoptosis induction, anti-angiogenesis, anti-metastasis, anti-inflammation

Editorial on the Research Topic

Current Aspects in Chemopreventive Strategies, Volume II

Despite extensive studies, cancer remains one of the most dreadful diagnoses and biggest challenges for human health all over the world, representing a leading cause of death in the industrialized countries. Various chemotherapeutic drugs, such as doxorubicin, tamoxifen and paclitaxel, have been used for the treatment of tumors for more than half a century; however, there are still no curative options currently available in clinical settings and the severe adverse effects of these drugs threaten the well-being of the patients seriously. Current evidence suggests that further knowledge is urgently needed to clarify the unknown properties and molecular mechanisms of action of various chemopreventive molecules. Such substances refer to the agents which are used for reducing the risk of carcinogenesis, or delaying development or recurrences of malignant disorders.

Several small molecules either of biological origin or from synthetic chemistry, such as cordycepin, mitomycin C, doxorubicin and methotrexate, have demonstrated great efficacy towards a variety of cancers. These agents are found to suppress the proliferation of cancer cells by various mechanisms such as apoptotic cell death, cancer cell cycle arrest, inhibition of angiogenesis and metastasis, induction of ROS generation etc. Few *in silico* tools, such as docking and QSAR (Quantitative structure-activity relationship) techniques, can be used to retrieve more comprehensive information about the targets and the action mechanisms of such molecules. However, there has been a vigorous need to explore the acute as well as the chronic toxicological effects of such chemopreventive molecules for further clinical implementation against diseases.

This Research Topic aims to highlight the ongoing advancement in chemopreventive and therapeutical approaches, as well as the promising role of the above-mentioned agents in the context of cancer prevention and therapy. In particular, it encompasses the research of Singh et al. about potential therapeutic significance of 4-(methylthio) butyl isothiocyanate (4-MTBITC) on modulation of glycolytic enzymes and hypoxia pathway in female rats. As increased glycolysis is known to be an indicator of malignancies (Zhong et al., 1999), inhibition of glycolytic enzymes by 4-MTBITC might be important in delaying the tumor progression. A review carried out by Kumar et al. describes chemopreventive and anticancer potential of kurarinone, presenting also isolation, bioavailability, metabolism and toxicity of this natural flavanone in different experimental models. Another research conducted by Singh et al. by using *Berberis aquifolium root* mother tincture (BAMT) shows multi-pronged therapeutic potential of this extract against HPV infection and cervical cancer. Infection with high-risk HPV subtypes is generally accepted as a risk factor for cervical carcinogenesis (Bharti et al., 2018), highlighting the potential role of anti-HPV action of BAMT in cervical cancer prevention. Abdalla et al. demonstrate safranal as a potent

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chemopreventive agent against hepatocellular carcinoma. Similarly in another study, Abdalla et al. explore antiangiogenic potential of safranal and propose its possible underlying mechanism in hepatocellular carcinoma (HCC). In a further article, Baba et al. review the dual role of TGF-β under different cellular conditions and its crosstalk with other signaling pathways in modulating cell fate. As a pleiotropic cytokine, TGF-β can act both as a tumor suppressor as well as tumor promoter depending on the context and stage of tumor progression (Yang and Moses, 2008). Fined-tuned modulation of this factor can therefore retard carcinogenesis process. Hakroush et al. describe a case report on Ado-trastuzumab emtansine uses in a breast cancer patient, analyzing nephrological aspects. As this therapy is widely applied in present-day oncological settings, clinicians should be aware of severe nephrological complications associated with administration of this antibody-drug conjugate. Mahapatra et al. suggest phenethylisothiocyanate as a potential chemosensitizing agent in light of acquired cisplatin resistance in cervical cancer and established its candidature for Phase I clinical trial. Shankar et al. and Farabegoli et al. describe a strategic approach in cancer chemoprevention using structurally diverse phytochemicals including curcumin, resveratrol, gingerol, epigallocatechin 3-gallate (EGCG), quercetin and grape seed procyanidins, among several other bioactive natural compounds, in the models of different cancer types. Hu et al. describe the contribution of lncRNAs and miRNAs in triple-negative breast cancer pathogenesis, proliferation, migration or malignancy. Finally, Rah et al. review the role of JAK/STAT pathway to improve the existing cancer therapies. Modulation of this signaling cascade might be important also in cancer prevention. We hope that you enjoy the reading of this article collection, composed in a topic that will probably be even more important in the coming years.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Modulatory Effect of 4-(methylthio) butyl Isothiocyanate Isolated From *Eruca Sativa* Thell. on DMBA Induced Overexpression of Hypoxia and Glycolytic Pathway in Sprague-Dawley Female Rats

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4-(methylthio)butyl isothiocyanate (4-MTBITC) is a hydrolytic product from the plant *Eruca sativa* Thell. In the present study, we explored the anti-cancer effect of 4-MTBITC against 7,12-dimethylbenz [a] anthracene (DMBA) induced breast cancer. Hypoxic conditions were developed using a single dose of 60 mg/kg DMBA. Hepatic and renal parameters were increased along with antioxidants in cancer-bearing rats which were lowered with the treatment of 4-MTBITC. Further, it inhibited the up-regulation of glycolytic enzymes caused by DMBA. The hypoxia pathway was evaluated using RT-PCR and it was found that the 40 mg/kg doses of 4-MTBITC statistically lowered the expression of HIF-1α. Akt/mTOR signaling pathway was one of the major pathways involved in 4-MTBITC-induced cell growth arrest by western blotting. Amino acid profiling serum-free plasma revealed the downregulation of specific amino acids required for vital components of fast-growing cancer cells. 4-MTBITC reduced the levels of serine, arginine, alanine, asparagines, and glutamic acid. Histological examination also showed neoplastic growth following DMBA doses. 4-MTBITC treated rats showed less infiltration and normal physiology. Our findings for the first time demonstrated the potential therapeutic significance of 4-MTBITC on modulation of glycolytic enzymes and hypoxia pathway in female rats.

Keywords: 4-(methylthio)butyl isothiocyanate, breast cancer, hypoxia-inducible factor, glycolytic enzymes, 7,12-dimethylbenz [a] anthracene

Abbreviations: HIF-1α, hypoxia-inducible factor-1α; PPP, pentose phosphate pathway; DMBA, 7,12-dimethylbenz[a]anthracene; PAH, polyaromatic hydrocarbons; AhR, Aryl hydrocarbon receptor; ARNT, ahR nuclear translocator; ROS, reactive oxygen species; mTOR, mammalian receptor of rapamycin; ITCs, isothiocyanates; GSLs, glucosinolates; 4-MTBITC, 4-(methylthio)butyl isothiocyanate; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; SOD, superoxide dismutase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PHD, prolyl hydroxylase; PTEN, phosphatase and tensin homolog; HSP-90α, heat shock protein-90α; FBPase, fructose-1,6-bisphosphatase; 6PGD, 6-phosphogluconate dehydrogenase; TNF-α, tumor necrosis factor-α; NF-kB, nuclear factor-kB; OPA, O-phthalaldehyde; MPA, mercapto propionic acid; FMOC, 9-fluorenylmethyl chloroformate; NBF, neutral buffered formalin; H&E, hematoxylin and Eosin; ANOVA, one-way analysis of variance; TCA, tricarboxylic acid cycle.

BACKGROUND

Many factors such as glycolytic enzymes and hypoxia-inducible factor-1α (HIF-1α) are known to be responsible for causing cancer. Increased glycolysis has been known as an indicator of cancer (Zhong et al., 1999). *Cancer* cells exhibit high aerobic glycolytic rates and produce high levels of lactate and pyruvate (Warburg effect). Lactate and pyruvate regulate hypoxia-inducible gene expression by stimulating the accumulation of HIF-1α (Lu et al., 2002). HIF-1α is a heterodimeric transcription factor that is a crucial regulator of the growing tumor's response to hypoxia. Hypoxia may occur when aberrant blood vessels are shut down by becoming compressed or obstructed by growth (a common feature observed during the growth of tumors) and the cells that become a hypoxic shift to glycolytic metabolism (Powis and Kirkpatrick, 2004). HIF-1α overexpression is considered a marker of highly aggressive disease and has been detected in the brain, bladder, breast, colon, ovarian, pancreatic, renal and prostate tumors (Talks et al., 2000). Clinical data also indicate that HIF-1α overexpression is associated with a poor prognosis of hepatocellular carcinoma (Li et al., 2011). HIF-1α has been shown to activate hypoxia-responsive genes which are implicated in numerous aspects of tumorigenesis and cancer progression including proliferation, metabolism, angiogenesis, invasion and metastasis (Luo et al., 2014).

The functional gain of oncogenes and loss of function of tumor suppressor genes are the main characteristic of cancer cells (Hanahan and Weinberg, 2011). This causes uncontrolled proliferation to form a solid mass. So to maintain the energy level for dividing cells, a continuous supply of anabolic building blocks and energy carriers are established. Alterations of metabolic pathways were added to the six cancer hallmarks by Hanahan and Weinberg (Hanahan and Weinberg, 2011). These pathways contain up-regulation of glycolysis, mitochondrial biogenesis, lipid and amino acid metabolism, pentose phosphate pathway (PPP) and macromolecule biosynthesis (Singh et al., 2017).

7,12-dimethylbenz [a]anthracene (DMBA) is commonly employed as a model of polycyclic aromatic hydrocarbons (PAHs)–induced breast carcinogenesis due to its powerful carcinogenic and immunosuppressive effects (Jung et al., 2006). It causes carcinogenesis by causing or promoting mutations in the genes involved. When DMBA is given, it causes the cytosolic aryl hydrocarbon receptor (AhR) to be translocated into the nucleus, where it joins the AhR nuclear translocator (ARNT) protein to create an AhR/ARNT complex, which causes the cytochrome P450 enzyme to upregulate, metabolizing DMBA into an intermediate mutagenic epoxide that forms a DNA adduct that causes mutations (Trombino et al., 2000). Moreover, it also induces oxidative stress by generating reactive oxygen species (ROS) which plays a significant role in carcinogenesis (Karnam et al., 2017).

Understanding therapy resistance and successful treatment require identifying oncogenes and their related potential pathways. Through receptor tyrosine kinases, activated PI3K phosphorylates PIP2 to PIP3 (Thapa et al., 2015). Phosphorylation of Akt by PIP3 activates the mechanistic target of rapamycin (mTOR) which initiates signaling that stimulates cell growth and protein synthesis. As a result of the severity of cancer, we were compelled to look for an alternate supplement to treat cancer, as chemotherapy has several drawbacks. Dietary regimens and potent natural products are effective tools for lowering breast cancer mortality (Krishnamoorthy and Sankaran, 2016). Natural agents have recently gained a lot of interest due to their antioxidant and anticancer capabilities (Kaur et al., 2002; Kaur et al., 2005). Because 80% of the world's population utilizes herbs to treat ailments in some form, WHO recommends using scientifically validated medicinal plants in primary health care after assessing quality, efficacy, and safety (Ekor, 2014).

Epidemiological studies demonstrate that the risk of cancer can be reduced significantly with the intake of cruciferous vegetables (Thimmulappa et al., 2002). This has been ascribed to the presence of secondary metabolites such as isothiocyanates (ITCs) from these plants which have the potential of cancer preventive activity (Verhoeven et al., 1996). These ITCs are hydrolytic products of glucosinolates (GSLs), which are most abundant in the reproductive parts of cruciferous plants in the maximum amount (Brown et al., 2003). Seeds of *Eruca sativa* Thell. also known as rocket salad contains a high amount of 4-(methylthio)butyl isothiocyanate (4-MTBITC) (Arora et al., 2018). Chemopreventive property of 4-MTBITC was documented for the first time in 1996 by Nastruzzi and others (Nastruzzi et al., 1996). Following this, a series of studies were done on different cancer cell lines (IMR-32, MCF-7, HeLa, MDA-MB-231, Caco-2, A549, HepG2, Hep3B, RT4, J82, UMUC3, etc) and different model organisms (swiss albino mice, SCID mice, Sprague Dawley rats) for *in-vivo* studies (Abbaoui et al., 2017; Prełowska et al., 2017). However, no report has been published on the modulatory effect of 4-MTBITC on differential expression of the glycolytic enzymes under hypoxic conditions in mammary tissues.

In the present study, we sought to demonstrate if the 4-MTBITC could act as a novel anti-tumor agent by altering the glycolytic pathway and hypoxia-regulating genes in breast cancer-bearing rats.

MATERIALS AND METHODS

Isolation and Characterization

4-MTBITC was isolated from the seeds of plant *Eruca sativa* using the method described by Arora et al. (2016) with some modifications. The isolated compound was analyzed on UHPLC (Shimadzu, Kyoto, Japan).

Ethical Statement

The rats were housed in the Central Animal Facility under standard animal husbandry conditions. The experimental protocol was approved by the Animal Ethical Committee of Guru Nanak Dev University (GNDU), Amritsar. The rules and regulations of control and supervision of experimental animals (CPCSEA) were followed according to the Ministry of Environment and Forests (File No. 226/CPCSEA/2014/17), Government of India. All experiments were performed following ethical standards.

Animal Study

Experimental Animal Model

Twenty-four female Sprague-Dawley rats were obtained from the National Institute of Pharmaceutical Education and Research, Mohali, Punjab. After 2 weeks of acclimatization, rats were divided randomly into four groups (n = 6). Initially twelve rats were administered with a single 60 mg/kg dose of DMBA, a dose sufficient to make 100% tumor incidence in the control group (Kumar et al., 2017; Sheokand, Navik and Bansal, 2019). After 90 days of DMBA administration when tumor volume measured about 20 mm^3 the animals were divided in experimental groups as follows: group A (administered orally with sunflower oil), group B (breast cancer-induced rats), group C (administered orally with 40 mg/kg 4-MTBITC in sunflower oil for the duration same as in group D), group D (breast cancer-induced rats were treated with 40 mg/kg oral doses of 4-MTBITC for 1 month on alternative days). 4-MTBITC was gavaged for total 15 days in 1 month. 40 mg/kg dose was selected from acute and subchronic toxicity studies and duration of repeated dose was selected from pharmacokinetic studies. Rats were palpated every day for mammary tumors. The quantitative measurements were done using caliper scale and equation: $\pi/6(a)^{2*}(b)$. Throughout the treatment, animals were observed for morbidity or mortality. The body weight of the animals was recorded before the start of the experiment, weekly during the experiment, and at the end of the experiment.

Biochemical Analyses

Blood samples were collected on the last day of the studies from the retro-orbital plexus and centrifuged at 3,000 rpm for 15 min. The serum was stored at -80°C for further analysis. Serum aliquots were used to estimate the different biochemical parameters like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), direct bilirubin, total bilirubin, creatinine, triglycerides, and cholesterol using readymade ERBA kits.

The weighed samples of liver were homogenized in ice-cold Tris-HCl buffer (pH 8.0) for 5 min and centrifuged at 8,000 rpm for 15 min at 4°C. The supernatant was stored at -80°C for enzyme analysis. Superoxide dismutase (SOD) was measured according to the method of Marklund and Marklund (1974). The method is based on the ability of the SOD enzyme to inhibit the phenazine methosulphate mediated reduction in nitroblue tetrazolium dye. Reduced glutathione levels were assessed according to the method of (Moron et al. (1979). The method is based on the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione to produce a yellow color. MDA content was estimated according to the method provided by Ohkawa et al. (1979). Hydroxyl radicals were quantified according to Gutteridge et al. (1981). Hydroxyl radicals were estimated by their reaction with 2-deoxy ribose resulting in the formation of TBA reacting species.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIzol (Thermo Fisher). Briefly, 100 mg frozen tissue was weighed and minced in pestle mortar using liquid nitrogen. Instantly, TRIzol was added and centrifuged. Chloroform was used to induce phase separation. Isopropanol was used to precipitate the RNA. Isolated RNA was washed thrice with 70% ethanol. DNA was removed using DNase treatment. The extracted RNA was quantified and qualified using a NanoDrop spectrophotometer. Finally, total RNA was normalized to avoid false changes in gene expression. Using Luna One-Step RT-qPCR (New England BioLabs), the gene expression of hexokinase, phosphoglucose isomerase, aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate isomerase, phosphoglycerate mutase, enolase, pyruvate kinase, HIF-1α, HIF-1β, prolyl hydroxylase (PHD), mTOR, phosphatase and tensin homolog (PTEN), Heat shock protein-90α (HSP-90α), and succinate dehydrogenase was recorded. Primer sequences are enlisted in **Supplementary Table S1**. The relative mRNA expression of each target was normalized to 18 S.

Estimation of Lactate Content and Other Enzymes

Lactate content was estimated using YSI 1500 L sport electron probe according to the manufacturer's instructions. Briefly, 25 μL whole blood was injected into an automated YSI 1500 L unit for immediate analysis. Lactate dehydrogenase was estimated by measuring the reduction of NADH into NAD^+ according to the protocol of King (King, 1965). The activity of fructose-1,6-bisphosphatase (FBPase) was measured according to the method of Taketa and Pogell and 6-phosphogluconate dehydrogenase (6PGD) was measured spectrophotometrically according to the method given by Ben-Bassat and Goldberg (Taketa and Pogell, 1965; Ben-Bassat and Goldberg, 1980).

Western Blot Analysis

Total protein in breast tissue homogenate was estimated using a readymade total protein estimation kit (ERBA). Approximately 40 μg protein was loaded on each well of polyacrylamide gel and separated by SDS-PAGE. The resolved proteins were transferred to the polyvinylidene difluoride membrane (0.2 μm) by electrophoresis. Then the membranes were blocked by 5% milk powder in 50 mM Tris-HCL, 150 mM NaCl, and 0.1% Tween 20 at room temperature for 1 h. The membrane was then immunoblotted for overnight at 4°C with either a rabbit monoclonal anti-HIF-1α (Cell Signaling Technology, United States), a rabbit polyclonal anti-Akt (Cell Signaling Technology, United States), a rabbit polyclonal anti-mTOR (Cell Signaling Technology, United States), a rabbit polyclonal anti-TNF-α (Tumor necrosis factor-α) (Cell Signaling Technology, United States), p21 (Cell Signaling Technology, United States), a rabbit monoclonal anti-PHD-2/Egln1 (Cell Signaling Technology, United States), a rabbit monoclonal anti-p53 (Cell Signaling Technology, United States), a rabbit monoclonal anti-NF-kB-p65 (Nuclear factor-kB) (Cell Signaling Technology, United States) or a rabbit polyclonal anti-β-Actin (Cell Signaling Technology, United States) diluted at 1/1,000–1/10,000 in TBS tween 0.1%. Subsequently, membranes were washed thrice with 50 mM phosphate-

buffered saline and 0.1% tween-20. Following incubation with horseradish peroxidase-conjugated anti-rat secondary antibody diluted at 1/1,000 for 1 h at room temperature. The blots were developed using ECL chemilluminicance substrate solution. Autoradiographic signals were captured on a Genegenious imaging system using the Genesnap software.

Enzyme-Linked Immunosorbent Assay

For the estimation of adiponectin, leptin, insulin, and IL6, fresh plasma was used. Respective rat ELISA kits (RayBiotech, United States) were used according to the manufacturer's instructions. 96 well Elisa plate was read on a multimode microplate reader (Sunergy HT, BioTek).

Amino Acid Analysis

Sample Preparation

Samples (liver) were prepared by digesting tissue homogenates in 6 N HCl along with 0.1% phenol in tightly closed 25 ml reagent bottles at 110°C for 24 h. Further, the samples were dried using a vacuum evaporator (Buchi) and reconstituted in 0.1 mol/L HCl solution for analysis. A total of 19 amino acid standards were analyzed including aspartic acid, glutamic acid, asparagines, serine, histidine, glutamine, glycine, threonine, arginine, alanine, tyrosine, cysteine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and lysine and their linearity of response across six-point serial dilution was plotted.

Chromatographic System

The HPLC-based amino acid analyzer consisted of Nexera X2, Shimadzu coupled with Shimadzu RF-20A prominence fluorescence detector was used. The HPLC separation of derivatized amino acids required the two mobile phases. Mobile phase A consists of 20 mmol/L potassium phosphate buffer (pH 6.5) and mobile phase B consists of 45:40:15 acetonitrile:methanol: water. All buffers were filtered through 0.2 µm filtered and degassed by sonication. The derivatized amino acids were separated on a Phenomenex Luna C18 column (250 mm X 4.6 mm id., 5 µm particle size). The chromatographic separation was performed at 35°C. Fluorometric measurement was conducted with an excitement wavelength of 350 nm and an emission wavelength of 450 nm and a gain at 100. The flow rate of the mobile phase was 0.95 ml/min throughout the analysis and sample injection volume for derivatization was 1 µL. The gradient conditions were as follows: initial conditions are 2% mobile phase A; from time 0.01–2 min the gradient changes to 90% solvent A and 10% solvent B; from 2 to 33.40 min the gradient changes to 43% solvent A and 51% solvent B; from 33.40 to 33.50 min the gradient changes to 100% solvent A and 0% solvent B; from 33.50 to 39.30 min the gradient remains same as 100% solvent A and 0% solvent B; from 39.30 to 39.40 min the gradient changes to 90% solvent A and 10% solvent B; from 39.4 to 45 min the gradient was maintained to 90% solvent A and 10% solvent B.

The HPLC run time for the separation of the derivatized amino acids in the sample or standard is 50 min. The amino acid derivatization reagents were prepared freshly each day. *O*-phthalaldehyde (OPA) was prepared by dissolving 10 mg of OPA in 0.3 ml methanol and mixed completely by sonication. Then 0.7 ml borate buffer and 4 ml HPLC grade water were added to make a working solution. Mercapto propionic acid (MPA) was prepared by adding 10 µL in 10 ml of 0.1 mol/L borate buffer. 9-fluorenylmethyl chloroformate (FMOC) was prepared by adding 4 mg into 20 ml of acetonitrile. The derivatization of amino acids in the samples and standards was performed in an automated fashion using a Nexera SIL-30AC autosampler kept at 15°C. The needle of the autosampler was rinsed with 50% methanol/water between injections. During chromatography of the sample, the derivatization of the next sample is started so that there is no delay in the injection of the next sample.

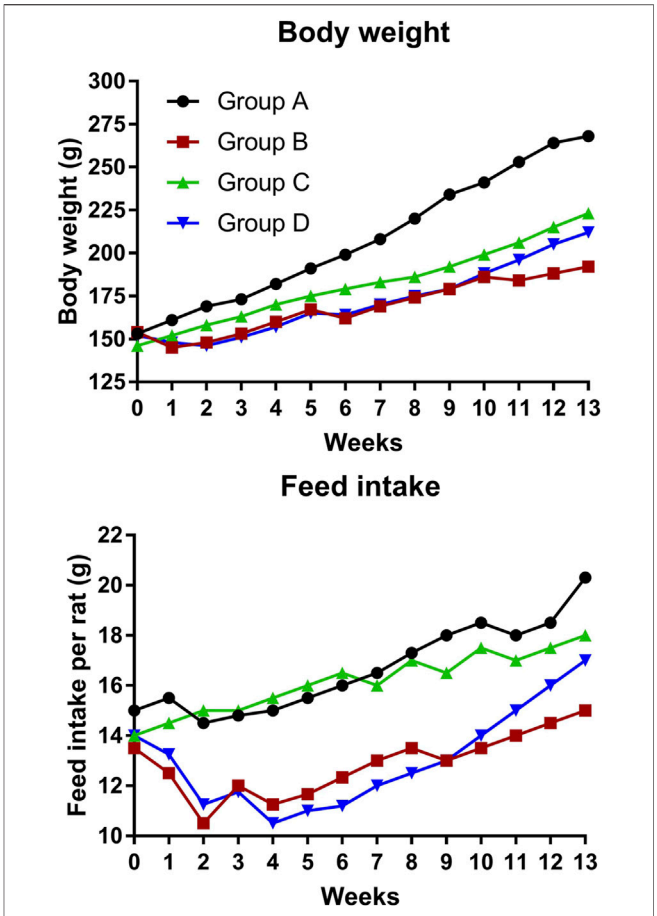

FIGURE 1 | Bodyweight transitions and feed intake changes during the animal study.

Histopathology

At the time of scheduled euthanasia, all animals underwent a full gross necropsy examination. Mammary tissue and tumor masses were preserved in 10% neutral buffered formalin (NBF) for each rat. All collected tissues were examined for histopathological changes with hematoxylin and eosin (H&E) staining after full fixation in 10% NBF. The tissues were trimmed, embedded in paraffin, sectioned, mounted on microscope slides, and stained

TABLE 1 | Effect of 4-(methylthio)butyl isothiocyanate on hepatic, renal, and lipid profile in control and experimental group of animals. SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase. All the values are represented as mean ± SEM for six animals. Significant levels are $^{**}p < 0.01$ when compared with group A and $\#\#p < 0.01$ when compared with group B.

Parameters	Group A	Group B	Group C	Group D	% change in group B compared to group A	% change in group B compared to group A
SGOT (IU/L)	25.66 ± 1.69	67.05 ± 2.69**	27.69 ± 1.43	27.17 ± 1.28##	161	−59
SGPT (IU/L)	53.66 ± 2.93	100.33 ± 5.65**	59.54 ± 5.23	70.36 ± 4.72##	87	−30
Direct bilirubin (mg/dl)	0.03 ± 0.001	0.07 ± 0.003**	0.05 ± 0.004	0.05 ± 0.002##	133	−29
Total bilirubin (mg/dl)	0.12 ± 0.01	0.19 ± 0.01**	0.12 ± 0.01	0.14 ± 0.01##	58	−26
Creatinine (mg/dl)	0.94 ± 0.05	1.61 ± 0.06**	0.99 ± 0.1	1.18 ± 0.1##	71	−27
Triglyceride (mg/dl)	65.12 ± 3.89	106.48 ± 4.79**	60.73 ± 2.42	67.63 ± 3.64##	63	−36
Cholesterol (mg/dl)	48.17 ± 4.83	103.58 ± 4.06**	57.77 ± 7.82	72.24 ± 2.93##	115	−30
Total Protein (mg/g tissue)	392.25 ± 15.69	300.02 ± 9.53**	357.71 ± 16.36	342.97 ± 22.19	23	−14

with hematoxylin and eosin. The paraffin-embedded blocks were sectioned 3 μm thin and stained with H&E.

Statistical Analysis

Data were analyzed statistically using a one-way analysis of variance (ANOVA) in GraphPad Prism 8. All results are presented as mean ± SEM from at least three independent experiments. Dunnett's multiple comparison test was used to compare multiple groups. For amino acid analysis ANOVA with sidak multiple comparison test was used. A difference was considered significant at the $p < 0.05$ level.

RESULTS

Metabolic Parameters

Figure 1 depicts the effect of 4-MTBITC on body weight and feed intake. In group D, the body weight was gained after the treatment of 4-MTBITC as compared to group B, the feed intake was also observed to increase after the treatment of 4-MTBITC.

Hepatic, Renal, and Lipid Profile

Serum parameters were recorded for all the rats. It was found that the parameters for hepatic (SGOT (161% at $p < 0.01$), SGPT (87% at $p < 0.01$), direct bilirubin (133% at $p < 0.01$), total bilirubin (58% at $p < 0.01$)), renal (creatinine (71% at $p < 0.01$)), and lipid profile (triglyceride (63% at $p < 0.01$), cholesterol (115% at $p < 0.01$)) were significantly enhanced in rats treated with DMBA (group B) compared to control group. In group D the levels of SGOT were brought down by 59% ($p < 0.01$), SGPT by 30% ($p < 0.01$), direct bilirubin by 29% ($p < 0.01$), total bilirubin by 26% ($p < 0.01$), creatinine by 27% ($p < 0.01$), triglyceride by 36% ($p < 0.01$) and cholesterol by 30% ($p < 0.01$) compared to group B (**Table 1**).

Oxidative Stress Parameters

Table 2 depicts the effect of DMBA and 4-MTBITC on antioxidant levels in experimental animals. In group B the activity of SOD was decreased by 33% ($p < 0.05$) and levels of reduced glutathione by 17% ($p < 0.01$) compared to normal rats. On the other hand, in group D the activity of SOD was increased by 112% ($p < 0.01$) and the levels of glutathione were increased by 112% ($p < 0.01$) compared to group B. DMBA treatment markedly increased the levels of MDA (122% at $p < 0.01$) and hydroxyl levels (73% at $p < 0.01$) in group B compared to group A. Oral doses of 4-MTBITC reduced the levels of MDA by 31% ($p < 0.01$) and hydroxyl radicals by 30% ($p < 0.01$) in group D compared to group A.

3.4. Modulation of Glycolytic Pathways

Figure 2 depicts the effect of DMBA and 4-MTBITC on gene expression analysis of glycolytic enzymes. The percent change in mRNA expression of hexokinase (768% at $p < 0.001$), phosphoglucose isomerase (380% at $p < 0.001$), phosphofructokinase (76% at $p < 0.01$), aldolase (143% at $p < 0.001$), triosephosphate isomerase (119% at $p < 0.05$), GAPDH (219% at $p < 0.001$), phosphoglycerate kinase (57% at $p < 0.02$), phosphoglycerate mutase (102% at $p < 0.01$), enolase (60% at $p < 0.01$), and pyruvate kinase (897% at $p < 0.001$) was significantly increased after the treatment of DMBA as compared to control. The counter-treatment of 4-MTBITC significantly decreased the gene expression of hexokinase (59% at $p < 0.01$), phosphoglucose isomerase (66% at $p < 0.01$), aldolase (54% at $p < 0.001$), GAPDH (45% at $p < 0.01$), phosphoglycerate mutase (79% at $p < 0.001$), enolase (45% at $p < 0.01$) and pyruvate kinase (64% at $p < 0.001$) as compared to group B. 4-MTBITC treatment in group D non-significantly decreased the percent change in gene expression of phosphofructokinase (26%), triosephosphate isomerase (29%) and phosphoglycerate kinase (11%) as compared to group B.

Hypoxia Pathway Evaluation

Having determined that 4-MTBITC disrupts the glycolytic pathway, we wanted to determine if 4-MTBITC attenuated hypoxia-inducible transcription. Evaluation of HIF-1α, HIF-1β, PHD, mTOR, PTEN, HSP-90α mRNA expression was done using RT-PCR. There a significant overexpression of HIF-1α (223% at $p < 0.001$) and mTOR (1,391% at $p < 0.001$) and downregulation in PHD (79% at $p < 0.01$) was observed after the treatment of DMBA in group B as compared to group A while an insignificant increase in HIF-1β (60%) and HSP-90α (39%) and decrease in PTEN (44%) was observed. Following the treatment of 4-

TABLE 2 | Effect of 4-(methylthio)butyl isothiocyanate on enzymatic and non-enzymatic antioxidant levels in control and experimental group of animals. SOD: Superoxide dismutase. All the values are represented as mean ± SEM for six animals. Significant levels are *$p < 0.05$, **$p < 0.01$ when compared with group A and ##$p < 0.01$ when compared with group B.

Parameters	Group A	Group B	Group C	Group D	% change in group B compared to group A	% change in group D compared to group B
SOD (IU/min/mg protein)	4.70 ± 0.30	3.13 ± 0.36*	5.48 ± 0.38	6.64 ± 0.36##	–33	112
Reduced glutathione (μM/g tissue)	417.98 ± 21.87	242.18 ± 10.48**	348.72 ± 26.44	514.34 ± 35.20##	–17	112
MDA (nM of MDA released/mg protein)	0.55 ± 0.05	1.22 ± 0.13**	0.53 ± 0.04	0.84 ± 0.07##	122	–31
Hydroxyl radicals (nmol/g tissue)	2.40 ± 0.17	4.16 ± 0.28**	2.34 ± 0.20	2.89 ± 0.14##	73	–30

MTBITC in group D the upregulated expression of HIF-1α (86% at $p < 0.001$), and mTOR (5% at $p < 0.001$) was lowered while PHD (443% at $p < 0.05$) was upregulated (**Figure 3**). Moreover, HIF-1β (15%), PTEN (101%), and HSP-90α (18%) were insignificantly changed.

Modulation of Lactate Production and Other Enzymes

The activity of lactate dehydrogenase (145% at $p < 0.001$) and 6-PGD (138% at $p < 0.001$) was increased in DMBA treated rats as compared to group A. Following the treatment of 4-MTBITC in group D, the activity of lactate dehydrogenase was decreased by 59% at $p < 0.001$ and 6-PGD by 48% at $p < 0.001$ as compared to group B (**Figures 4A,B**). Succinate dehydrogenase, an essential enzyme of the tricarboxylic acid cycle (TCA cycle), was found to be significantly downregulated (42% at $p < 0.05$) in group B as compared to group A (**Figure 4C**). 4-MTBITC treatment increase the expression of succinate dehydrogenase by 123% at $p < 0.01$ as compared to group D. The activity of FBPase responsible for conversion of fructose 1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis for storage of glucose moieties was observed to be significantly decreased (40% at $p < 0.01$) under hypoxic conditions of group B compared to group A (**Figure 4D**). A significant increase (50% at $p < 0.05$) was observed in group D as compared to group B following the treatment of 4-MTBITC. Furthermore, the lactate content was quantified in all the experimental animals and it was found that the DMBA increased the lactate levels (214% at $p < 0.001$) which were then significantly reduced (27% at $p < 0.001$) by 4-MTBITC in group D as compared to group B (**Figure 4E**).

Inflammation and Glucose Regulation

Adiponectin is known for its overall energy homeostasis and metabolism. Significant elevation (200% at $p < 0.01$) in serum leptin and a decrease in adiponectin levels (44% at $p < 0.01$) were observed in group B compared to vehicle control rats (**Figures 5A,B**). 4-MTBITC treatment attenuated DMBA induced alteration in leptin (29% at $p < 0.05$) and adiponectin (52% at $p < 0.01$) levels compared to group B. It was observed that the levels of insulin were significantly increased (94% at $p < 0.01$) in cancer-bearing rats as compared to control (**Figure 5C**). Further, the treatment of 4-MTBITC (group D) reduced (40% at $p < 0.05$) the insulin significantly from group B. IL-6 a pleiotropic cytokine related to inflammation was seen significantly enhanced (241% at $p < 0.01$) in group B as compared to the control group (**Figure 5D**). 4-MTBITC was able to reduce (42% at $p < 0.01$) the IL-6 levels significantly as compared to group B.

Modulation of Amino Acid Profile

The mean normalized amino acid concentrations of all the groups were investigated (**Supplementary Figure S2**). The average levels of serine (169% at $p < 0.001$), arginine (37% at $p < 0.05$), alanine (81% at $p < 0.001$), asparagine (43% at $p < 0.01$) and glutamic acid (145% at $p < 0.001$) were increased in animals bearing cancer (group B). Treatment of 4-MTBITC in group D decreased the levels of serine by 31% ($p < 0.05$), arginine by 54% ($p < 0.001$), alanine by 43% ($p < 0.001$), asparagines by 26% ($p < 0.05$) and glutamic acid by 24% ($p < 0.05$) as compared to group B (**Table 3**). On the other hand, the concentrations of tryptophan (40% at $p < 0.01$) were decreased in response to a carcinogen (group B) and increased by 4-MTBITC (53% at $p < 0.05$).

Protein Expression Analysis

The protein expression of Akt, mTOR, TNF-α, p21, PHD, p53, and NF-kB was evaluated using western blot analysis with normalization against Actin (**Figure 6**). DMBA induced the overexpression of cell proliferation markers Akt (119%), mTOR (722)%, and NF-kB (81%) in group B as compared to group A which is then lowered (20, 43 and 51% respectively) by the treatment of 4-MTBITC in group D as compared to group B (**Figures 6B,C,G**). Low expression of tumor suppressor proteins p21 (75%) and p53 (60%) was observed following the treatment of DMBA (**Figures 6E,H**). Counteraction of 4-MTBITC was seen to elevate the expression of p21 by 54% and p53 by 122%. TNF-α an inflammatory cytokine responsible for apoptosis/necrosis was seen decreased by 72% in group B compared to group A while increased by 57% following the treatment of 4-MTBITC in group D (**Figure 6D**). PHD, responsible for oxygen-dependent degradation of HIF-1α subunit by forming a complex with VHL was seen decreased by 65% following the treatment of carcinogen (group B) compared to group A (**Figure 6F**). Treatment of 4-MTBITC in group D

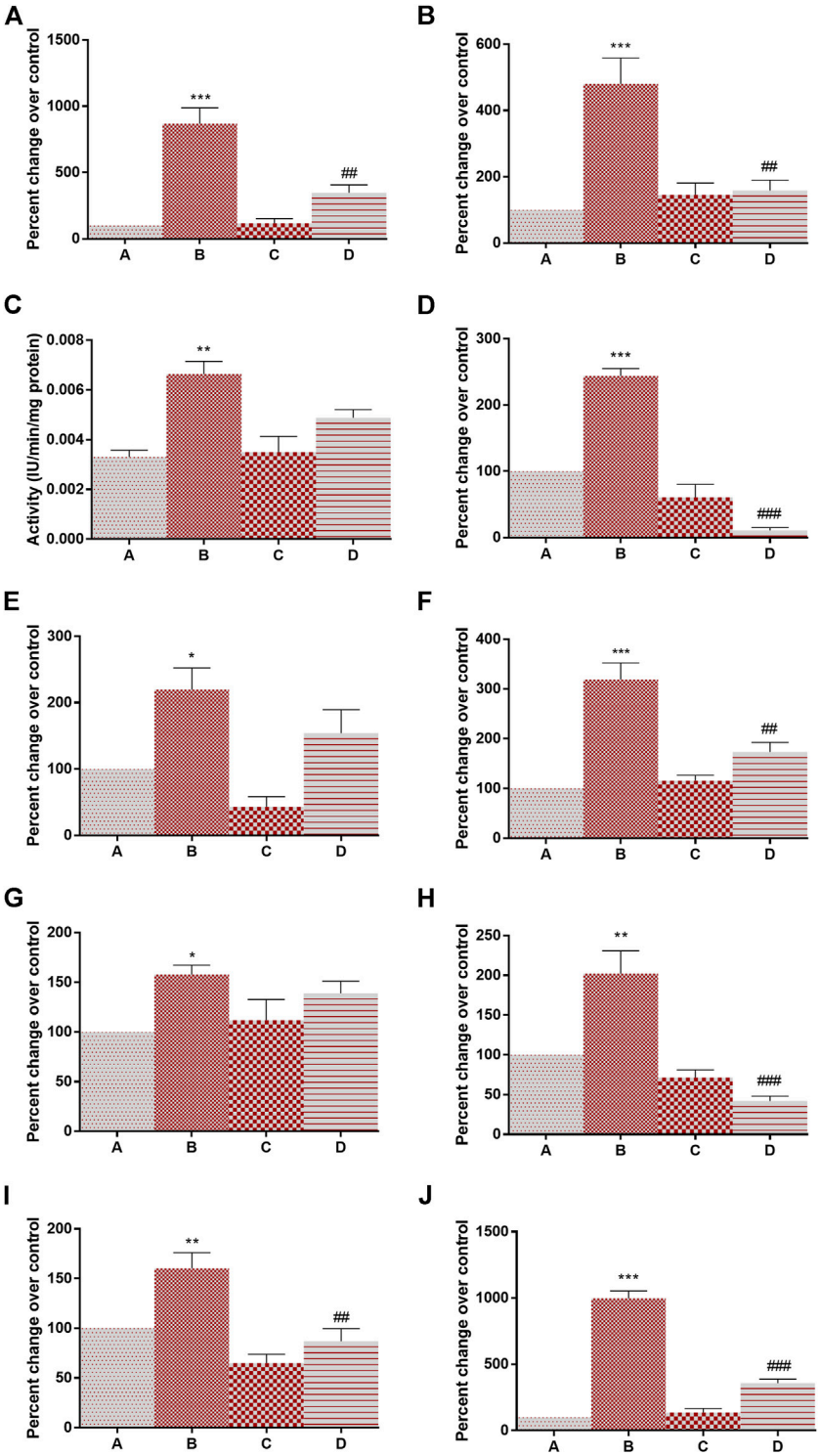

FIGURE 2 | Effect of 4-(methylthio)butyl isothiocyanate on gene expression analysis of glycolytic enzymes in the control and experimental group of animals. **(A)** Hexokinase, **(B)** Phosphoglucose isomerase, **(C)** phosphofructokinase, **(D)** Aldolase, **(E)** Triosphosphate isomerase, **(F)** Glyceraldehyde 3-phosphate dehydrogenase, **(G)** Phosphoglycerate kinase, **(H)** Phosphoglycerate mutase, **(I)** Enolase, **(J)** Pyruvate kinase. All the values are represented as mean ± SEM (n = 3). Significant levels are $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with group A and $\#p < 0.05$, $\#\#p < 0.01$ when compared with group B.

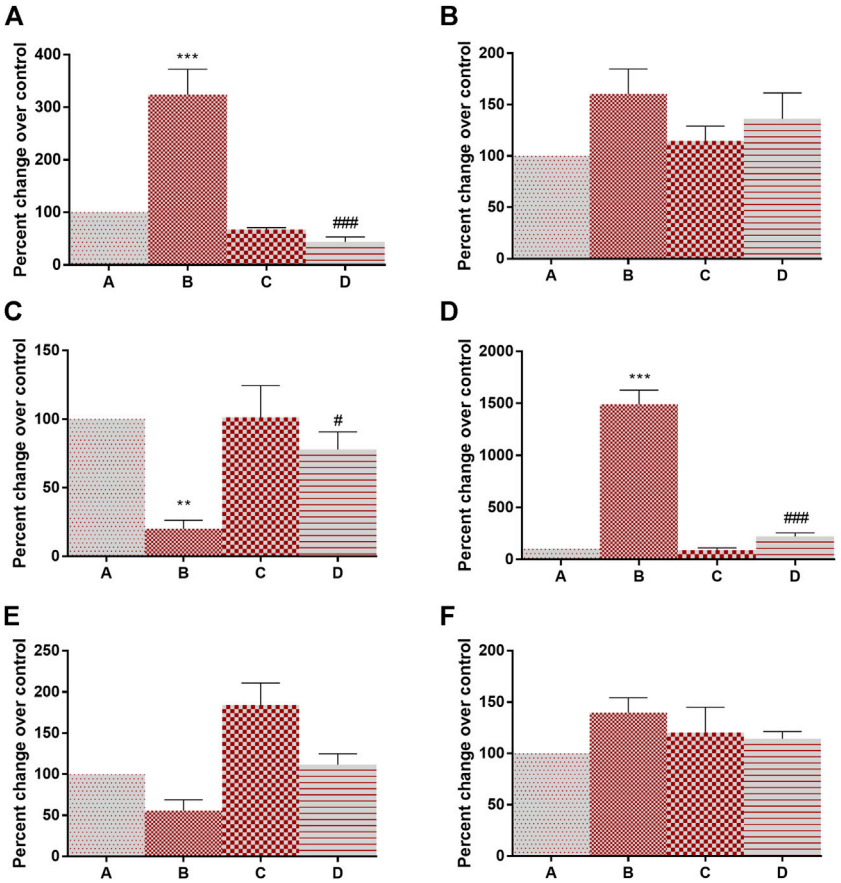

FIGURE 3 | Gene expression analysis of hypoxia-inducible factor-associated genes in control and experimental group of animals. **(A)** Hypoxia inducible factor-1α, **(B)** Hypoxia inducible factor-1β, **(C)** Prolyl hydroxylase, **(D)** Mammalian target of rapamycin, **(E)** phosphatase and tensin homolog, **(F)** Heat shock protein-90α. All the values are represented as mean ± SEM (n = 3). Significant levels are $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with group A and $\#p < 0.05$, $\#\#\#p < 0.001$ when compared with group B.

inhibited the degradation of PHD and restored the protein expression by 133% as compared to group B.

Histopathology

Mammary tissue was sliced and washed with ice-cold PBS. Examination of group A showed dense collagenous tissue, blood vessels, and islands of glandular tissue surrounded by dense fibrous and adipose tissue (**Figure 7**). Administration of rats with DMBA (group B) revealed a neoplastic growth made up of trabeculae, ducts, and nests of malignant ductal cells. The stroma was desmoplastic and infiltrated by lymphocytic infiltrates mainly lymphocytes. In addition examination of mammary gland tissues of rats treated with 4-MTBITC after DMBA (group D) administration showed low infiltrating components.

DISCUSSION

Chemoprevention is the use of plant, synthetic, or biological chemicals to prevent, kill, or reverse the progression of premalignant cells from their initial stages to invasive disease. Mammary tumor induction by DMBA is one of the most popular methods for researching different aspects of breast cancer among various animal models (Barros et al., 2004). The present study was conducted to evaluate the modulation of hypoxia and glycolytic pathways as anti-breast cancer activity of 4-MTBITC in DMBA induced rats. The development of ROS and the peroxidation of membrane lipids in DMBA-induced mammary carcinoma-bearing animals resulted in a substantial rise in tumors (Davis and Kuttan, 2001). Increased antioxidant levels protect cells from oxidative damage while preserving regular structural and biochemical processes. The ubiquitous enzymes cytoplasmic SOD protect cells from free radicals generated during carcinogen metabolism. 4-MTBITC therapy resulted in a substantial rise in the rate of SOD enzyme, showing that 4-MTBITC has free radical scavenging activity by its stimulation. Breast cancer-bearing rats had higher levels of mammary lipid peroxidation products, which play a role in tumor development by combining with DNA to form MDA–DNA adducts, which cause genetic changes and contribute to cancer. Reduction in the levels of MDA content following 4-MTBITC treatment was very likely due to its anti-lipid peroxidative properties. GSH is an important antioxidant that breaks the cycle of

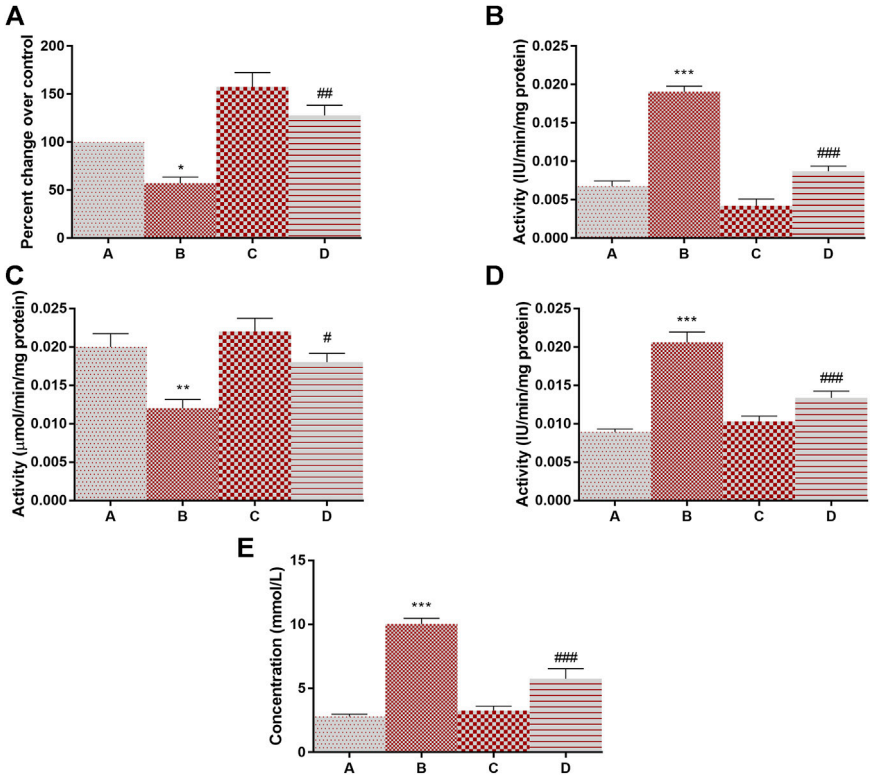

FIGURE 4 | The activity of succinate dehydrogenase, lactate dehydrogenase, fructose-1,6-bisphosphatase, phosphogluconate dehydrogenase, and levels of lactate content in response to 4-(methylthio)butyl isothiocyanate and DMBA. **(A)** Succinate dehydrogenase, **(B)** Lactate dehydrogenase, **(C)** Fructose-1,6-bisphosphatase, **(D)** Phosphogluconate dehydrogenase, **(E)** Lactate content. All the values are represented as mean ± SEM (n = 3). Significant levels are $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ when compared with group A and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ when compared with group B.

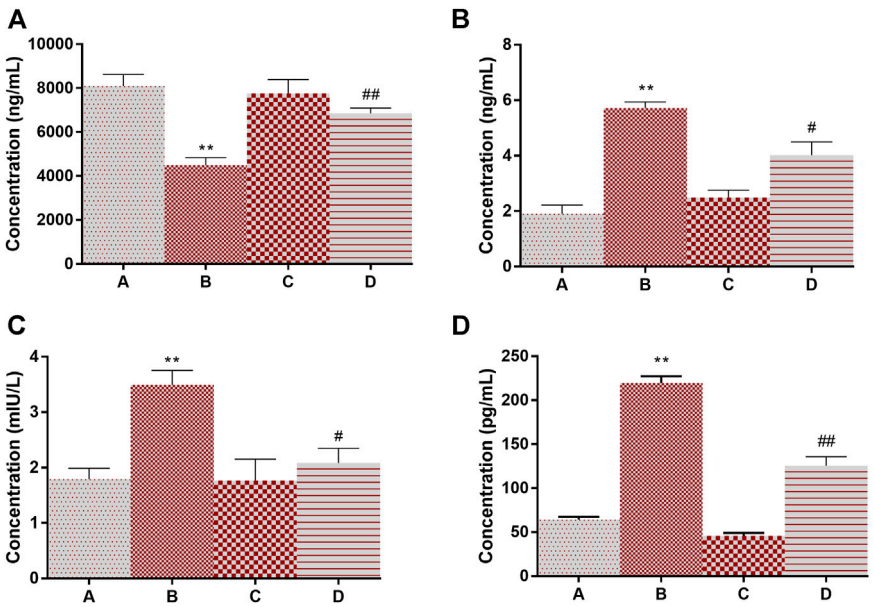

FIGURE 5 | Modulation of levels of adiponectin, leptin, insulin, and IL6 in response to 4-(methylthio)butyl isothiocyanate and DMBA. **(A)** Adiponectin, **(B)** Leptin, **(C)** Insulin, **(D)** IL-6. $**p < 0.01$ when compared with group A and $\#p < 0.05$, $\#\#p < 0.01$ when compared with group B.

TABLE 3 | Concentration (nmol/ml) of recorded amino acids in different groups. Data represents mean ± SEM. One way ANOVA with sidak multiple comparison test was applied. Significant levels are *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with group A and #$p < 0.05$, ###$p < 0.01$ when compared with group B.

Amino acids	Group A	Group B	Group C	Group D	% change in group B compared to group A	% change in group D compared to group B
Glutamine	4.98 ± 0.33	4.64 ± 0.43	4.9 ± 0.36	5.8 ± 0.35	–6	25
Threonine	8.28 ± 0.19	8.59 ± 0.07	7.85 ± 0.05	8.61 ± 0.46	4	–0.2
Alanine	10.21 ± 0.71	18.55 ± 0.79***	7.32 ± 0.34	10.59 ± 1.22###	81	–43
Arginine	11.31 ± 0.6	15.54 ± 1.39*	11.96 ± 0.36	7.19 ± 0.74###	37	–54
Asparagine	6.26 ± 0.34	8.95 ± 0.65**	5.48 ± 0.13	6.65 ± 0.64#	43	–26
Aspartic Acid	11.58 ± 0.34	10.12 ± 0.31	12.92 ± 0.88	11.38 ± 0.6	–13	12
Glutamic Acid	6.4 ± 0.39	15.68 ± 1.72***	10.09 ± 0.4	11.83 ± 0.71#	145	-24
Glycine	8.72 ± 0.34	8.31 ± 0.29	8.3 ± 0.05	8.01 ± 0.11	–5	–4
Histidine	6.35 ± 0.25	7.84 ± 0.42	7.79 ± 0.02	9.36 ± 0.75	23	19
Valine	5.33 ± 0.65	7.17 ± 0.22	5.1 ± 0.54	8.74 ± 0.99	34	22
Isoleucine	10.13 ± 0.06	8.09 ± 0.1	7.84 ± 1.12	7.28 ± 0.48	–20	–10
Methionine	14.91 ± 0.36	13.68 ± 0.61	14.41 ± 0.36	14.34 ± 0.52	–8	5
Phenylalanine	9.01 ± 0.34	7.91 ± 0.46	7.69 ± 0.07	8.83 ± 0.08	–12	12
Serine	4.44 ± 0.32	11.93 ± 1.32***	4.08 ± 1.2	8.21 ± 0.46#	169	–31
Tryptophan	13.79 ± 0.65	8.22 ± 0.60**	11.04 ± 1.68	12.61 ± 0.75#	–40	53
Tyrosine	18.51 ± 0.04	17.54 ± 0.54	17.08 ± 1.36	17.87 ± 0.07	–5	2
Cysteine	8.09 ± 0.36	8.15 ± 0.06	8.41 ± 0.03	8.69 ± 0.34	0.7	7
Leucine	6.31 ± 0.59	7.9 ± 0.59	7.42 ± 1.22	6.37 ± 0.34	25	–19
Lysine	2.39 ± 0.53	4.33 ± 0.58	4.04 ± 0.78	4.57 ± 0.35	–69	5

a free radical movement to prevent the peroxidation of polyunsaturated fatty acids which can act as a carcinogenesis promoter (Adly, 2010). We observed that the level of GSH was low in DMBA treated rats which were significantly upregulated in 4-MTBITC treated cancer-bearing rats. Hepatic profile screening revealed the injury caused by an oral dose of DMBA which was further lowered with the treatment of 4-MTBITC. Before performing this anticancer study we performed a 14 days single dose acute toxicity study (OECD-423) from which the LD50 was came out to be 500 mg/kg. We then performed long-term toxicity studies using 4-MTBITC with different concentrations and it was found that the daily doses of 4-MTBITC for 28 and 90 days caused serious hepatic injury specifically to the female rats. We also performed pharmacokinetic studies which revealed its slow elimination (Ke = 0.0036 min^{-1}) from blood plasma and hence in the current study, the frequency of dose was lowered to reduce such side effects (Unpublished data).

Tumor formation, development, angiogenesis, and metastasis are also thought to be influenced by inflammation. The main molecular players in the inflammation-to-cancer axis are proinflammatory mediators such as TNF-α and IL-6. TNF-α, an inflammatory cytokine that is strongly expressed in breast carcinomas, was reduced by 4-MTBITC. The inducible transcription factor Akt, mTOR, p21, and p53 regulates the expression of several genes involved in inflammation, proliferation, and apoptosis. 4-MTBITC inhibited the expression of genes NF-kB, and IL-6, which control the immune response to infection. It also inhibited the expression of cell proliferative markers such as Akt, mTOR, p21, and p53.

A high level of plasma leptin and a low level of adiponectin was observed following the DMBA administration. Adiponectin represses proliferation in breast cancer cells through inactivation of p44/42 MAPK protein 1 and 3 expressions, a stimulation of AMPK activity by phosphorylation at Thr172, and a decrease in Akt phosphorylation (Thr308) associated with an increased expression of LKB1 leading to a reduction of mTOR activity as evidenced by reduced phosphorylation of S6K (Taliaferro-Smith et al., 2009). Leptin induces the PI3K/Akt survival pathway by activating the phosphorylation of Akt Thr308 or Akt Ser473 and by stimulating the protein expression of PKC-alpha, or leptin activates the MAPK pathway by inducing ERK1 and ERK2 phosphorylation, or leptin rapidly and directly stimulates the STAT3 pathway and the up-regulate c-myc (one target gene of STAT3), at both mRNA and protein levels (Binai et al., 2010). As compared to DMBA treatment the levels were significantly reverted to normal with the treatment of 4-MTBITC.

There are multiple mechanisms by which HIF-1 mediates adaptive metabolic responses to hypoxia, such as increased glycolytic flux and decreased TCA flux, to minimize mitochondrial ROS production. HIF-1α a prime regulator of cell signaling in the absence of oxygen was upregulated in cancer-bearing rats. PHD an important gene that regulated the expression of genes involved in the hypoxia pathway was seen downregulated in cancer-bearing rats. In normal oxygen levels, glucose converts to pyruvate and enters the TCA cycle for the synthesis of ATP. As the oxygen level lowers the glucose converted to pyruvate will not enter the TCA cycle moreover lactate will be produced to lower the free radical formation from the electron transport chain. This lactate will serve as an instant energy source for neighboring cells. Lactic acid produced from lactate helps cancer cells to dissolve the extracellular matrix and finally metastasize to other organs. HIF-1α represses succinate dehydrogenase an important TCA enzyme that can limit the

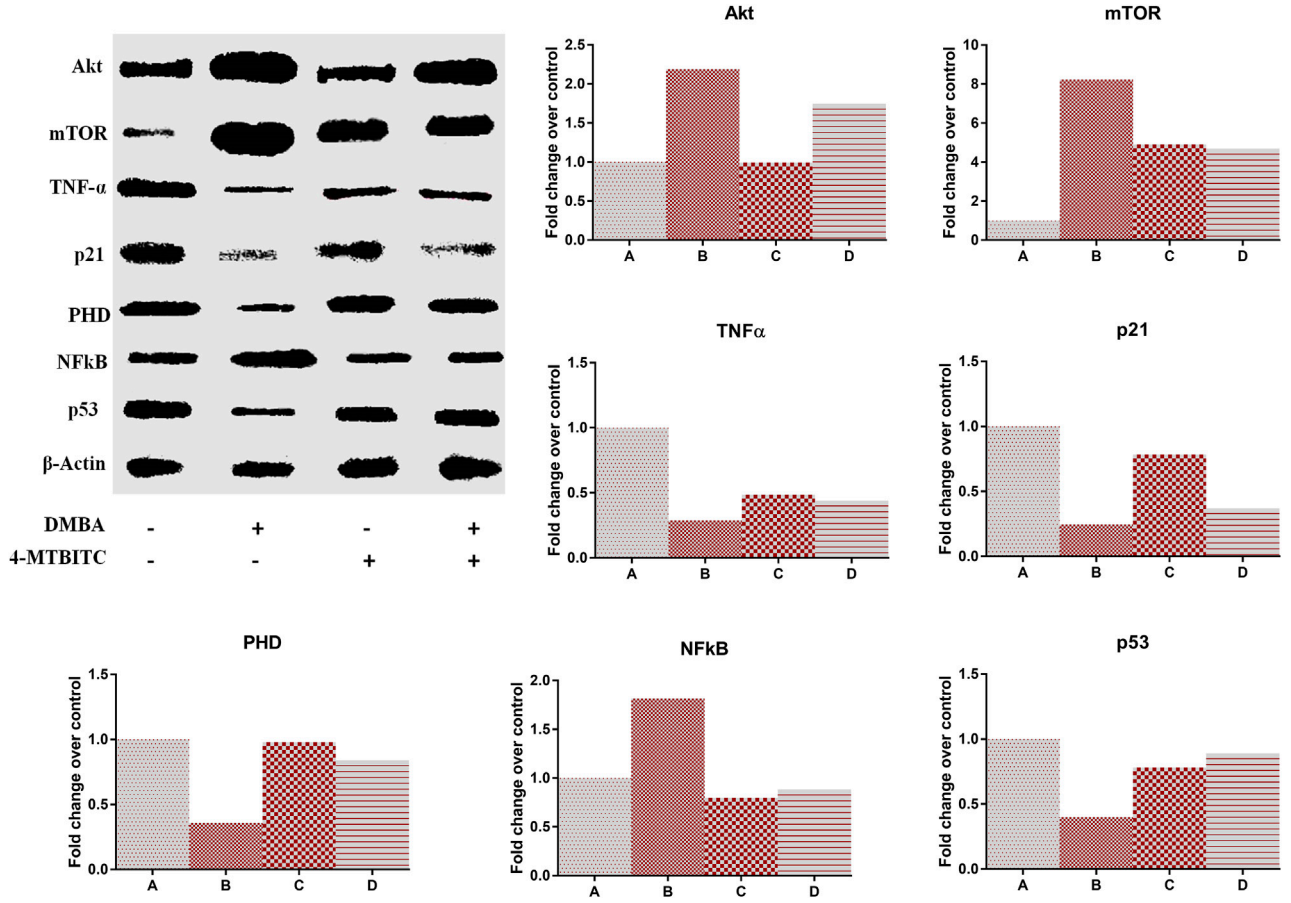

FIGURE 6 | Western blot analysis of different protein markers in response to the treatment of 4-(methylthio)butyl isothiocyanate and DMBA. **(A)** Representative expression of protein markers, **(B)** Akt, **(C)** Mammalian target of rapamycin, (D) Tumor necrosis factor-α, **(E)** p21, F Prolyl hydroxylase, **(G)** Nuclear factor-kB, **(H)** p53.

content of mitochondrial oxaloacetate required for aspartate biogenesis (Meléndez-Rodríguez et al., 2019).

Gluconeogenesis is the reverse of glycolysis through which glucose can be generated from a non-carbohydrate source. FBPase is a rate-limiting key enzyme that converts fructose-1,6-bisphosphate to fructose-6-phosphate. Loss of FBPase contributes to the development, promotion, and progression of multiple cancers, including basal-like breast cancer, whereas FBPase functions as a tumor suppressor by increasing oxidative phosphorylation and ROS levels, which are harmful to cancer cells (Dong et al., 2013). The activity of FBPase was seen enhanced with the treatment of 4-MTBITC as compared to cancer-bearing rats.

PPP pathway is critical for oxidative stress and the synthesis of nucleotides in the metabolism of breast cancer cells. 6PGD is a central enzyme in PPP's oxidative branch that is active in nucleotide biosynthesis and redox status maintenance. DMBA induced the overactivation of this enzyme to withstand the increased requirement of nucleotides for proliferative cells which then reduced following the 4-MTBITC.

Amino acids are readily measured in blood, saliva, and urine, they may be useful biomarkers for diagnosis and screening during treatment. Serine is synthesized from glycolysis intermediate 3-phosphoglycerate through several enzymatic reactions. Serine is also involved in the production of antioxidants, which help cancer cells survive in low-oxygen conditions. The removal of serine causes breast cancer cells to proliferate less, an effect that can be entirely reversed by reintroducing it. Out of other amino acids, glutamate is the one that is consumed by cancer cells at the highest rate. Glutamic acid is the precursor of glutamine converted by glutaminase synthetase.

Blockage of arginine uptake results in apoptotic cell death in cancer cells (Abdelmagid et al., 2011). Nitric oxide is another metabolic consequence of arginine metabolism. Nitric oxide promotes angiogenesis and limits host immune response to promote tumor growth (Choudhari et al., 2013). Alanine is another important amino acid that is synthesized from pyruvate in the mitochondrial matrix (Groen et al., 1982). It is therefore expected that in hypoxic conditions more alanine is synthesized from pyruvate by competing with the pyruvate dehydrogenase. Recent studies suggest that the activity of aminotransferases is required for the formation of extracellular matrix in metastatic breast cancer cells (Elia et al., 2019). Asparagine is known as an amino acid exchange factor that regulates mTOR activation, nucleotide synthesis,

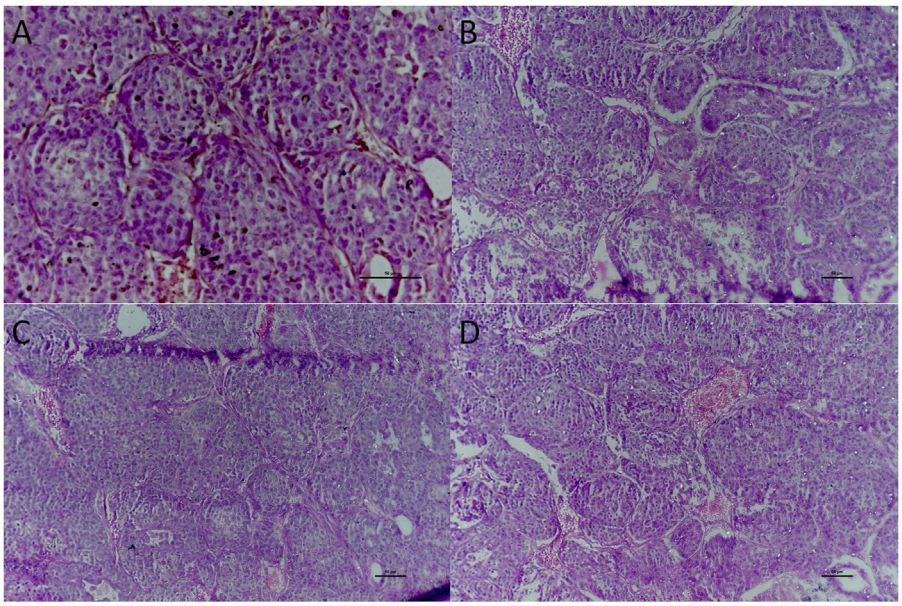

FIGURE 7 | Histological examination of the mammary gland tissue of studied groups. **(A)** Group A x400 treated as control; **(B)** Group B x200 treated with DMBA; **(C)** Group C x200 treated with 4-(methylthio)butyl isothiocyanate; **(D)** Group D x200 treated with DMBA and then 4-(methylthio)butyl isothiocyanate.

proliferation, and metastasis (Knott et al., 2018). Asparagine is a metabolic dead end and may therefore be more available for exchange with extracellular amino acids (Krall et al., 2016). Along with other amino acids, asparagines preferably exchange with serine/threonine suggesting its indirect participation in energy production through the TCA cycle (Amelio et al., 2014; Krall et al., 2016). In the human body, glutamic acid is converted to glutamine by an energy-dependent reaction with ammonia using glutamine synthetase (Kulkarni et al., 2005). One of the most common amino acids in the human body is L-glutamine, a derivative of L-glutamic acid. Glutamine participates in the PPP pathway to withstand the high requirement of nucleotides by synthesizing purine and pyrimidines. It also transports to the mitochondrial matrix where glutaminase converts it into glutamate. This glutamate participates in the TCA cycle through conversion to α-ketoglutarate. Under hypoxic conditions, α-ketoglutarate can undergo reductive carboxylation to generate isocitrate which converts to citrate (Mullen et al., 2012). Citrate synthesis helps in lipogenesis. Amino acids are also helpful to boost the immune system. Tryptophan catabolism is known for its involvement in immune response modulation. The low plasma concentration of tryptophan in cancer indicates its rapid catabolism resulting in immunosuppression. This impairs the ability of dendritic cells and regulatory T cells to target and eliminate cancer cells (Greene et al., 2019). In the present study, amino acid profiling of serum-free plasma showed that the amino acids required to meet the high energy consumption for proliferative cells were affected by the treatment of 4-MTBITC. These alterations were confirmed by histopathological changes also.

Based on these, we speculated that the 4-MTBITC modulate DMBA induced glycolytic pathway and hypoxia pathway by downregulating the expression of HIF-1α and mTOR along with some amino acids that are needed for cancer growth.

CONCLUSION

In conclusion, our study indicates for the first time that 4-MTBITC displays modulatory effect on DMBA induced key hypoxia-inducible pathway and metabolic pathway including glycolysis. It also modulates the expression of a central link of the proliferative pathway known as mTOR and Akt followed by modulation of p21, lactate production as well as amino acid metabolism involved in cancer cell proliferation. However, the direct interaction of 4-MTBITC with HIF-1α or glycolytic enzymes that may produce pharmacological response through *in-vitro* assay needs to be done. In-silico computational modeling may also help for further investigations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee, Guru Nanak Dev University, Amritsar.

AUTHOR CONTRIBUTIONS

Study design: SA, BS; Study conduct: DS, ST, DS; Data collection; Data analysis: DS, ST; Data interpretation: DS, SA, BS; Drafting manuscript: DS; Approval of the final manuscript: All the authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Five-Decade Update on Chemopreventive and Other Pharmacological Potential of Kurarinone: a Natural Flavanone

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In the present article we present an update on the role of chemoprevention and other pharmacological activities reported on kurarinone, a natural flavanone (from 1970 to 2021). To the best of our knowledge this is the first and exhaustive review of kurarinone. The literature was obtained from different search engine platforms including PubMed. Kurarinone possesses anticancer potential against cervical, lung (non-small and small), hepatic, esophageal, breast, gastric, cervical, and prostate cancer cells. *In vivo* anticancer potential of kurarinone has been extensively studied in lungs (non-small and small) using experimental xenograft models. In *in vitro* anticancer studies, kurarinone showed IC_{50} in the range of 2–62 μM while *in vivo* efficacy was studied in the range of 20–500 mg/kg body weight of the experimental organism. The phytochemical showed higher selectivity toward cancer cells in comparison to respective normal cells. kurarinone inhibits cell cycle progression in G2/M and Sub-G1 phase in a cancer-specific context. It induces apoptosis in cancer cells by modulating molecular players involved in apoptosis/anti-apoptotic processes such as NF-κB, caspase 3/8/9/12, Bcl2, Bcl-XL, etc. The phytochemical inhibits metastasis in cancer cells by modulating the protein expression of Vimentin, N-cadherin, E-cadherin, MMP2, MMP3, and MMP9. It produces a cytostatic effect by modulating p21, p27, Cyclin D1, and Cyclin A proteins in cancer cells. Kurarinone possesses stress-mediated anticancer activity and modulates STAT3 and Akt pathways. Besides, the literature showed that kurarinone possesses anti-inflammatory, anti-drug resistance, anti-microbial (fungal, yeast, bacteria, and Coronavirus), channel and transporter modulation, neuroprotection, and estrogenic activities as well as tyrosinase/diacylglycerol acyltransferase/glucosidase/aldose reductase/human carboxylesterases 2 inhibitory potential. Kurarinone also showed therapeutic potential in the clinical study. Further, we also discussed the isolation, bioavailability, metabolism, and toxicity of Kurarinone in experimental models.

Keywords: kurarinone, flavanone, anticancer, apoptosis, migration, pharmacological activity

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INTRODUCTION

Plant-based secondary metabolites are indirectly helpful for the growth and development of the plants, but it is of direct importance for humans. The plant-based phytochemical/secondary metabolites can be categorized in various groups majorly based on their structural skeleton and functional groups (Kumar, 2014). Our research group reported the pharmaceutical activities of different plants and/or phytochemicals (Kumar and Pandey 2013; Mishra et al., 2013; Kumar et al., 2014; Kushwaha et al., 2019a; Kushwaha et al., 2020a; Kushwaha et al., 2020b; Kushwaha et al., 2021). Flavonoids are a group of polyphenolic compounds that occur widely in plants. They contain a benzo-γ-pyrone structure comprised of two benzene rings (A and B), connected with a heterocyclic pyran ring (C). Flavonoids are classified as flavone, flavanol, flavanone, and others. These groups may differ from each other based on the oxidation level and type of substitution group at the C ring. They are well known for their pharmacological potential such as anticancer, antioxidant, neuroprotective, hepatoprotective, immune-modulatory, antimicrobial, antidiabetic, etc (Kushwaha et al., 2019b). Kurarinone is a natural flavanone found in different plants and possesses various pharmacological activities including chemoprevention efficacy. Literature showed anticancer, anti-fungal, anti-bacterial, anti-Corona virus, neuroprotective, anti-drug resistance, antioxidant, and anti-inflammatory potential of kurarinone. In the present review, we discuss the chemoprevention potential of kurarinone and the underlying regulatory mechanism in detail. The various pharmacological activities such as Ca^{+} channel and glucose transporter activity modulation, metabolic enzyme, and xenobiotic metabolism enzyme inhibition potential are reviewed. The bioavailability and toxicity of a therapeutic agent are of prime concern. Thus, we also reviewed the studies based on these parameters of kurarinone.

Kurarinone Chemistry and Natural Sources

Kurarinone is a naturally occurring prenylated flavanone. Komatsu et al. (1970) for the first time isolated kurarinone from the methanolic root extract of *Sophora angustifolia* (Fabaceae). Briefly, the extract was chromatographed and eluted using acetone:hexane (1:1 ratio). The eluted material was subjected to thin-layer chromatography and the relatively slower-moving fraction was taken and re-chromatographed using chloroform:methanol (95:5 ratio) as elution solvent which yielded 15 g kurarinone (started with 20 kg raw plant material). Kurarinone was obtained as a colorless crystalline substance. The structure was established using UV, IR, and NMR techniques (Komatsu et al., 1970). Later, Yamahara et al. (1990) isolated the kurarinone from *Sophora flavescens* root extract, which became a choice of isolation source for the scientist in the two decades. Isolation of kurarinone from different sources and plant parts has been summarized in **Table 1**. Kurarinone contains a lavandulyl group at the C-8 position and a methoxy group at the C-5 position along with hydroxyl groups at positions C-2, C-4, and C-7. Chung et al. (2004) reported that the lavandulyl group and the positions of the hydroxyl group are important for the diacylglycerol acyltransferase inhibitory activity of kurarinone. Son et al. (2003) reported that the lavandulyl group and methoxy groups of kurarinone play important roles in tyrosinase inhibitory activity.

Anticancer Potential

Kang et al. (2000) first reported the anticancer efficacy of *Sophara flavescens*. To isolate the compounds, the methanol extract was prepared and partitioned between dichloromethane and aqueous methanol. Further through re-chromatography kurarinone was isolated in a sub-fraction. The methanol extract of the *S. flavescens* root and isolated compounds showed potential anticancer efficacy against HL-60 cells (human myeloid leukemia). Kurarinone depicted 18.5 μM IC_{50} in comparison to the standard drug cisplatin (2.3 μM IC_{50}) (Kang et al., 2000). It has been reported that chemotherapy induces the nuclear factor NF-κB pathway which in turn results in the activation of survival signaling and molecular events involved in anti-apoptosis. By doing this, the NF-κB pathway activation plays an important role in cancer drug resistance. Bcl2, an

TABLE 1 | Kurarinone isolation from different sources.

S. No	Source	Solvent	Part/type	Ref
1	*Sphora flavescens Ait*	Ethyl acetate extract	Root	Yamahara et al. (1990)
2	*Gentiana macrophylla*	Aqueous acetone	Root	Tan et al. (1996)
3	*S. flavescens Ait*	—	Root	Kang et al. (2000)
4	*S. flavescens Ait*	Dichloromethane fraction	—	Kim et al. (2003)
5	*Albizzia julibrissin* (Leguminosae)	EtOAc fraction of the MeOH extract	—	Jung et al. (2004)
6	*Sophora flavescens*	Polyphenolic extract	Root	De Naeyer et al. (2004)
7	*S. flavescens Ait*	MeOH extracts	—	Lee et al. (2005)
8	*S. flavescens Ait*	—	—	Zhang et al. (2007)
9	*S. flavescens*	—	Root	Li et al. (2008)
10	*S. flavescens*	—	—	Ma et al. (2013)
11	*S. tonkinensis*	—	—	He et al. (2013)
12	*S. flavescens*	—	Root	Zhang et al. (2013)
13	*S. flavescens*	—	—	Zhang et al. (2016a)
14	*S. flavescents*	—	Flower	Zhang et al. (2016b)
15	Traditional Chinese Medicine Xin-Su-Ning capsule (XSNC)	—	—	Guo et al. (2018)
16	*S. flavescens*	—	—	Zhou et al. (2018)

important NF-κB pathway target gene, is involved in anti-apoptotic events and drug resistance in clinical oncology. Han et al. (2007) studied the efficacy of kurarinone on NF-κB pathway and apoptosis induction. Further, the study explored the effect of test samples on the activity of different receptor tyrosine kinases involved in clinical oncology. The *in vitro* apoptosis induction potential of the kurarinone containing extract was studied in lung and esophageal carcinoma cell lines (H460 and Eca-109, respectively). The *in vivo* apoptosis induction potential of isolated kurarinone alone and in combination with Taxol (standard anticancer drug) was studied in the lung cancer cell line xenograft model. At a dose of 100 mg/kg body weight per day, kurarinone decreased the expression of Bcl2 protein and up regulated the levels of caspase 8 and 3 in the experimental rat model. Kurarinone showed dose-dependent (5.8 μg/ml IC_{50}) inhibitory potential on NF-κB pathway activation in lipopolysaccharide induced pathway activation in an experimental model. Kurarinone inhibited induced IκBα phosphorylation (regulates NF-κB nuclear translocation) in HEK293 (kidney) cells at 100 μg/ml concentration. Kurarinone inhibited EGFR and Her-2 phosphorylation (in A431 and MDA-MB-453 cell lines) at 20 μg/ml concentration. The EGFR activity was also inhibited in *in vivo* experimental rats at 100 and 500 mg/kg body weight treatment with 14 and 40% inhibition efficacy, respectively. Moreover, kurarinone showed inhibition of KDR activity with 2.3 μM IC_{50} (Han et al., 2007).

Sun et al. (2008) studied the anti-tumor efficacy of *S. flavescens* flavonoids in *in vitro* (A549, SPC-A-1, NCI-H460 cell lines) and *in vivo* (lung xenograft model) cancer models. The study showed that the phytochemical did not produce toxicity in the experimental rats up to 750 mg/kg bodyweight treatment. The flavonoids showed significant anti-tumor potential in lung cancer in *in vitro* and *in vivo* models at test concentrations/treatment. The study concluded that *S. flavescens* flavonoids such as kurarinone may be developed as novel anti-tumor candidates (Sun et al., 2008). Berghe et al. (2011) studied the mechanistic aspect of TNF-α induced NF-κB pathway activation in fibroblast L929sA cells. Kurarinone inhibited TNF-α induced IL-6 mRNA expression in transfected L929sA cells significantly at 4 and 40 μM concentrations. Further, the TNF-α induced promoter activity of different genes (IL6, IL8, E-sel, PGK. and NF-κB) were decreased in the presence of kurarinone at 10, 25, 50, and 100 μg/ml concentrations. Kurarinone did not affect the TNF-induced NF-κB binding to DNA but it significantly inhibits p42/p44 ERK phosphorylation thereby inhibiting the downstream effector molecules (p90RSK and target S6RP) at test concentrations. Further, the toxicity of kurarinone was studied in low and high metastatic *in vitro* breast cancer models (MCF7/6 and MDA-MB-231 cells, respectively). Dose-dependent activity was observed in the test cells (Berghe et al., 2011). In an interesting study, Shi et al. (2012a) studied the microbiologically transformed nor-kurarinone compounds for their anticancer potential. A total of seven compounds (kurarinone was one of them) was formed by the action of *Cunninghamella blakesleana* on the test compound and the structure was confirmed using NMR and MS techniques. Kurarinone showed toxicity against Hela and A375 cell lines with an IC_{50} of 36 and 62 μM, respectively (Shi et al., 2012a) (**Figure 1**). The same research group obtained some new glycosylated compounds by transforming kurarinone in the presence of *Cunninghamella* spp. One of the transformed products named kurarinone-7-O-β-glucoside showed 8.7 μmol/ IC_{50} against Hela cells (Shi et al., 2012b).

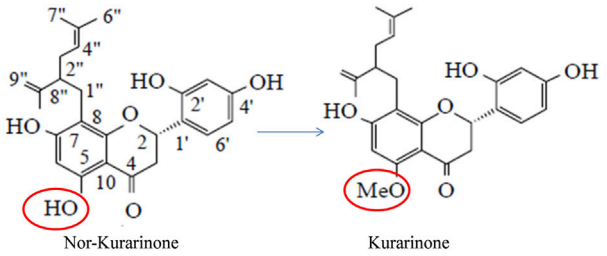

FIGURE 1 | Structural difference between nor-kurarinone and kurarinone.

Seo et al. (2012) reported the effect of kurarinone on TRAIL (TNF-related apoptosis inducing ligand) induced apoptosis and associated mechanism in Hela cells. Results showed that kurarinone exerts apoptosis induction potential in a caspase-dependent manner at 5 μM concentration. It showed effect neither on Bcl2 and inhibitor of apoptosis (IAP) family proteins nor on death receptors (DR4 and 5) induced cytotoxicity. TRAIL is known to induce apoptosis through TRAIL-R1/R2 (DR4/5) transmembrane receptors. Further results showed that kurarinone has the ability to potentiate the apoptosis induction potential of TRAIL via inhibiting the NF-κB mediated cFLIP (FLICE-inhibitory protein long form) expression (Seo et al., 2012). TRAIL is an important anticancer agent, which induces apoptosis in different types of cancer cells. Gastric cells are known to be less sensitive to TRAIL induced apoptosis. Zhou et al. (2015) studied the effect of kurarinone and TRAIL co-treatment in gastric cells (SGC7901). Kurarinone showed significant cytotoxicity to gastric cells at 10 μM or higher concentrations. Co-treatment with the TRAIL (50 ng/ml), kurarinone showed toxicity even at lower concentrations (5 μM). Similarly, the co-treatment significantly increased the cleaved caspase-3 and PARP proteins in comparison to treatment alone. Further, the co-treatment arrested the gastric cells in the G2/M phase and decreased the cyclin B1 and cyclin A protein expression. Kurarinone-TRAIL treatment decreased the apoptotic regulator proteins (Mcl-1 and c-FLIP) at mRNA and protein level. The result showed that the co-treatment decreases the phosphorylation of STAT3 protein which is required for the expression of Mcl-1 and c-FLIP proteins (Zhou et al., 2015).

Recently, Yang et al. (2018) studied in detail the anticancer efficacy of kurarinone in non-small cell lung cancer (NSCLC) *in vitro* and *in vivo* models. The author proposed the underlying apoptosis induction mechanism of kurarinone in NSCLC cells (A549). Kurarinone showed little toxicity on normal human bronchial epithelial cells (BEAS-2B) at 5–25 μM concentration. The phytochemical produced dose-dependent apoptosis induction potential by decreasing the Bcl2-Bax protein ratio, activating caspase 9/3, decreasing Grp78 expression, inhibiting

caspase 12/7, and suppressing the Akt activity at 5, 10, and 25 μM concentrations. Kurarinone did not produce toxicity in experimental rats and produced anti-cancer efficacy in the A549 xenograft rat model at a dose of 20 and 40 mg/kg body weight. Kurarinone treatment significantly reduced the tumor weight and volume in comparison to the non-treated group in 27 days of treatment (Yang et al., 2018). In a different study, Chung et al. (2019) studied the anticancer effect of kurarinone in small-cell lung cancer (SCLC) cells (H1688 and H146) and deduced the underlying mechanism. Kurarinone showed 12.5 and 30.4 μM IC_{50} for H1688 and H146 cancer cell lines, respectively. Early and late apoptotic cell population was increased in kurarinone treated cells. The cleaved PARP level was increased in kurarinone treated cells at 6.25, 12.5, and 25 μM concentrations. Further, the change in cleaved caspase 3, Bcl-2 and Bcl-XL proteins in the presence of kurarinone revealed mitochondria and receptor mediated apoptosis induction in SCLC cells. Kurarinone increased the sub-G1 population of H1688 cells up to 60% at test concentrations. The phytochemical treatment at 3.125, 6.25, and 12.5 μM concentrations increased the E-cadherin level and decreased the vimentin, N-cadherin, and MMP3/2/9 which indicates the epithelial-mesenchymal transition potential in kurarinone (Chung et al., 2019).

Activating transcriptional factor 4 (ATF4) is an important protein that senses the various stress in the cell (especially ER stress). After activation, it induces stress-relieving and apoptotic genes. Activation of ATF4 by pharmacological agents is a good strategy to target cancer. Nishikawa et al. (2019) reported the effect of kurarinone on ATF4 activation and the cytostatic effect of kurarinone in prostate cancer cells. Kurarinone was isolated from the acetone extract of the *S. flavescens*. A dose dependent anticancer activity was observed in a prostate cancer cell line (PC3) at 10–50 μM concentration (IC_{50} 24.7 μM). A 2.02 selectivity index score was obtained by analyzing the cytotoxic activity of kurarinone in PC3 and normal human diploid fibroblast (TIG3 cells). The index showed the high selectivity of kurarinone toward cancer cells. It showed ATF4 activation and increased expression of its downstream effectors TRB3 in a time (6 and 10 h) and dose (20 and 50 μM) dependent manner at the protein level in prostate cancer cell line (PC3). Kurarinone also increased the TRB3 promoter activity at test concentrations. Kurarinone induced ATF4 activation through the PERK-eIF2α pathway, which was revealed by the activation of PERK by its phosphorylation in the presence of the phytochemical. Further, the cytostatic effect of kurarinone was observed in prostate cancer cells, which was evident by the increased p21 and p27 levels and decrease in cyclin D1 and cyclin A protein expression at 20 and 50 μM concentration (Nishikawa et al., 2019). Recently, Liang et al. (2021) predicted the potential anticancer efficacy of the ingredients of "Compound Kushen Injection" used for lung cancer. The study utilized the network pharmacology approach to find the drug targets, pathway prediction, and protein-protein network analysis for the identified active ingredients including kurarinone (Liang et al., 2021). Pre-clinical anticancer studies on kurarinone from the year 2000 to date show indifferent cancer experimental models, which have been summarized in **Figure 2A**. The effect of kurarinone on various hallmarks of cancer (**Figure 2B**) indicates its diversified mode of action in cancer cells. The various anticancer mechanism of kurarinone is depicted in **Figure 3**.

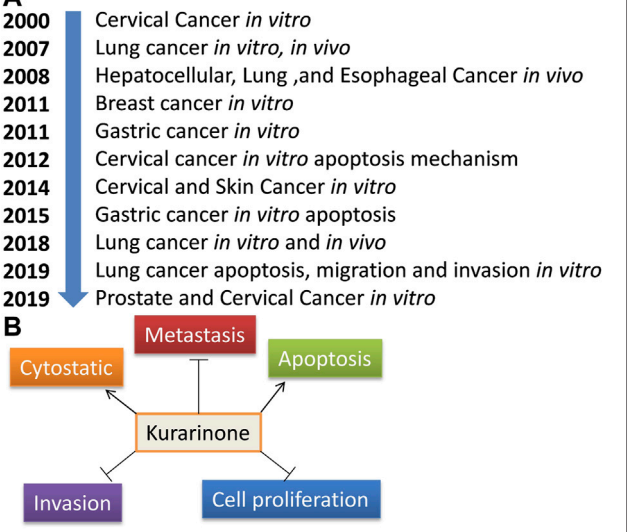

FIGURE 2 | Anticancer studies on kurarinone. **(A)** Year-wise anti-cancer studies on kurarinone, **(B)** cancer hallmarks target by kurarinone.

Anti-Inflammatory and Immune Response

Chi et al. (2001) studied the effect of kurarinone on eicosanoid generating enzymes such as cyclooxygenase 1 and 2 (COX-1 and COX-2) as well as 5- and 12-lipooxygenase (5-LOX and 12-LOX) in bovine platelet and polymorphonuclear leukocytes and monocyte/macrophage cell line (RAW 264.7). Kurarinone inhibited the COX and LOX enzymes in micromolar concentrations. Kurarinone showed better efficacy against the COX-1 enzyme (Chi et al., 2001) (**Figure 4**). Kim et al. (2002) showed that kurarinone was not able to down-regulate the COX-2 induction in LPS treated RAW cells up to 25 μM concentration. Han et al. (2010) studied the nitric oxide (NO) production, reactive oxygen species (ROS) generation, inflammatory cytokine expression, NF-kB activity, and MAP kinases phosphorylation of kurarinone in lipopolysaccharide (LPS) induced monocyte/macrophage cell line (RAW 264.7). The NO and ROS mediated stress modulates the cytokine production by regulating the NF-kB pathway. The NF-kB translocates to the nucleus and initiates the transcription of target genes responsible for different molecular events (such as stress-response, peptide/cytokine/chemokine secretion, apoptosis inhibitory proteins, etc.). Kurarinone decreased ROS production, NO radical generation, and iNOS protein expression in LPS induced RAW 264.7 cells at micromolar concentration. Similarly, kurarinone reduced the expression of LPS induced expression of inflammatory genes (CCL2, TNF-α, IL-1β, and iNOS) at mRNA level in 24 h treatment. Further, kurarinone decreased the LPS induced

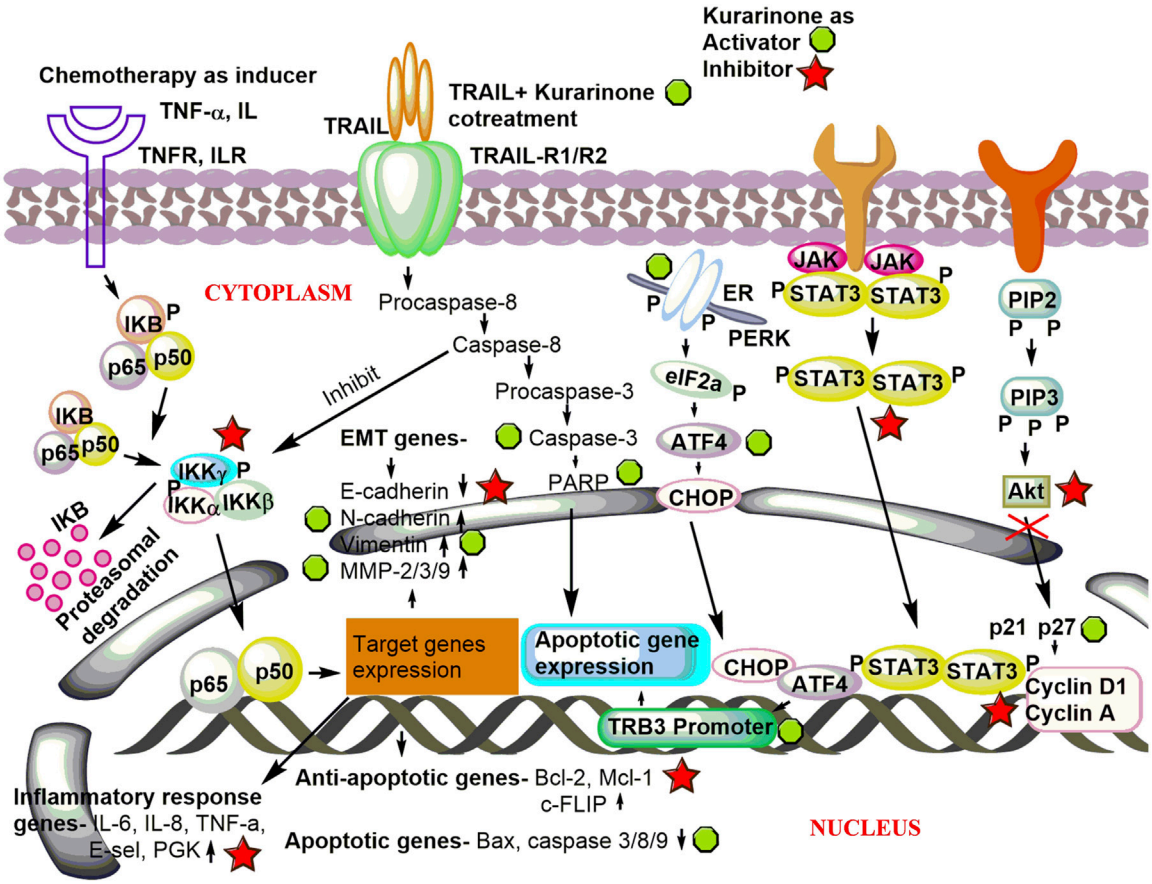

FIGURE 3 | Underlying anticancer mechanism of kurarinone. Kurarinone induces apoptosis and cytostatic effects in cancer cells and inhibits invasion, metastasis, and cellular proliferation. TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor; IL, interleukin; ILR, interleukin receptor; TRAIL, TNF-related apoptosis inducing ligand; TRAIL-R1/R2, TNF-related apoptosis inducing ligand-receptor 1/2; EMT, epithelial mesenchymal transition; MMP, matrix metalloproteinase; PGK, phosphoglycerate kinase; cellular FLICE (FADD-like IL-1β-converting enzyme), inhibitory protein; Mcl-1, myeloid leukemia and chlamydia; PARP, poly (ADP-ribose) polymerase; ER, endoplasmic reticulum; PERK, protein kinase R (PRK) like endoplasmic reticulum; eIF2a, eukaryotic translation initiation factor 2A; ATF4, activating transcriptional factor 4; C/EBP, homologous protein; TRB3, Tribbles homolog 3; JAK-STAT, Janus kinase-signal transducer and activator of transcription; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; Akt, protein kinase B.

phosphorylation of different MAP kinases (ERK1/2, JNK, and p38) in test cell lines and NF-kB activation (Han et al., 2010).

Sahlan et al. (2019) studied the *in vivo* anti-inflammatory potential of propolis from *Tetragronula sp*. and characterized the compounds present in it. The anti-inflammatory potential of the micro-capsulated propolis was studied in the carrageenan-induced rat's paw inflammation model. LC-MS/MS analysis of the sample revealed that the presence of kurarinone is an active ingredient in propolis. Kurarinone containing propolis produced significant *in vivo* anti-inflammatory potential (Sahlan et al., 2019). Chen et al. (2020) studied the mechanistic efficacy of *S. flavescens* ethanolic extract in dextran sodium sulfate-induced ulcerative colitis rats. Ulcerative colitis (UC) is an immunological disease; if not treated, it may lead to colon cancer. The different pharmacodynamics parameters related to UC were studied. The UHPLC-MS/MS based analysis of the test extract showed the presence of kurarinone in the extract. The study concluded that the compounds such as kurarinone can alter the UT-related pathophysiological conditions (colon length and healthiness of colon tissue, bleeding, etc.) in an experimental rat model (Chen et al., 2020). Kim et al. (2013) studied the immune response inhibitory potential of kurarinone in psoriasis-like skin disease and contact dermatitis experimental chronic inflammatory skin models. The study showed that the phytochemical can decrease the expression of inflammatory molecules (cytokines/interleukins and inflammatory enzymes) and thereby inhibit the JAK/STAT signaling and T-cell receptor pathways. Besides, kurarinone also suppressed the inhibition of the differentiation of CD4(+) T cells in the experimental models. Xie et al. (2018) studied the clinical parameters such as inflammation, demyelination, T helper cells sub-population in sections of the spinal cord, and splenocytes of the multiple sclerosis experimental models. The study reported that kurarinone (100 mg/kg/day) has therapeutic potential against multiple sclerosis mainly by inhibiting Th1 and Th17 cellular proliferation and differentiation.

Nishikawa et al. (2020) studied the anti-inflammatory mode of action of kurarinone *in vitro* using RAW264.7 and HaCaT cells. Kurarinone showed KEAP1 (kelch-like ECH-associated protein

1) down regulation mediated Nrf2 stabilization in a dose dependent manner (at 20–50 μM concentration). Translocation of Nrf2 to the nucleus results in the expression of antioxidant genes (such as heme oxygenase-1 or HO-1) which initiates the detoxification process. Moreover, the study revealed that kurarinone has the potential to inhibit the LPS induced inflammation in RAW264.7 by suppressing the inflammatory mediators through the HO-1 enzyme (Nishikawa et al., 2020). Recently, Tang et al. (2021) studied the effect of kurarinone and its mode of action in collagen induced arthritis mice model. The kurarinone treated experimental group showed decreased serum and paw tissue levels of TNF-α, IL-6, IFN-γ, and IL-17A in collagen-induced arthritis (CIA) mice at 100 mg/kg/day dose. It increased the expression of antioxidant proteins (SOD and GSH-Px) and decreased the MDA production and hydrogen peroxide in paw tissues. Further, the study showed that kurarinone increased the Nrf2 and OH-1 protein expression and decreased the KEAP1 expression in the experimental mice. The study showed that kurarinone exerts the anti-inflammatory action by altering the Th1 and Th17 cell differentiation, increasing antioxidant level and alterations in the Nrf2 pathway in the arthritis animal model (Tang et al., 2021) (**Figure 3**).

Effect on Drug Resistance

Choi et al. (1999) tested the anticancer efficacy of the sophora flavanone including kurarinone on P-glycoprotein (Pgp) expressing human colon cancer cell line (HCT15). The effect of kurarinone was also studied on the multi-drug resistance subline of the HCT15 cells (HCT15/CL02). They found that kurarinone showed less activity against HCT15/CL02 cells in comparison to HCT15 cells. Moreover, they reported that combining the treatment with the standard anticancer drug and Pgp inhibitor (Verapamil) also did not increase the cytotoxicity of kurarinone in the test cells. At non-toxic concentrations the kurarinone was not able to increase the paclitaxel mediated cytotoxicity as well as cytoplasmic accumulation of rhodamine 123 dye in the drug resistant cell line. The author concluded that the kurarinoe has no effect on PgP mediated drug resistance in colon cancer cells (Choi et al., 1999). Chen et al. (2005) studied the anti-bacterial efficacy in methicillin and vancomycin antibiotic resistant bacteria *Staphylococcus aureus* (MRSA) and *Vancomycin-resistant enterococci* (VRE), respectively. These two bacteria are among the most common causative agents for lethal hospital infections. They isolated the kurarinone from the roots of *S. flavescens* using HPLC and characterized the compound using NMR spectroscopy. Kurarinone showed 2 μg/ml MIC against both tested drug-resistant bacteria. The author also reported the dose dependent anti-drug resistant potential in kurarinone against MRSA and VRE bacteria (Chen et al., 2005).

Antimicrobial (Fungal, Yeast, Virus, Bacteria) Activity

Tan et al. (1996) isolated kurarinone from aqueous-acetone root extracts of *Gentiana macrophylla* (Family) using the LC-UV-mass spectrometry method. They reported that kurarinone is active against plant pathogenic fungus and human pathogenic yeast, namely, *Cladosporium cucumerinum* and *Candida albicans*, respectively. The minimum inhibitory concentration (MIC) of kurarinone against the *C. cucumerinum* and *C. albicans* was 5 μg. In the same study, Miconazole and Propiconazole standard growth inhibitors against *C. cucumerinum* and *C. albicans* showed 0.1 and 0.001 μg MIC, respectively (Tan et al., 1996). Sohn et al. (2004) studied the antimicrobial activity of kurarinone (isolated from *S. flavescens* root) against bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus epidermis*, and *S. aureus*) and fungus (*Candida albicans* and *Saccaromyces cerevisiae*) using micro-dilution method-based MIC calculation. Kurarinone showed 60 and 100 μg/ml MIC against all the test fungus and bacteria, respectively. The antibacterial activity was compared with respective standard antibacterial and antifungal compounds such as Ampicillin, Erythromycin, Amphotericin B, Miconazole, and 5-Fluorocytosine (Sohn et al., 2004). Ryu et al. (2008) reported antibacterial activity of kurarinone against Gram-positive/negative bacteria using the paper-disk diffusion method. The *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli* were used to assess the antibacterial activity. The study was performed on the kurarinone isolated from the chloroform fraction of *S. flavescens* methanolic root extract. Kurarinone showed potential antibacterial against *B. subtilis*, *B. cereus*, and *S. aureus* (≈15 zone of inhibition, ZOI). Kurarinone did not show inhibitory potential against *E. coli*. The antibacterial activity was compared with the standard antibacterial agent, Ampicillin (Ryu et al., 2008). In an interesting study, Chong et al. (2013) reported the antibacterial activity of kurarinone nanoparticles deposited onto a filter material. The antimicrobial potential durability and nanoparticles morphology was assessed over a time period of 5 months. The study was designed to tackle the toxicity and infection related problems that arise from airborne biological particles such as bacteria. The ethanolic solution of freeze-dried *S. flavescens* whole plant powder and Gram-positive/negative bacteria (Gram-positive *Staphylococcus epidermidis* and *Escherichia coli*) were used for the study. The bacterial inactivation percentage was calculated by using the $(CFU_{experiment}/CFU_{control}) \times 100$. $CFU_{experiment}$ and $CFU_{control}$ represented the concentration of bacterial colonies obtained from the test and control groups, respectively. The major components in the ethanolic fraction of the test material were studied, and kurarinone was one of them. The study showed that kurarinone chemical degradation was significantly lower in comparison to other test compounds over the 5-month period. The study established the use of natural product nanoparticles for the antimicrobial potential of filters (Chong et al., 2013).

Recently, Min et al. (2020) reported the anti-human coronavirus infection potential in kurarinone. The human coronavirus-OC43 (HCoV-OC43) infected lung fibroblast cell line (MRC-5) was utilized to study the anti-corona virus infection. The cellular toxicity, quantification of virus RNA copy number, viral protein expression, quantification of cytokine at mRNA level, and other parameters were studied in

transfected MRC-5 cells treated with kurarinone. The test compound inhibited the growth of virus infected fibroblast cell line at ≈3.5 μM IC_{50} concentration (Min et al., 2020). The cells were incubated with kurarinone at 3.5 μM for 4 days and then studied for the virus-induced cytopathic effect using light microscopy. The results were compared with the non-treated and remdesivir treated groups. The result revealed promising potential in kurarinone against virus-induced cytopathic effect in transfected fibroblast. qRT-PCR and Western blot analysis revealed that kurarinone significantly reduced the expression of viral proteins at mRNA and protein levels in the transfected cells. Further, the time-of-addition assay showed virus-induced cytopathic effect of kurarinone was significant (in co-administered and post-viral administered treatment) (Min et al., 2020). Impairment in autophagy in host cells is an important phenomenon in virus-induced cytopathic effect. Studies showed that the kurarinone has potential to mitigate the virus-induced autophagy by modulating the expression of LC3-II/LC3-I ratio at 5 μM (Min et al., 2020).

Antioxidant Activity

Jung et al. (2004) first assessed the antioxidant potential of kurarinone isolated from the ethanolic fraction of the methanol extract of *Albizzia julibrissin* (Leguminosae). The antioxidant potential was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Jung et al., 2004). Later, Piao et al. (2006) reported *in vitro* radical scavenging potential of kurarinone in biochemical and cell culture experiments. The kurarinone was isolated from aqueous, methylene chloride, and butanol fractions of *S. flavescens* root methanol extract using bioactivity-guided fractionation and isolation approach. The radical scavenging potential was tested using DPPH radical scavenging assay. The protective efficacy of kurarinone against free radical (2,2′-azobis(2-amidinopropane) dihydrochloride or (AAPH)-induced damage in kidney cells (LLC-PK_1). Kurarinone (butanol fraction) showed potential dose dependent DPPH radical scavenging activity (7.73 μg/ml IC_{50}). The butanol fraction produced dose dependent protective efficacy in AAPH induced oxidative damage in LLC-PK_1 cells. The non-treated and AAPH treated cells were considered as control experimental setup to compare the results. AAPH decreased the LLC-PK_1 cell viability by 60%. Kurarinone treatment restored the cell viability by 70–90% at 5–50 μg/ml concentration (Piao et al., 2006).

Jeong et al. (2008) studied the copper-induced low-density lipoprotein (LDL) oxidation of *S. flavescens* root isolated kurarinone. The LDL was isolated from human plasma and oxidized with copper to produced oxidized LDL. Conjugate diene formation, malondialdehyde (MDA) estimation, and REM (relative electrophoretic mobility) the assay was performed to assess the anti-oxidative effect of kurarinone in cu-induced LDL oxidation model. Dose and time dependent decreased MDA (IC_{50} 14.5 μM) and diene production (at 5 μM), respectively, were observed in a kurarinone treated experimental group (Jeong et al., 2008). REM assay showed that at higher concentrations (20–80 μM) kurarinone inhibited the formation of oxidized LDL (from wild type non-oxidized LDL). Further, the effect of kurarinone on LDL oxidation was also studied by assessing the fragmentation of lipoprotein apoB-100 using the SDS-PAGE technique. Results showed 56–89% protection against apoB-100 oxidative modification at 40–160 μM concentration in SDS-PAGE based assay. The study revealed the apoB-10 fragmentation protection against LDL oxidation (Jeong et al., 2008). Zhou et al. (2018) assessed the antioxidant potential of kurarinone containing *S. flavescens* in ultrasonic-assisted optimized isolated flavonoid fraction. The HPLC analysis of the fraction revealed the presence of kurarinone as one of the major constituents of *S. flavescens* extract. The fraction was subjected to test the antioxidant potential in terms of DPPH radical and hydroxyl radical scavenging activity. Dose dependent antioxidant activity was observed with 0.984 and 1.084 mg/g IC_{50} in DPPH and OH radical scavenging assay, respectively (Zhou et al., 2018).

Neuroprotective Efficacy

Jeong et al. (2008) studied the neuroprotective efficacy of flavones isolated from *S. flavescens* in glutamate induced experimental model. The glutamate mediated neurotoxicity (oxidative stress) was induced in immortalized mouse hippocampal cell line (HT22). The heme oxygenase (HO)-1 activity and ROS generation were studied in *S. flavescens* flavones treated HT22 cells. Kurarinone did not show the effect on (HO)-1 activity and ROS generation in the experimental setup (Jeong et al., 2008). Park et al. (2009) studied neuroprotective effects of *S. flavescens* ethyl acetate extract (alkaloid free) by studying its efficacy against focal cerebral ischemia (FCI) in the experimental rat model. HPLC analysis of the solvent-portioned extract (by using various solvents such as water, hexane, etc.) revealed ≈46% kurarinone in the test material. The middle cerebral artery occlusion (MCAO) method was to induce FCI in the Sprague-Dawley rats. The extract showed dose dependent decrease in the sodium nitroprusside induced cell mortality in SH-SY5Y (neuronal) cells. Kurarinone containing test extract pre-treatment (0.2–10 μg/ml) increased the apoptotic population (by modulating the protein expression of caspase-3 and extent of DNA fragmentation) which was decreased in the sodium nitroprusside treated group. After the satisfactory results in the *in vitro* study, the author studied the neuroprotective efficacy of the test sample in the MCAO model. Results showed that kurarinone containing test extract significantly reduced the severity of neurological deficits in the experimental rats (Park et al., 2009).

Channel and Transporter Activity Modulation

Voltage-gated Ca^+ channel modulates the concentration of calcium ions into the cells by checking their passage across the cell membrane and thereby affect muscle contraction. Yamahara et al. (1990) studied the muscle relaxation potential of *S. flavescens* root methanolic (MT) and ethanolic (ET) fractions in the thoracic aorta of rabbits and rats. The potassium chloride (50 mM) was used to induce contraction in the test sample and then the relaxation efficacy of *S. flavescens* root fractions was

studied. The results were compared with the papaverine (10^{-4} M) induced contraction (considered as 100%). The MT and ET fractions at 25–50 μg/ml concentration exert 40–60% muscle relaxation. After portioning the ET fraction in butanol, water, and ET fractions, the ET fraction showed 100% muscle relaxation. To identify the active ingredient the ET fraction was further sub-fractionated into nine parts. Out of which the second sub-fraction was identified as kurarinone, and showed about 100% muscle relaxation in rabbit and rat aorta (Komatsu et al., 1970; Yamahara et al., 1990).

Sodium-glucose cotransporter (SGLT) is known to absorb/re-absorb the glucose molecules into the cells. The SGLT1 and SGLT2 are responsible for the absorption of dietary glucose and reabsorption of body glucose in the proximal tubule, respectively. The inhibition of the SGLT transporter is an attractive target for type 2 diabetes. Sato et al. (2007) studied the SGLT1 and SGLT2 transporter inhibition potential of *S. flavescens* root extracts. Initially, the methanolic extract was portioned into water and ethyl alcohol. The alcoholic fraction was further sub-fractionated into 10 fractions out of which fraction 5 yielded kurarinone. To study the SGLT inhibitory potential of the isolated compounds, [^{14}C]methyl-a-D-glucopyranoside uptake was assessed in hSGLT1 or hSGLT2 expressing monkey kidney derived fibroblast like cells (COS-1). At 50 μM, kurarinone containing fraction showed ≈100% SGLT1 and SGLT2 inhibition efficacy. In a further experiment, kurarinone exerted 50% inhibitory potential against GSLT1 and SGLT2 with IC_{50} of 10.4 and 1.7 μM, respectively (Sato et al., 2007).

Gamma-aminobutyric acid type A ($GABA_A$) receptors are involved in neurotransmission inhibition through the influx of calcium ions in response to the binding of γ -aminobutyric acid to the receptor. $GABA_A$ receptor inhibitors are used to treat different neuronal pathophysiological conditions. Yang et al. (2011) studied the $GABA_A$ receptors potentiation of kurarinone in *Xenopus* oocytes. The ethanol extract of *S. flavescens* root was prepared and the micro fractionation was done using HPLC to obtain 22 fractions. The kurarinone was identified in fraction 9 and showed maximum potentiation (100% at 10 μM concentration) of $GABA_A$ receptors, which were transiently expressed in stages I-IV *Xenopus* oocytes (Yang et al., 2011).

The BK_{Ca} (large conductance Ca-activated K-channel) channel is involved in the relaxation process of the urinary bladder smooth muscle. In over-reactive bladders, the channel possesses therapeutic target potential to control micturition frequency (Lee et al., 2016). Lee et al. (2018) screened ≈800 natural compounds for their BK_{Ca} potentiation efficacy by using cell-based fluorescence assay in hyperactive mutant BK_{Ca} channel expressing AD-293 cells (derived from human embryonic kidney cells). At 5 μM kurarinone increased the BK_{Ca} channel activity as evident by the increase in fluorescence. The mechanistic study revealed that the kurarinone stabilizes the open conformation of the channel. Moreover, the study also reported that the kurarinone treatment decreases the bladder contraction in rats having over-activated urinary bladders and thereby decreased the micturition frequency (Lee et al., 2018).

In diabetic patients, use of SGLT inhibitors may create a urinary tract infection (UTI) problem as the increased glucose concentration in the urine favors bacterial growth. The bacteria used to attach with the host cells by Type 1 pili (which possess FimH protein). Mashraqi et al. (2021) studied the human SGLT transporter and bacterial FimH protein inhibition potential of natural flavonoids using computer aided drug discovery approach. It has been postulated that the natural compound possessing the inhibitory action against both targets may be used as potential anti-diabetic agents having kisser side effects. In the study kurarinone showed potential binding (−7 kcal/mole) against an SGLT transporter but moderate binding (about −4 kcal/mole) with the FimH protein (Mashraqi et al., 2021).

Tyrosinase Inhibition Potential

Tyrosinase enzyme is an attractive target for disease associated with local hyperpigmentation as well as in the cosmetic industry for skin whitening. The enzyme modulates the melanin biosynthesis and possesses 1-tyrosine hydroxylase and 1-dopa oxidase activities. Son et al. (2003) prepared *S. flavescens* root methanol extract, and after portioning (using water, dichloro-methane, and ethyl acetate (ET)) the fractions were tested for tyrosinase inhibitory activity. The most potent ET fraction was further fractionated in eight sub-fractions. The kurarinone was identified in one of the sub-fractions and showed potent *in vitro* tyrosinase potential with 1.3 μM IC_{50} concentrations (Son et al., 2003). In a different study, Kim et al. (2003) reported significant dose dependent mushroom tyrosinase inhibitory efficacy (≈100%) in kurarinone at 1–50 μM concentration. Ryu et al. (2008) studied the tyrosinase inhibition potential of kurarinone in the *S. flavescencs* methanol extract isolated compounds. The result showed that kurarinone inhibited (IC_{50} 2.2 μM) L-tyrosine oxidation in a dose dependent manner but did not fully inhibit the tyrosinase enzyme activity. Enzyme kinetics study showed that kurarinone decreased the *Vmax* value with increasing concentration. The mushroom tyrosinase enzyme inhibition assay revealed kurarinone as a noncompetitive inhibitor with 4.1 μM inhibition constant (Ryu et al., 2008).

Other Pharmacological Potential

The accumulation of triacylglycerol in the body is related to several pathophysiological conditions such as coronary heart disease, obesity, diabetes, and hypertriglyceridemia. Diacylglycerol acyltransferase (DGAT) is a committed step in triacylglycerol synthesis, which makes it a suitable target for the management of the abovementioned disease in patients. Chung et al. (2004) studied the DGAT inhibitory potential of kurarinone. In the spectrophotometric method, kurarinone showed dose dependent DGAT inhibition potential with 10.9 μM IC_{50} in microsomal rat liver fractions. Further, the study on Raji cells showed that kurarinone inhibit free long chain in fatty acid based lipid synthesis at 3–10 μM concentrations (Chung et al., 2004). Protein tyrosine phosphatase 1B is known to inhibit the insulin signaling pathway and thus possess potential as an anti-diabetic therapeutic target. Sasaki et al. (2014) reported that kurarinone inhibited the protein with 41.68 μM IC_{50}.

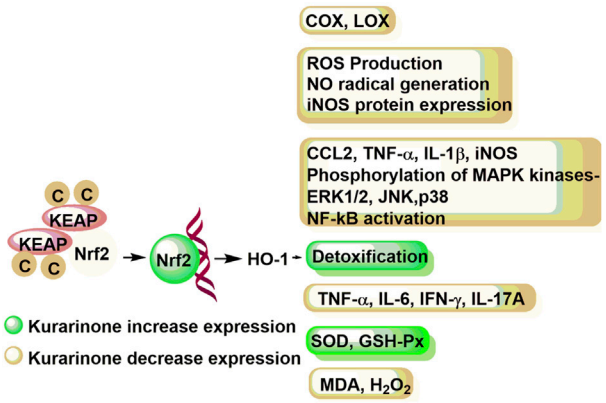

FIGURE 4 | Immune response modulation potential of kurarinone at the molecular level. COX, cyclooxygenase; LOX, lipoxygenase; ROS, reactive oxygen species; NO, nitric oxide; iNOS, inducible nitric oxide synthase; CCL2, C-C motif chemokine ligand 2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MAPK, mitogen activated protein kinase; ERL1/2, extracellular signal regulated kinase 1/2; JNK, c-Jun N-terminal kinase; NF-κB, necrosis factor-κB; IL-6, interleukin-6; IL-17A, interleukin-17A; SOD, superoxide dismutase; GSH-Px, plasma glutathione peroxidase; MDA, melanoma differentiation associated protein; H_2O_2, hydrogen peroxide; KEAP, Kelch-like-ECH-associated-protein; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1.

De Naeyer et al. (2004) studied the estrogenic potential of kurarinone. The kurarinone was obtained from the bioactivity-guided sub-fractionation of the *S. flavescens* phenolic extract. The activity was studied in the Ishikawa Var-I bioassay and yeast model. Kurarinone showed potent dose dependent estrogenic activity among the test compounds with 4.6 and 1.6 μM EC_{50} in the yeast screen and Ishikawa Var-I bioassay (De Naeyer et al., 2004).

Glucosidase is an important enzyme related to digestion of carbohydrates, glycoprotein synthesis, and degradation of glycoconjugates. Glucosidase inhibitors are well studied for type 2 diabetes, cancer, and other diseases. Kim et al. (2006) studied the glycosidase inhibition potential of kurarinone isolated from *S. flavescens* root extract and sub-fractionation. The glycosidase activity was assessed for α-glucosidase, β-galactosidase, α/β amylase, and invertase enzymes. Kurarinone showed about 99 and 54% α-glucosidase and β amylase inhibition potential with 45 and 980 μM IC_{50} concentrations, respectively. Further, the enzyme kinetics study showed that kurarinone is a noncompetitive inhibitor of α-glucosidase with 6.8 μM K_i (inhibitory constant) (Kim et al., 2006).

Gao et al. (2007) studied the effect of kurarinone on renal trans-differentiation and interstitial fibrosis in the experimental rat model. The renal interstitial fibrosis rat model was utilized and kurarinone treatment was done at 100 mg/kg body weight. The serum biomarkers (such as creatinine, protein content, albumin, blood urea nitrogen, etc.), pathological markers (in renal tissue), and molecular markers (TGF-β1, αSMA, Smad3, collagen I, etc.) of the disease were studied using appropriate techniques. The kurarinone treated experimental group showed significant down regulated TGF-β1 and collagen I expressions. The study concluded that kurarinone may exert the anti-fibrosis effect through Smad3 down expression (Gao et al., 2007).

Aldose reductase (AR) an NADPH-dependent oxidoreductase converts excess glucose in sorbitol and ultimately in fructose. Later, their accumulation hampers the normal metabolic process and creates complications in diabetic patients. Excess amounts of sorbitol and fructose also produce reactive dicarbonyl species much related to AGE (advanced glycation end products) formation. Jung et al. (2008) reported the AR (rat lens and human recombinant ARs) and AGE inhibition potential in kurarinone using the spectrophotometric method. Kurarinone showed about 31–65% rat lens AR inhibition potential at 0.4–2 μg/ml concentration with 2.99 μM IC_{50}. The human recombinant AR inhibition potential was assessed at 1 and 5 μg/ml, which showed 45 and 75% inhibition of the AR with 3.81 μM IC_{50}. Kurarinone did not exert the AGE inhibitory efficacy (Jung et al., 2008).

Phytochemicals are known to increase osteoblastic cell proliferation and alkaline phosphate activity both *in vitro* and *in vivo*. Plant product-based management of bone associated diseases such as osteoporosis is a cost-effective strategy. Keeping these facts in mind, Wang et al. (2011) studied the osteogenic effect of flavonoids isolated from *Drynaria fortune*. The study isolated kurarinone from the plant extract which did not show the osteogenic effect at all the test concentrations (10–1,000 nm) in the osteoblastic UMR 106 cells. At higher concentrations, kurarinone increased the ALP activity about 74% (Wang et al., 2011). Xanthine oxidase is an important drug target used for gout, hyperuricemia, ischemic tissue/vascular injuries, inflammation related diseases, and myocardial infarction. Suzuki et al. (2013) studied the xanthine oxidase inhibition potential of kurarinone. The result showed that kurarinone was active against the enzyme only at a higher concentration (100 μM) (Suzuki et al., 2013).

Human carboxylesterases 2 (hCE2) is an important type I xenobiotic metabolism enzyme involved in the ester group metabolism. It detoxifies several environmental toxins in the body as well as hydrolyzes the ester group present in several therapeutic agents. Song et al. (2019) reported the hCE2 inhibitory potential of kurarinone *in vitro*. Kurarinone showed more than 90% hCE2 inhibition potential with 1.46 μM IC_{50} concentration. The activity was dose dependent. Kurarinone was found to be an uncompetitive type inhibitor of hCE2 with 1.73 μM inhibition constant (K_i) (Song et al., 2019).

Bioavailability, Metabolism, and Toxicity

Sophora flavescens is a medicinal herb and possesses hepato-protective phytochemicals. Zhixue capsule, a Chinese herbal prescription, encompasses *S. flavescens* extract. The capsule was found to exert dose dependent hepatotoxicity in primary rat hepatocytes (Yu et al., 2013). The *S. flavescens* possess hepato-protective phytochemicals, so how can it show hepato-toxicity? To solve this puzzle, Yu et al. (2013) studied the hepatotoxicity of the *S. flavescens* phytoconstituents in experimental rats. The rats were administered with the *S. flavescens* methanol extract at 1.25 and 2.5 g/kg body weight, twice a day. The treatment was carried out for 3 days. After the treatment, serum alanine transaminase (ALT), AST, and liver histopathology were studied. The result showed an increased concentration of the enzyme markers and

damaged liver histology. The extract was sub-fractionated into eight fractions using the semi-preparative HPLC technique. The hepatotoxic effect of kurarinone (subfraction) in rat primary hepatocyte and HL-7702 cells (at 50, 100, and 200 μg/ml concentration) showed significant toxicity (≈30 and 48 μM IC_{50}, respectively) (Yu et al., 2013). Jiang et al. (2017) studied the mechanism of kurarinone toxicity in *in vitro* and *in vivo* experimental models. The rats were administered with 1.25 and 2.5 g/kg kurarinone for 14 days. The *in vitro* test in HEK293 cells showed that kurarinone glucuronide get internalized into the cells through OATP1B3 transporter, which is responsible for the entry of therapeutic molecules in the hepatic cells. Inside the hepatocyte, kurarinone inhibits the PPAR-α pathway and reduces L-carnitine which leads to lipid accumulation and liver cell injury (Jiang et al., 2017).

Zhang et al. (2015) developed a method to determine the kurarinone concentration in biological fluids (rat plasma) using UPLC-MS/MS technique. The kurarinone was administered at a dose of 10 mg/kg body weight. The result showed that kurarinone was found for about 1 h in the plasma; after that, the concentration decreased very fast and becomes zero at the sixth hour (Zhang et al., 2015). A similar study was performed by Yang et al. (2016), which administered a higher amount of kurarinone (25 and 500 mg/kg by weight) and the stability in rat plasma was monitored for 12 h (Yang et al., 2016). In a similar but different study, Huang et al. (2020) developed a UPLC-MS/MS based method to detect the kurarinone levels in dog plasma. In this study, kurarinone was administered at 2 and 20 mg/kg body weight, and the blood plasma level was monitored for 25 h (Huang et al., 2020).

S. flavescens extract has been reported for its effect on xenobiotic metabolism enzyme modulation. For the first time, Qin et al. (2020) studied the interaction of kurarinone with cytochrome P450 and UDP-glucuronosyl transferase enzymes in the liver microsomes and recombinant human supersomes. The result showed that kurarinone inhibited UDP-glucuronosyl transferase (UGT1A1/A6) and cytochrome P450 (CYP2C9, 1A2, and 2D6) effectively at 100 μM concentrations (Qin et al., 2020). In a different study, Zhang et al. (2016) reported the mode of kurarinone metabolism in human liver microsomes. The study showed that it is metabolized in liver microsomes by the glucuronidation detoxification pathway. The study provided important information about the safe usage of kurarinone as a therapeutic molecule.

Clinical Study and Patents

In an important clinical study, Pan et al. (2005) studied the combinatorial effect of kurarinone and interferon alpha-1b (IFNa-1b) in chronic hepatitis B patients. The kurarinone and IFNa-1b were added with the conventional hepatitis B treatment to the patients (Group A). Further, Groups B and C represented the addition of kurarinone and IFNa-1b alone with the treatment, respectively. The treatment was carried out for 6 months and during the next 6 months follow up of the patients was carried out to study the disease associated pathological markers such as liver histology, tissue, and serum TGF-β levels, ALT, etc. The result showed that only Group A significantly lowered the liver fibrosis scores and mitigated the pathophysiological markers (Pan et al., 2005). Natural formulation containing kurarinone has been patented for its therapeutic efficacy against hepatitis and cancer (patent number CN1970001B and CN1961898A, respectively). The fact substantiates the therapeutic efficacy of kurarinone.

CONCLUSION AND FUTURE PROSPECTS

An extensive literature survey on kurarinone (from its first isolation in 1970 to date) revealed the potent therapeutic potential in kurarinone against various disease/ailments. The present review summarizes the chemoprevention and other pharmacological activities of kurarinone (a natural flavanone). It exhibits cell proliferation inhibition, cell cycle arrest, apoptosis induction, anti-metastasis, and stress-induced cytotoxicity in different cancer cells. Kurarinone is an important ingredient of several Chinese medicinal products. The present review activities justify the traditional use of kurarinone in the medicinal system. Although some authors reported hepatotoxicity of kurarinone, but in most of the studies authors showed cancer cell selectivity property in it. Further, a clinical study also showed the non-toxic therapeutic property in kurarinone. Thus, the targeted study should be designed to study the bioavailability and toxicity profile of kurarinone in a pre-clinical and clinical setup. Most of the *in vivo* mechanistic anticancer potential was studied in the lung cancer experimental model, leaving other devastating cancers such as breast, colon, etc. This creates a large thrust area for further research in other cancers at pre-clinical and clinical levels. The literature is silent about the effect of kurarinone on other hallmarks of cancer such as angiogenesis, cancer stemness, etc. Experimental studies showed that kurarinone has the potential to inhibit NF-κB activation directly and indirectly (by lowering TNF-α induction) in disease models such as inflammatory disease (collagen-induced arthritis) and cancer. In arthritis, higher expression of inflammatory molecules (interleukins and TNF-α) activates the NF-κB inflammatory pathway which results in the disease progression. In cancer, NF-κB activation is known to inhibit the apoptotic process which results in cancer cell survival. Thus, the literature indicates that the inhibition of the NF-κB mediated pathway is one of the important mechanisms behind the pharmacological potential of kurarinone. The pharmacological potential of the phytochemical in other diseases that are associated with the NF-κB inflammatory pathway should be studied in appropriate *in vitro* and *in vivo* models. Furthermore, the literature showed that kurarinone can inhibit NF-κB, JAK/STAT, and Akt pathways in different disease experimental models. These pathways are well-known therapeutic targets for cancer and inflammatory diseases. Activation of JAK/STAT, and Akt pathways are well correlated with the tumor initiation, progression, and metastasis. Inflammatory

molecules are known to up-regulate the notch signaling pathway which results in various pathological conditions including cancer. Thus, kurarinone might inhibit the notch signaling mediated pathological effects by regulating the NF-κB inhibition mediated inflammatory response. Although the literature showed antioxidant potential in kurarinone, its neutraceutical and/or food supplementation potential has not yet been established. Thus, the *in vitro* and *in vivo* studies regarding neutraceutical/food supplementation potential of the phytochemicals are very much required. The results of the studies might open the transformation of kurarinone from a therapeutic molecule to a potential neutraceutical. Moreover, the metabolism and xenobiotic metabolic enzyme inhibition mediated in-depth efficacy in the associated disease model have not been fully investigated well. Overall, the present study provides a comprehensive update on the therapeutic potential of kurarinone, which may foster the discovery and development of novel therapeutic agents for the treatment of various diseases including cancer.

AUTHOR CONTRIBUTIONS

SK designed the study, drafted and wrote the article. PK, KP, AS, and MS searched literature and compiled the data. KP, AS, and PK prepared the figures and tables. All authors read and approved the final article. HT reviewed the article critically.

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GLOSSARY

αSMA αsmooth muscle actin

AST aspartate aminotransferase

eIF2a eukaryotic translation initiation factor 2 α

EGFR epidermal growth factor receptor

ERK1/2 extracellular signal regulated kinase 1/2

Grp78 glucose-regulated protein

GSH-Px plasma glutathione peroxidase

Her-2 human epidermal growth factor receptor-2

HEK293 human embryonic kidney 293

H_2O_2 hydrogen peroxide

IL-6/8/17A interleukin-6/8/17A

iNOS inducible nitric oxide synthase

JAK/STAT Janus kinase/signal transducer and activator of transcription

JNK c-Jun N-terminal kinase

KDR kinase insert domain receptor

KEAP Kelch-like-ECH-associated-protein

LPS lipopolysaccharide

Mcl-1 myeloid leukemia and chlamydia

MDA melanoma differentiation associated protein

MIC minimum inhibitory concentration

MMP3/2/9 matrix metalloproteinase

NADPH nicotinamide adenine dinucleotide phosphate

NF-κB necrosis factor-κB

NO nitric oxide

Nrf2 nuclear factor erythroid 2-related factor 2

OATP1B3 organic anion transporting polypeptide 1B3

PARP poly (ADP-ribose) polymerase

PERK-eIF2α protein kinase R (PRK) like endoplasmic reticulum

PGK phosphoglycerate kinase

p90RSK serine/threonine-protein kinase

ROS reactive oxygen species

SOD superoxide dismutase

S6RP S6 ribosomal protein

TGF-β1 Tumor growth factor β1

Th1 type 1T helper

Th17 type 17T helper

TNF-α tumor necrosis factor-α

TRB3 tribbles homolog

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Targeting Aberrant Expression of STAT3 and AP-1 Oncogenic Transcription Factors and HPV Oncoproteins in Cervical Cancer by *Berberis aquifolium*

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Background: Present study examines phytochemical preparation that uses berberine's plant source *B. aquifolium* root for availability of similar anti-cervical cancer (CaCx) and anti-HPV activities to facilitate repurposing of the *B. aquifolium* based drug in the treatment of CaCx.

Purpose: To evaluate therapeutic potential of different concentrations of ethanolic extract of *B. aquifolium root* mother tincture (BAMT) against HPV-positive (HPV16: SiHa, HPV18: HeLa) and HPV-negative (C33a) CaCx cell lines at molecular oncogenic level.

Materials and Methods: BAMT was screened for anti-proliferative activity by MTT assay. Cell cycle progression was analyzed by flowcytometry. Then, the expression level of STAT3, AP-1, HPV E6 and E7 was detected by immunoblotting, whereas nuclear localization was observed by fluorescence microscopy. Phytochemicals reportedly available in BAMT were examined for their inhibitory action on HPV16 E6 by *in silico* molecular docking.

Results: BAMT induced a dose-dependent decline in CaCx cell viability in all cell types tested. Flowcytometric evaluation of BAMT-treated cells showed a small but specific cell growth arrest in G1-phase. BAMT-treatment resulted in reduced protein expression of key transcription factors, STAT3 with a decline of its active form pSTAT3 (Y705); and components of AP-1 complex, JunB and c-Jun. Immunocytochemistry revealed that BAMT did not prevent the entry of remnant active transcription factor to the nucleus, but loss of overall transcription factor activity resulted in reduced availability of transcription factors in the cancer cells. These changes were accompanied by gradual loss of HPV E6 and E7 protein in BAMT-treated HPV-positive cells. Molecular docking of reported active phytochemicals in *B. aquifolium* root was performed, which indicated a potential

Abbreviations: BA, *Berberis aquifolium*; CaCx, cervical cancer; HPV, human papillomavirus; MT, mother tincture; OD, optical density; PI, propidium iodide; VC, vehicle control.

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interference of HPV16 E6's interaction with pivotal cellular targets p53, E6AP or both by constituent phytochemicals. Among these, berberine, palmatine and magnoflorine showed highest E6 inhibitory potential.

Conclusion: Overall, BAMT showed multi-pronged therapeutic potential against HPV infection and cervical cancer and the study described the underlying molecular mechanism of its action.

Keywords: Cervical cancer, Berberis aquifolium, Cell cycle, STAT3, AP-1, Oncoprotein, HPV16 E6, Molecular Docking

INTRODUCTION

Berberis aquifolium commonly known as Oregon grape or Mahonia is a widely used medicinal plant by native North American Indian tribes, in Chinese medicine and in many other traditional medicines (Duke and Ayensu 1985; Satyavathi et al., 1987; Moerman 1998). The root and root bark is used as laxative, diuretic, cholagogue, alterative, and blood tonic (Bown and Herb Society of America. 1995; Chevallier 1996). *B. aquifolium* root extracts are used to treat psoriasis, syphilis, haemorrhages, gastric disorders, sore throats, and bloodshot eyes. *B. aquifolium* is extensively used in alternative medicine system (Boericke 2001). Mother tincture prepared from *B. aquifolium* root is a remedy for the skin, chronic catarrhal affections, secondary syphilis, hepatic torpor, and lassitude.

Briefly, CaCx is the fourth leading cancer of women with an annual incidence of 570,000 and mortality 311,000 (Arbyn et al., 2020). To make things worse, over 85% CaCx cases and resultant deaths are reported from low resource countries. Contrastingly, China and India alone collectively contributed more than a third of the global burden. CaCx is caused by persistent infection of high-risk HPV primarily HPV16 (> 50% cases) and followed by HPV18 (~15% cases) (IARC 2012). HPV codes for two key oncoproteins E6 and E7 that drive cervical carcinogenesis by physically interacting and targeting functions of p53 and pRB, respectively (Yeo-Teh et al., 2018). Loss of p53 and pRb are both key players in cell cycle dysregulation. Among these two, E6 plays a more dominant role by making a complex with p53 in association with host-derived E6-associated protein (E6AP) (Scheffner et al., 1990; Huibregtse et al., 1991). 3D-structure of co-crystallized E6-E6AP-p53 revealed binding of E6 to the conserved domain of E6AP occurs *via* the LxxLL consensus sequence (Martinez-Zapien et al., 2016). E6 and E6AP together form a heterodimer that degrades p53.

Expression of E6/E7 is tightly controlled by early promoter located downstream to an untranslated Upstream Regulatory Region (URR) that contains *cis*-regulatory elements for binding of viral regulatory protein E2 and host-derived transcription factors like AP-1, STAT3, among several others (Shishodia et al., 2018). AP-1 is a redox sensitive transcription factor (Angel et al., 1987), whereas STAT3 is a pro-carcinogenic and pro-inflammatory transcription factor (Bromberg et al., 1999). Both the transcription factors are found overexpressed and constitutively active in the cervical cancer cells and their expression and activity increased with disease severity (Prusty and Das 2005; Shukla et al., 2010). These transcription factors played a functional regulatory role in the expression of viral oncogenes (Shukla et al., 2013). Experimental targeting of E6/E7 activity/expression by targeting viral transcription using synthetic pyrrolidine-dithiocarbamate (PDTC) (Rosl et al., 1997), or natural anti-oxidants (curcumin, berberine or other phytochemicals), anti-sense oligos, RNA interference or by different genome targeting approaches induced remarkable growth inhibition, cell cycle arrest and induction of apoptosis/senescence in CaCx cells (reviewed in (Bhartih et al., 2018)).

Despite being a highly preventable disease due to viral etiology, availability of prophylactic vaccines and substantial understanding of the disease mechanism leading to several preclinical leads (Bharti et al., 2018), there is no clinically-available anti-HPV therapeutics as yet. Therefore, taking leads obtained from berberine, we explored preparations in clinical practice that may potentially contain berberine as one of the constituent. In this regard, *B. aquifolium* mother tincture (BAMT) which is an ethanolic extract of *B. aquifolium* root, was studied in the current investigation. Apart from berberine, *B. aquifolium* root contains several other medicinally-useful constituents like berbamine, hydrastine, jatrorrhizine, magnoflorine, oxyacanthine, columbamine, obamegine, aromoline, and palmatine (USDA 1992-2016) that can potentially act against HPV and/or CaCx. Therefore, in the present investigation, we examined if phytomedicine based on *B. aquifolium* could be repurposed for treatment of CaCx by using well-established cell and molecular biology and bioinformatics assays. We evaluated cell viability, cell cycle, effects on transcription regulators of viral oncogenes, and expression of oncoprotein E6 and E7. Further, we examined the molecular interaction of constituent phytochemicals *in silico* on HPV16 E6 and its ability to interact with corresponding cellular targets that promote cervical carcinogenesis.

MATERIALS AND METHODS

Chemical and Reagent

B. aquifolium MT (ethanol content 70% in water; batch no.–UM180120 (3/2018)) were procured from authoritative sources SBL–Sharda Boiron Laboratories Private Limited, India; a GMP-certified firm. The alcohol (ethanol) utilized to prepare the drug was used at corresponding concentration uniformly as negative control. The cell culture media, trypsin-

EDTA and penicillin-streptomycin solution were procured from HiMedia (India). MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), DAPI (4′,6-diamidino-2-phenylindole) and all other reagents were of analytical grades and were procured from Sigma-Aldrich Chemicals unless specified.

Cell Culture

Human CaCx cell lines with known HPV positivity for HPV type 16 - SiHa and HPV type 18 - HeLa; and HPV negative C33a were originally procured from ATCC and were maintained in DMEM/MEM supplemented with 10% FBS and 1% antibiotic (penicillin and streptomycin) as *mycoplasma* contamination-free cultures. All cell lines were maintained in 5% CO_2 incubator at 37°C. The cell lines were tested for HPV positivity and genotype by using HPV consensus L1 and HPV type-specific PCR periodically for ensuring authenticity and rule out cross contamination (**Supplementary Figure SF1**).

MTT Cell Viability Assay

Cell viability assay was performed as described earlier (Bharti et al., 2003) with minor modifications. Briefly, CaCx cells (2×10^3) were seeded in triplicate in a tissue culture grade 96-well plate in a final volume of 0.1 ml overnight at 37°C in a CO_2 incubator and subjected to drug treatment as indicated by diluting in complete medium (10–0.01%). Ethanol at corresponding strength was used uniformly as vehicle control to treat CaCx cells. Thereafter, 0.025 ml of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (5 mg/ml in PBS) was added to each well. After 2 h incubation at 37°C, the culture medium was removed and 100 µl lysis buffer (20% SDS; 50% dimethylformamide) was added and incubated overnight at 37°C for solubilisation of formazan crystals. The optical density (OD) at 570 nm was measured using a 96-well Multiscanner Autoreader (Biotek, United States). BAMT was inherently colored and hence, interfered with colorimetric assays at higher concentrations. Therefore, cell-free reagent controls were uniformly used to subtract reagent OD. The percentage of viable cells was calculated using the following formula:

$$\text{Percent Cell Viability} = (\text{OD of the experiment samples/OD of the control}) \times 100.$$

Calculation of IC_{50} of the homoeopathic preparations: IC_{50} of the drug was calculated with non-linear regression analysis on log transformed normalized values of BAMT dosage points using Prism (Version 8.0.2; GraphPad Software, CA, United States).

Cell Cycle Analysis by Flowcytometry

Cell cycle analysis was performed by RNase and propidium iodide (PI) staining followed by flowcytometry as described earlier (Bharti et al., 2003) with some minor modifications. Briefly, CaCx cells were seeded into 6-well plates at a density of 5×10^4 cells/well. Cells were treated with indicated concentrations of BAMT or vehicle control. At completion of treatment, the cells were harvested by trypsinization using 0.3 ml of 1X Trypsin-EDTA solution, washed with PBS containing 10% serum, fixed in 70% ethanol. After overnight incubation at −20°C, cells were washed and stained with PI by suspending in staining buffer (10 µg/ml (BD Biosciences, United States), 0.5% Tween-20; 0.1% RNAase) for 40 min at 4°C in dark. The cells were analysed by FACSAriaIII equipped with FACSDiva software (BD Biosciences). A total of 10,000 events were acquired for each sample. For analysis, the cells were gated to exclude cell debris, cell doublets and cell clumps to identify the single cell population first using PI width vs. PI area. The gates were applied to the PI histogram plot.

Immunoblotting

Levels of different proteins were evaluated by immunoblotting as described earlier (Shukla et al., 2010). Briefly, CaCx cells (1×10^6 cells/100 mm plate) were treated as described. At the end of treatment, the cells were scraped, collected and washed with ice cold 1X PBS. Total cellular proteins were extracted by re-suspending the pellet in the cell lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM Na_3VO_4). Lysates were spun at 14,000 rpm in a microfuge for 10 min to remove insoluble material and clear supernatant for each sample was collected. The concentration of proteins was determined by spectrophotometric method and the proteins were stored in aliquots at −80°C till further use. Proteins (40 µg/lane) were resolved in 10–12% polyacrylamide gel using 2X Laemmli buffer (100 mM Tris-HCL pH 8.0, 20 mM EDTA pH 8.0, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue) and transferred to PVDF membranes (0.22 µM; Millipore Corp, Bedford, MA, United States) by wet transfer method. During PAGE, border lanes were loaded with Precision Plus Protein Dual Color Standards (Bio-Rad, United States; Catalog# 161–0374) protein pre-stained marker. Blot was cut between 25 and 37 kDa bands. Upper blot (with ≥37 kDa proteins) was used for detection of transcription factors and lower blot (with ≤25 kDa proteins) was used for detection of HPVE6/E7 proteins. The membranes were blocked in 5% BSA (prepared in 0.1% Tween-20 in Tris-Borate Saline) and probed with specific antibodies in a probing-stripping-reprobing cycle**.** Upper blot was first incubated overnight at 4°C in the pre-standardized dilution of primary antibody against pSTAT3(Y705), and subsequently re-probed with anti-STAT3, anti-JunB, anti-c-Jun, and β-actin. Similarly, lower blot was incubated first with HPV16/18 E6 and subsequently re-probed with either HPV16 E7 or HPV18 E7 depending upon the cell type. Antibodies and their specific dilution in the blocking solution used in different assays are described in **Supplementary Table S1**. Absence of leftover signal following stripping was ascertained before the next reprobing cycle. During each probing, blots were washed, incubated with horse reddish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG secondary antibodies and visualized by Luminol detection kit (Santa Cruz Biotech, United States) under Amersham Imager 600 (GE Life Sciences ABI, Sweden). The western blot membranes were stripped at each interval using mild stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20 pH-2.2) for 15 min at room temperature followed by

re-blocking. β-actin expression was used as an internal control. The quantitative densitometric analysis of the bands was performed using ImageJ software (NIH, United States).

Immunocytochemistry and Fluorescence Microscopy

Subcellular localization of various proteins was determined by ICC as described earlier (Bharti et al., 2003) with minor modifications. CaCx cells were seeded on coverslips in 6 well plates at a density of 5,000 cells/well. After overnight incubation, the cells were treated. When the treatment got over, the medium was removed, and cells were rinsed with 1X PBS for 3 times. Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in 1X PBS. Cells were blocked with 5% BSA in 1X PBS for 1 h. Cells were incubated with primary antibodies (**Supplementary Table ST1**) for 3 h followed by incubation with fluorescently tagged secondary antibodies for 1 h. Counter staining was done with DAPI at a concentration of 50 ng/ml. Finally, the coverslips were mounted on a microscope slide with Fluor mount as mounting media. Preparations were visualized using a ZEISS ImagerZ2 microscope. Fluorescence intensity analyses were performed using ImageJ software (U.S. National Institutes of Health).

In Silico Molecular Docking of Phytochemicals to HPV16 E6 Molecule

Preparation of HPV16 E6 Structures

Two recent protein structures of HPV16 E6 i.e. chain B of 6SJA (aa7-158; resolution 1.5 Å) that represents native conformation of E6 (non-p53 interacting) (Suarez et al. 2019) and chain F of 4XR8 (aa8-158; resolution 2.25 Å) that represents p53-interacting conformation of E6 (Martinez-Zapien et al. 2016) were downloaded from RCSB PDB website (http://www.rcsb.org/pdb/home/home.do). The analysable part of Chain B (aa7-aa140) and chain F (aa7-aa143) corresponding to aa14 -aa147 and aa14 -aa150, respectively in UniProtKB Reference sequence for E6 P03126 were used for analysis. Chain B of 6SJA differed from reference protein at position 54 as it had ARG instead of PHE. The original crystal structures were processed in PyMOL to remove non-E6 components of the structure and chain B of 6SJA and chain F of 4XR8 structure were isolated. Chain B of 6SJA and chain F of 4XR8 structure were independently used to dock phytochemical ligands. Apart from RCSB PDB database, Online file format converting tools (https://cactus.nci.nih.gov/translate/), NCBI (https://www.ncbi.nlm.nih.gov/), Online Alignment Tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and softwares AutoDock Tools 1.5.6 (ADT) (http://autodock.scripps.edu/resources/adt), PyMOL (https://www.PyMOL.org/) and Discovery Studio (http://accelrys.com/products/collaborative-science/biovia-discoverystudio/visualization-download.php) were used in present study. The protein structures were processed before docking by ADT. The water molecules were deleted, non-polar hydrogens added into carbon atoms of the E6 molecule and Kollman charges were also assigned. Gasteiger charges were assigned and torsions degrees of freedom were allocated by ADT.

Preparation of Ligands

The phytochemicals likely to be present in the root of *Berberis aquifolium* were identified from Dr. Duke's Phytochemical and Ethnobotanical database (USDA 1992–2016). The molecular structures of the phytochemicals were retrieved from NCBI PubChem and converted to. pdb format using Online SMILES Translator and Structure File Generator. For each docking, the central atom and number of active bonds in ligand were detected and saved as. pdbqt file.

Molecular Docking

E6 structure and ligand molecules to be used for docking were defined, and the search space (Grid box) was specified using AutoGrid (part of the AutoDock package) around E6 molecule for a blind docking. A grid of 126, 126, and 126 points in the x, y, and z directions and the whole E6 molecule was centred by running the AutoGrid. Protein-ligand docking studies were performed using the AutoDock 4.2 program. The Genetic Algorithm was applied to model the interaction pattern between the E6 protein and the phytochemicals. For all docking procedures, 10 independent genetic algorithm runs with a population size of 150 were considered for each molecule under study. A maximum number of 2.5×10^5 energy evaluations, 27,000 maximum generations, a gene mutation rate of 0.02, and a crossover rate of 0.8 were used. AutoDock was run in order to prepare corresponding docking log file (.dlg) for further analysis. The E6 as rigid molecule, ligand and docking parameters were defined and AutoDock4 was launched. The resultant log file was then used for further deriving the information pertaining to Binding Energies (BE) and Inhibition Constants (IC) of different conformations. The macromolecule and ligand complex was saved in. pdb format to be analyse in PyMOL.

Visualization

The visualization of structure files was done using the graphical interface of the ADT tool and the PyMol molecular graphics system and Discovery Studio Visualizer.

Statistical Analysis

The data analysis was performed using the Microsoft Excel and IC50 was calculated using Prism (GraphPad). All cell culture experiments were carried out at least in 3 independent runs. Statistical significance of difference between the 2 test groups was analysed by the Student's *t*-test and multiple comparisons versus control group was assessed by analysis of variance. In all cases, p value ≤ 0.05 was considered to be significant. The distribution of data set was normalized using Shapiro-Wilk normality test ($p > 0.05$). Further, we performed One way ANOVA and post hoc test for multiple comparisons.

RESULTS

BAMT Induced a Dose-dependent Decline in CaCx Cell Viability

To assess the anti-cervical cancer response, cultures of CaCx cell lines, C33a, SiHa and HeLa were incubated with increasing

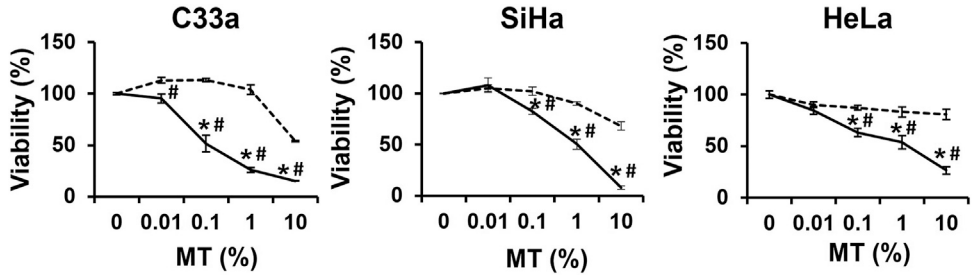

FIGURE 1 | Effect of *Berberis aquifolium* mother tincture (BAMT) on the viability of different CaCx cell lines. Each panel shows percent cell viability of different HPV negative (C33a) and HPV positive (SiHa and HeLa) CaCx cell lines treated with increasing concentrations (0.01, 0.1, 1 and 10%) of MT of *B. aquifolium* at 24 h. The cell viability was measured by MTT assay as described in methods. Cells were similarly treated with succussed ethanol strength for MT- 70%) at corresponding concentration of alcohol content in the drug as alcohol/vehicle control. The results are representative of three independent experiments with similar results, data represent mean ± SD with $^{*}p$ values ≤ 0.05 with respect to control. $^{\#}p$ values ≤ 0.05 compared to cultures treated with corresponding concentrations of vehicle control. Solid lines represent drug and broken lines represent vehicle control.

dilutions of the drug (BAMT) for 24 h and the cell viability was evaluated by MTT assay. The BAMT-treated CaCx cell cultures, irrespective of their HPV status, showed a dose-dependent loss of cell viability >**Figure 1**). The cytotoxic response was also observable at higher dilutions (0.01 and 0.1%) in all the cell types and showed a distinct difference from the cytotoxicity displayed by the VC (IC50: C33a (BAMT–0.17% vs. VC–9.09%), SiHa (BAMT–0.65% vs. VC–14.12%) and HeLa (BAMT–0.67% vs. VC–17.28%)).

BAMT Induces Cell Cycle Arrest at G0/G1 Checkpoint in CaCx Cells

Growth inhibitory effect of BAMT was investigated further to assess the distribution of cells in different phases of the cell cycle using flow cytometry. C33a SiHa and HeLa cells treated with BAMT (1 and 10%) or corresponding VC for 24 h showed a decline in the number of cells in S and G2/M phase and an equivalent increase in G0/G1 phase of the cell cycle (**Figure 2A**). Evaluation of changes in cell distribution in G1 phase in different experiments revealed a small but statistically significant increase in G0/G1 phase (**Figure 2B**). These differences were perceivable in live adherent cells at both the concentrations of BAMT tested (1 and 10%) as compared to cells treated with corresponding VC.

BAMT Downregulated Expression of Oncogenic Transcription Factors in CaCx Cells

Next, we examined the level of transcription factors, STAT3 and AP-1 proteins, JunB and c-Jun in BAMT-treated CaCx cells. BAMT-treated cells irrespective of their infecting HPV genotype showed a dose-dependent decline in the level of pSTAT3(Y705) and STAT3 (**Figure 3**). However, it was intriguing to note that overall STAT3 level that included non-phosphorylated STAT3 declined sharply in contrast to pSTAT3. Though, cells treated with VC also showed a similar dose-response with reduced pSTAT3/STAT3 but the reduction was of a lower magnitude. The pSTAT3/STAT3 levels of treated HeLa cells were erratic and decline of pSTAT3/STAT3 levels were seen at BAMT (5%). BAMT-treated SiHa cells, on the other hand, failed to show significant changes in JunB and c-Jun level with respect to untreated or VC-treated cells and the response was inconsistent, whereas a specific decline in JunB and c-Jun was recorded for BAMT-treated HeLa cells.

Effect of BAMT on Nuclear Localization of Oncogenic Transcription Factors in CaCx Cells

BAMT-treated SiHa cells immune-stained for different transcription factors were subjected to fluorescence microscopy to visualize the localization of these transcription factors within the cell and their translocation into the nucleus (**Figure 4**). BAMT-treated SiHa cells showed dose-dependent decline in overall pSTAT3 and STAT3 staining. Both pSTAT3, STAT3 staining in cytoplasm significantly declined, whereas a relatively-reduced but still prominent staining was detected in the nuclei. A concomitant reduction in the nuclear size of many of the live cells in culture was noted in some microscopic fields. Strong positivity of JunB in the nuclei and a diffused positivity of c-Jun well distributed in cytoplasm and nuclei was detected in control cells. Following BAMT-treatment, a marked decline in JunB and c-Jun was noted (**Figure 4C**). However, there was no notable re-distribution of these AP-1 components was observed in BAMT-treated cells at any strength of drug tested (**Figures 4A,B**).

BAMT Reduced the Expression of HPV Oncoproteins E6 and E7 in CaCx Cells

To assess the downstream effect of BAMT on level of viral oncoproteins in treated cells, the lower part of the membranes derived from immunoblotting experiments with proteins ≤25 kDa were used to measure transcription factor

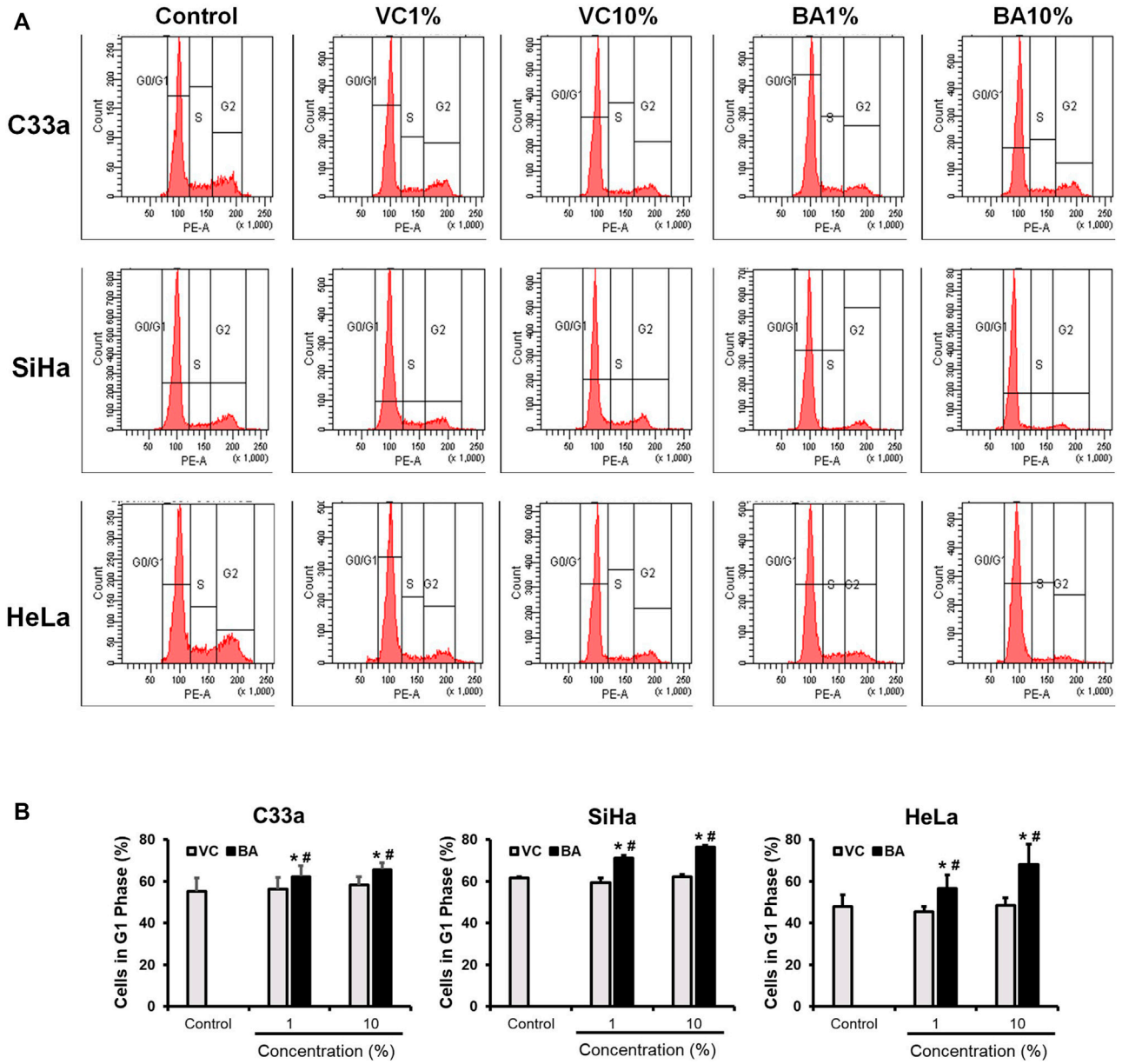

FIGURE 2 | Effect of BAMT on distribution of cervical cancer cells in different phases of the cell cycle. **(A)** Representative flowcytometric analysis showing histograms of propidium iodide-stained nuclei from BAMT-treated CaCx cells. Cell were gated for G1, S and G2/M phases. **(B)**. Cumulative analysis of total cell population in G1 phase after treatment with BAMT or alcohol. Data represent mean ± SD with $^{*}p$ values ≤ 0.05 with respect to control. $^{\#}p$ values ≤ 0.05 compared to cultures treated with corresponding vehicle control (VC).

expression were probed for HPV16/18 E6 and E7 (**Figures 5A,B**). A dose-dependent decline of both HPVE6 and E7 proteins in SiHa and HeLa cells was noted, but the response achieved statistical significance only at higher concentrations (2.5 and 5%). The inhibition of E6 and E7 expression was stronger for HeLa cells and was only marginally better than the VC in SiHa cells. Immunocytochemical staining of SiHa cells further confirmed the loss of E6 and E7 proteins (**Figure 5C**). However, the nuclear positivity of E6 and E7 was not affected in BAMT-treated cells.

Anti-Oncogenic Potential of Phytochemicals Presents in BAMT on HPV E6 Activity

The disparity between strong inhibitory effect of BAMT and moderate effect on HPV16 E6 and E7 was investigated further

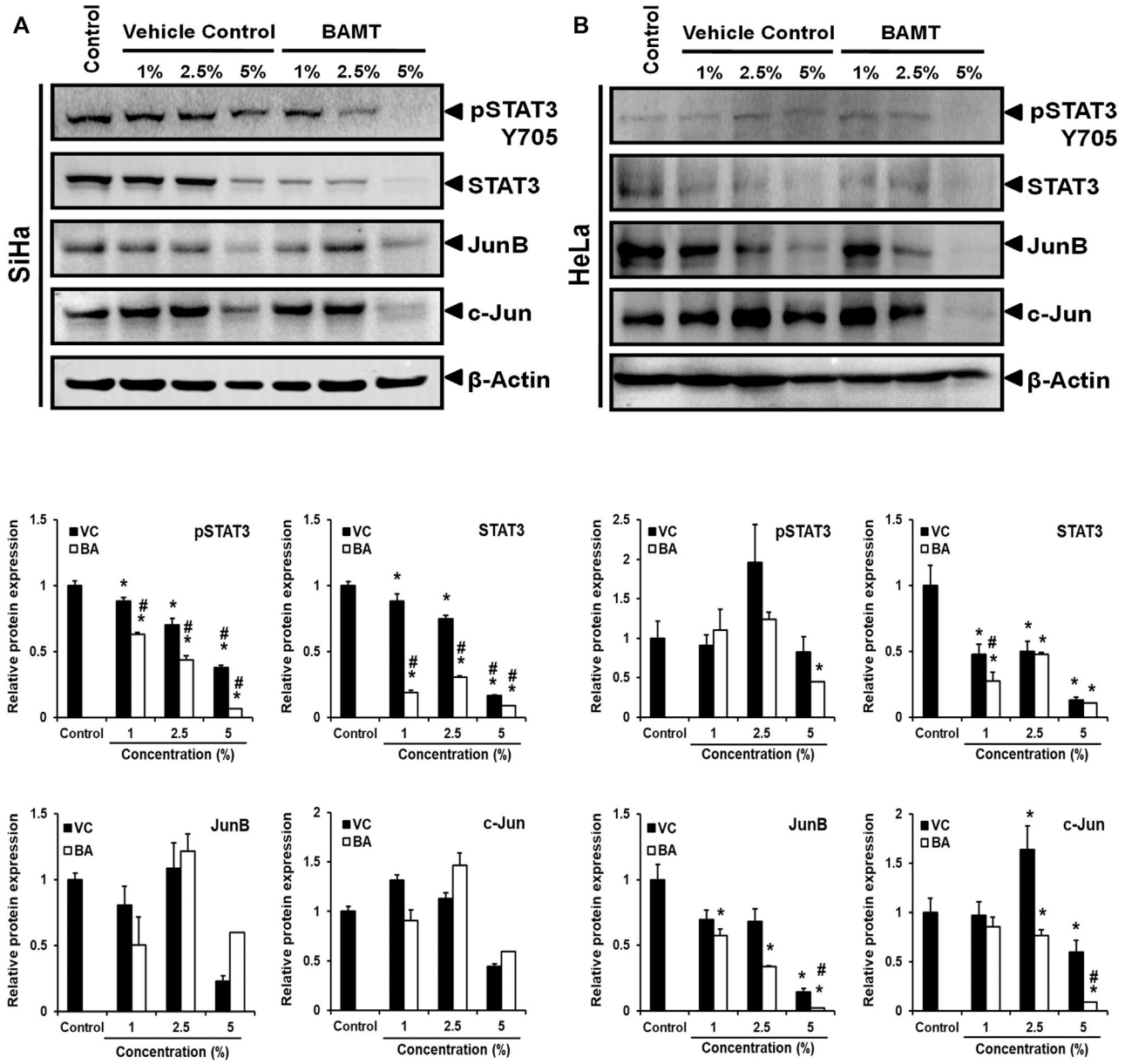

FIGURE 3 | Effect of BAMT on expression of pSTAT3, STAT3 and AP-1 components, JunB and c-Jun. **(A)**. Representative immunoblots of total cellular proteins (40 μg/lane) isolated from BAMT-treated SiHa **(A)** or HeLa cells **(B)** separated on 10% SDS-PAGE were transferred on PVDF membrane and first probed for pSTAT3(Y705), and then reprobed for STAT3, JunB, c-Jun and β-actin **(upper panels)**. Graphs **(Lower panels)** show aggregated normalized fold change in band intensities of pSTAT3, STAT3, Jun B and c-Jun obtained from densitometric analysis. Values are means ± S.D. (indicated as error bars) of the three independent measurements. *p value <0.05 vs. control. #p value <0.05 vs. vehicle control (VC).

using *in silico* molecular docking approach. Phytochemicals reportedly present in root of *B. aquifolium* root were downloaded and a blind molecular docking was performed against two most recent high-resolution crystal structures of HPV16 E6 present in PDB, 6SJA (chain B) and 4RX8 (chain F) **Supplementary Figures SF2A, SF2B**. Molecular docking characteristics of each phytochemical and the interacting amino acid residues with potential binding partners in the HPV16 E6 binding pocket were identified. The carcinogenically-relevant amino acid residues were determined using reported key interacting motifs and participating amino acid residue for p53, E6AP, PDZ, and Zn-binding domain (**Supplementary Table ST2**). Among the top 10 confirmations obtained for each phytochemical from docking experiments (**Supplementary Tables ST3, ST4**), only the best-ranked docking conformation with lowest

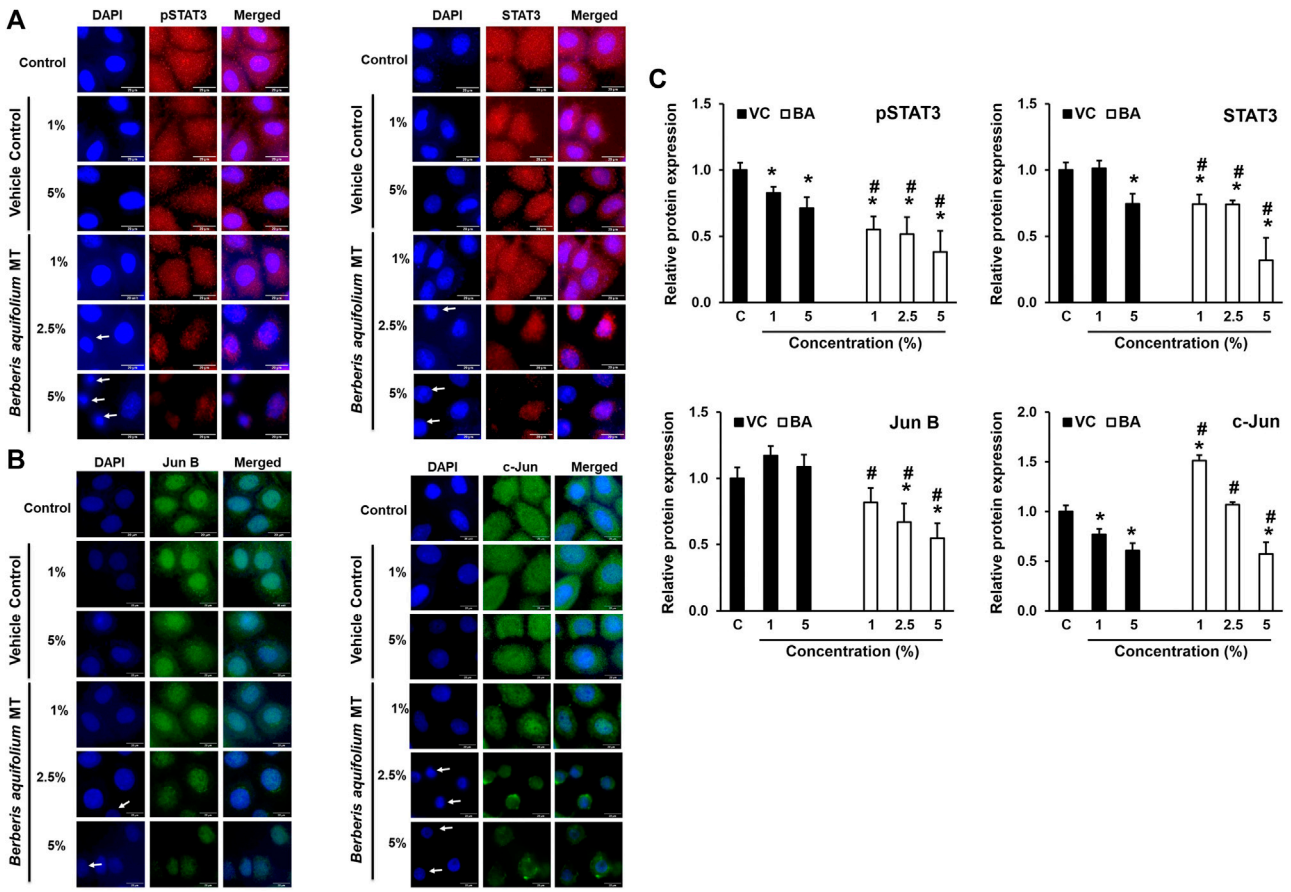

FIGURE 4 | Localization of pSTAT3, STAT3, JunB and c-Jun in BAMT-treated SiHa cells. Representative fluorescence photomicrographs showing distribution of indicated transcription factors in BAMT-treated and control cells by immunocytochemistry. Cells were fixed, stained with respective antibodies and detected with goat anti-mouse IgG-Alexa-594 (for pSTAT3Y705 and STAT3; Red; **(A)** and goat anti-rabbit-IgG-Alexa-488 (Jun B and c-Jun; Green; **(B)** and counterstained with DAPI (Blue). Arrow indicates nuclei with reduced size. The expression level was measured by ImageJ and plotted as mean ± SEM of the representative experiment out of three independent experiments **(C)**. Values were obtained by analyzing at least 30 cells per specimen. *p value < 0.05 vs. control. #p value < 0.05 vs. vehicle control (VC).

binding energy are described in **Table 1** Berberine showed a strong interaction with 6SJA but showed a moderate binding to 4XR8 structure. Notably, magnoflorine and palmatine showed a stronger specific interaction with E6 than berberine and manifested high negative binding energies and low inhibition constants (**Figure 6A**). Most of the interactions involved either p53 interacting pocket (columbamine), or E6AP interacting pocket of E6 (obamegine and aromoline), or both (berbamine, palmatine, jatrorrhizine, magnoflorine, oxyacanthine) with different affinities (**Table 1**). In case of E6 conformation 4XR8, most of the interactions involved amino acid residues that were common to p53 and E6AP binding pockets, though the corresponding negative binding energies were comparatively less than their respective interaction in E6 (6SJA) (**Table 1**). Cumulative analysis of all interacting amino acid residue revealed, L100 and R131 as the most targeted residues of E6 in both the structures that participated in interaction with p53 and E6AP, respectively (**Figure 6B**).

DISCUSSION

In this report, we show the presence of anti-CaCx and anti-HPV activity in the *B. aquifolium*-based herbal preparation. BAMT induced a dose-dependent decline in the viable CaCx cells irrespective of their HPV status. Remnant BAMT-treated cells showed G1 phase growth arrest. BAMT induced a decline in STAT3, JunB and c-Jun, however, their nuclear localization was not affected. Reduction in STAT3/pSTAT3 level was more consistent in SiHa cells, whereas, alterations in AP1 members were consistent in HeLa cells suggesting different targets/mechanisms of inhibition. Nevertheless, a decline in HPV E6/E7 expression in CaCx cells irrespective of the cell type was noted in association with loss of the transcription factors in BAMT-

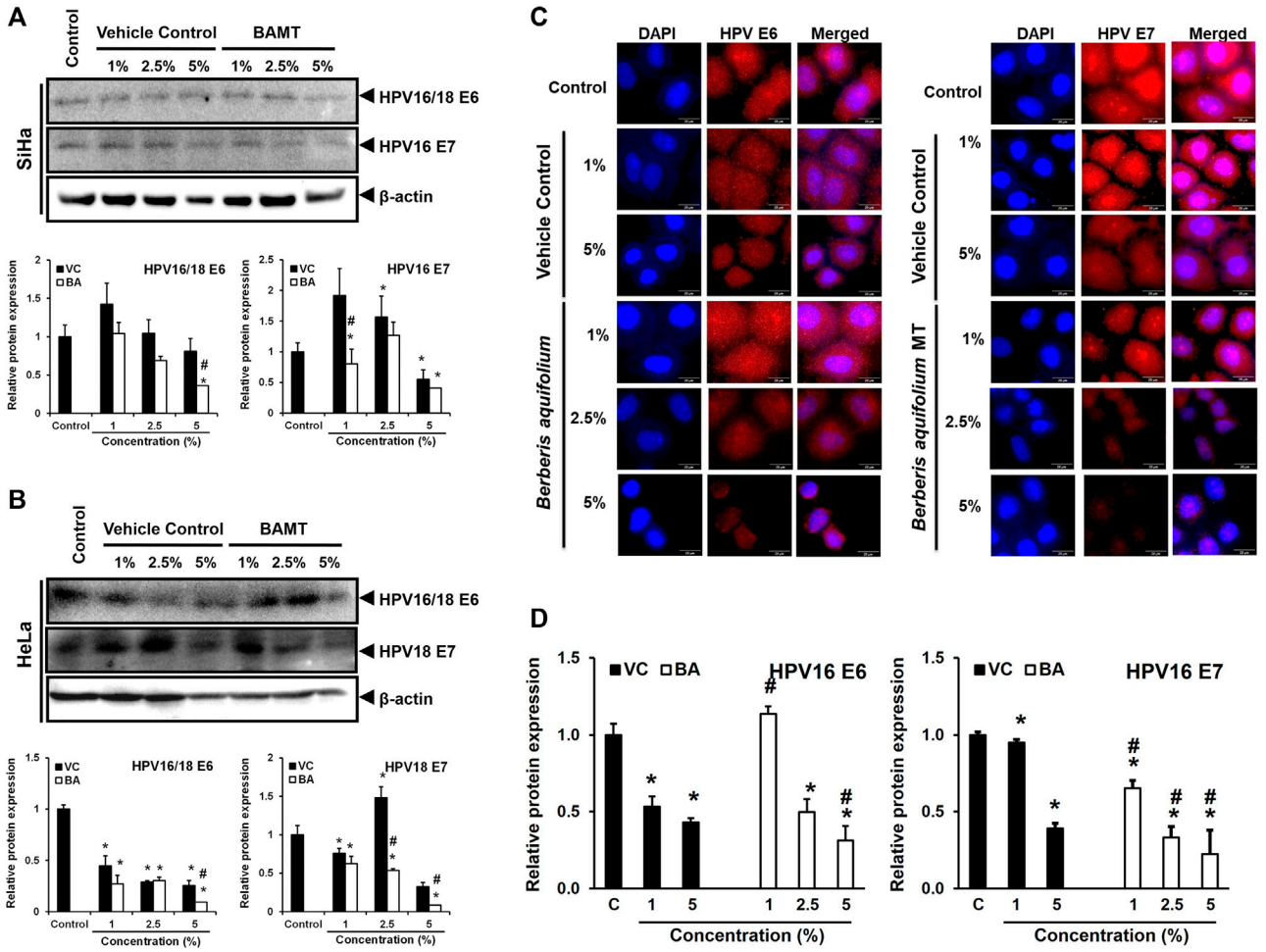

FIGURE 5 | Effect of BAMT on expression of HPV oncoproteins E6 and E7 and localization. A. and B. Lower blot (with ≤25 kDa proteins) of the representative experiment shown in **Figure 3**. BAMT-treated SiHa **(A)** or HeLa cells **(B)** were first probed for HPV E6, and then re-probed for HPV E7 **(upper panels)**. *β-actin of corresponding upper blot is reproduced for comparison. Graphs **(Lower panels)** show aggregated normalized fold change in band intensities of HPV E6 and HPV E7 obtained from densitometric analysis. Values are means ± S.D. (indicated as error bars) of the three independent measurements. **C. and D.** Representative fluorescence photomicrographs showing distribution of HPV16 E6 and E7 in BAMT-treated and control SiHa cells by immunocytochemistry **(C)**. Cells were fixed, stained with HPV16/18 E6 and HPV16 E7 antibodies and detected with goat anti-mouse IgG-Alexa-594 (Red) and counterstained with DAPI (Blue). The expression level was measured by ImageJ and plotted as mean ± SEM of the representative experiment out of three independent experiments **(D)**. Values were obtained by analyzing at least 30 cells per specimen. *p value < 0.05 vs. control. #p value < 0.05 vs. vehicle control (VC).

treated cells. Using *in silico* molecular docking we show for the first time presence of anti-HPV activity in phytochemicals of BAMT that could strongly interfere with the normal function of HPV16 E6 to bind p53 and E6AP. We identified leading BAMT-constituent molecules showing highest negative free energy for cooperative binding to HPV16 E6.

The cell viability study demonstrated that anti-CaCx activity reported in berberine (Jantova et al., 2003; Mahata et al., 2011; Chu et al., 2014; Saha and Khuda-Bukhsh 2014), is well represented in BAMT. Early studies showed *B. aquifolium* constituents to be effective in preventing cell growth of human keratinocytes during psoriasis (Muller et al., 1995), a pro-inflammatory hyperproliferative disease. However, direct effect of BAMT on cervical cancer has not been reported. In a study where HeLa cells were treated with *B. aquifolium* ethanolic extracts that resembled BAMT, showed similar growth inhibition with comparable IC_{50} values (Damjanović et al., 2016). Interestingly, berberine specifically targeted HPV-positive CaCx cells (Mahata et al., 2011), whereas, BAMT could prevent the growth of both HPV-positive and HPV-negative CaCx cells. The reasons behind broader spectrum of BAMT's response are not known. Besides berberine, ethanolic extracts of *B. aquifolium* root are known to contain high concentrations of other metabolites like berbamine, palmatine, hydrastine, jatrorrhizine, oxyacanthine, columbamine, obamegine, and aromoline (Godevac et al., 2018, USDA 1992–2016) that could possibly contribute to the anti-cancer effects of BAMT. BAMT has been reported to contain high

TABLE 1 | Molecular docking characteristics of various phytochemicals reportedly present in root of *B. aquifolium* with the 3D crystallographic structure of HPV16 E6 (6SJA and 4XR8) available on PDB and their interacting amino acid residues with potential binding partners in the HPV16 E6 binding pocket.

S. No	Ligand name[a]	Activities reported	Nature	PubChem CID	6SJA (chain B) (best ranked docking conformation)				4XR8 (chain F) (best ranked docking conformation)			
					BE (kcal/mol)	IC (μM)	AA residues[c]	Known E6 interacting partner(s)	BE (kcal/mol)	IC (μM)	AA residues[c]	Known E6 interacting partner(s)
1	Berberine	130	Alkaloid	CID 2353	−6.6	13.7	L50, *C51*, A61, V62, L67, S71, *S74*, *R102*, *R131*	*E6AP*	-5.1	193.4	L99, **L100**, G130, *R131*	**p53,** *E6AP*
2	Berbamine	38	Alkaloid	CID 275182	−6.1	31.7	*K11*, **D49**, S97, S98, L99, **L100**, P109, L110, S111, *K115*	**p53,** *E6AP*	-3.9	1,460	***R10***, D98, L99, **L100**, *R131*	**p53,** *E6AP*
3	Palmatine	17	Isoquiniline alkaloid	CID 19009	−6.7	12.6	L99, **L100**, I101, G130, *R131*, W132	**p53**,*E6AP*	-5.7	66.4	**F47**, **D49**, L50, *C51*, **L100**, *R102*, G130, *R131*, W132	**p53,** *E6AP*
4	Hydrastine	16	Alkaloid	CID 197835	−4.5	476.2	F45, L50, *C51*, V62, L67, *R102*	*E6AP*	-4.0	1,220	*K11*, P13, **Q14**, A46, **F47**, **D49**	**p53,** *E6AP*
5	Jatrorrhizine	13	Protoberberine alkaloid	CID 72323	−6.0	40.9	**L100**, *R131*, W132	**p53**, *E6AP*	-5.4	110.2	**Q6**, ***R10***, **Q14**, **E18**	**p53**, *E6AP*
6	Magnoflorine	11	Apophine alkaloid	CID 73337	−7.1	6.5	Y92, **L100**, *R102*, *R131*, W132	**p53**, *E6AP*	-5.6	80.9	**Q6**, ***R10***, **Q14**, **E18**	**p53**, *E6AP*
7	Oxycanthine	10	Alkaloid	CID 442333	−6.1	36.7	**L100**, G130, *R131*	**p53**, *E6AP*	-3.9	1,500	D98, L99, **L100**	**p53**
8	Columbamine	7	Berberine alkaloid	CID 72310	−6.4	21.8	L12, P13, C16, I23, A46, R47,**D49**	**p53**	-5.2	144.3	L99, **L100**, *R102*, *R131*, W132	**p53**, *E6AP*
9	Obamigine	4	Phenolic base	CID 441064	−5.6	83.9	*R129*, G130, *R131*	*E6AP*	-4.2	831.3	*K11*, P13, A46, **F47**, **D49**	**p53**, *E6AP*
10	Aromaline	2	Alkaloid	CID 362574	−6.2	30.5	*R129*, *R131*	*E6AP*	-4.1	1,020	K94, D98, L99, **L100**, *R131*, W132	**p53**, *E6AP*

[a]*From Dr. Duke's Phytochemical and Ethnobotanical database (USDA 1992–2016)*

[b]*High negative binding energy is indicative of non-specific interaction.*

[c]*Bold amino acid residues correspond to p53 binding pocket, italic amino acid residue corresponds to E6AP-binding pocket. BE: binding energy, IC: inhibitory constant.*

alkaloid content equivalent to 5.5 mg/ml of berberine but a net content of berberine is only 0.5 mg/ml (Ungurean et al., 2018). Therefore, the resultant effect seen in BAMT-treated CaCx cells could be multifactorial and could be contributed by phytochemicals other than berberine. Indeed, many phytochemical constituents like berbamine (Wang et al., 2009), palmatine (Hagiwara et al., 2015; Liu et al., 2020), hydrastine (Guo et al., 2016), jatrorrhizine (Liu et al., 2013), magnoflorine (Sun et al., 2020; Wei et al., 2020), and columbamine (Bao et al., 2012) have displayed anti-cancer properties in different tumor models.

Induction of cell death is a major contributor to reduced cell growth in BAMT-treated cells as observed in the MTT assay. We were particularly interested in cell cycling state of BAMT-treated cells, which survived BAMT and remained attached in cultures. These cultures showed a small but significant increase in proportions of CaCx cells in G1-phase in all cell types tested. Berberine is known to induce cell cycle arrest predominantly in G1-phase (Eom et al., 2008; Yan et al., 2011; Puthdeei et al., 2017; Gu et al., 2020). However, G2/M arrest in berberine-treated cancer cells is also reported (Lin et al., 2006; Mantena et al., 2006; El Khalki et al., 2020) and the phenomenon was found to vary with berberine's dose and the cell type. Interestingly, when berberine is used to treat Hela cells, G1 growth arrest was noted (Gu et al., 2020). Apart from berberine, berbamine (Zhang et al., 2018), jatrorrhizine (Liu et al., 2013) and hydrastine (Guo et al., 2016) are reported to induce G1 arrest, whereas, columbamine (Bao et al., 2012), palmatine (Liu et al., 2020) and magnoflorine (Sun et al., 2020) induced cell growth arrest in G2/M phase in different *in vitro* cell culture models. Despite variations in mechanisms of cell cycle arrest, our study shows that BAMT-induces G1 growth arrest that could be the resultant effect of multifactorial inhibition of cellular growth on different

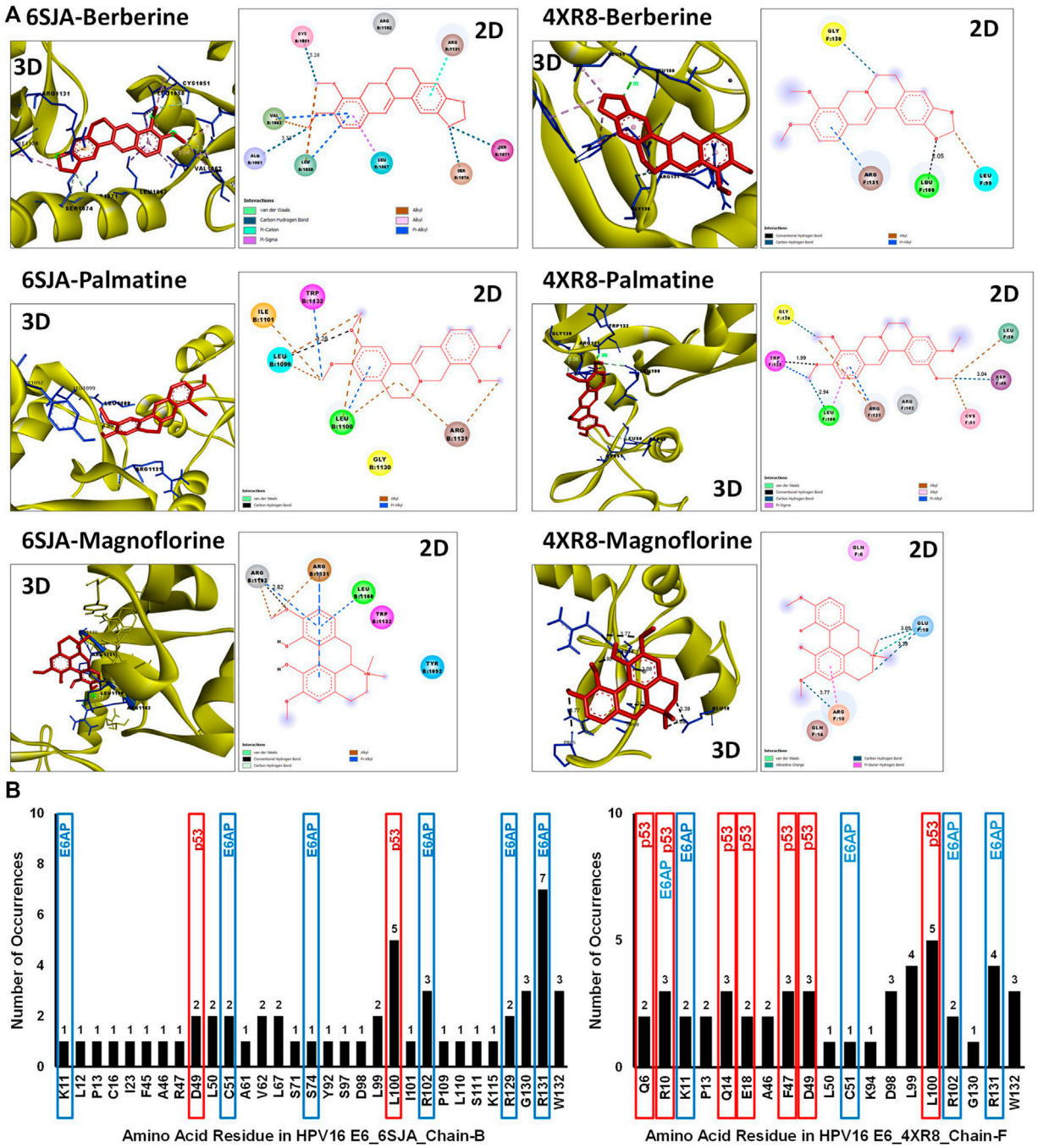

FIGURE 6 | Molecular docking of key phytochemicals reported in *B. aquifolium* root with HPV16 E6 and its impact of their binding on E6's carcinogenic interaction. **(A)**. Best ranked docking pose (3D) and a 2D simplified representation of the binding pockets of three best leads, berberine, palmatine and magnoflorine, in association with HPV16 E6 (based on 6SJA (**left panel**) or 4XR8 (**right panel**) crystal structure) showing interacting residues and non-covalent interactions. The hydrogen bonds are shown as dashed lines. **(B)**. Graphical representation of the cumulative data showing number of times an amino acid residue of HPV16 E6 (6SJA (**left panel**) or 4XR8 (**right panel**)) appeared in the binding pocket of different phytochemical ligands examined. The residues identified in molecular docking that are involved in key carcinogenic interactions with p53 or E6AP are highlighted with different colored boxes.

checkpoints, which could be less toxic as predicted earlier for other phytochemicals (Singh et al., 2002).

Our data revealed for the first time that BAMT-induced alterations in expression of STAT3 and AP-1 that control expression of viral oncogenes E6 and E7 and thereby contributing to BAMT's anti-CaCx activity. Notably, STAT3 inhibition was prominent in SiHa, whereas AP-1 inhibition prevailed in HeLa cells. BAMT-could not block the upstream

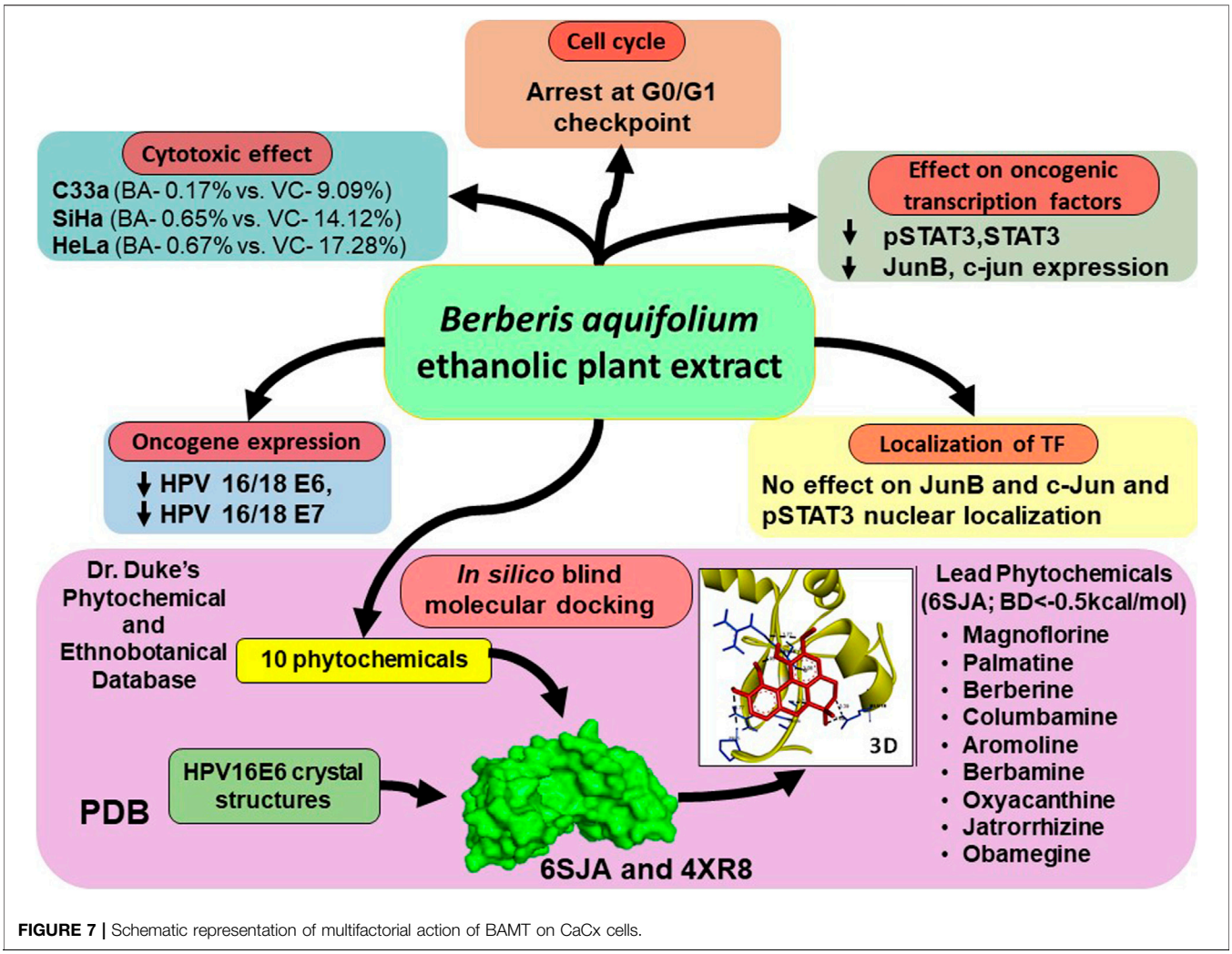

FIGURE 7 | Schematic representation of multifactorial action of BAMT on CaCx cells.

signalling that would have resulted in their cytoplasmic retention. This shows that BAMT exerted its inhibitory effects by different mechanisms in different cell lines. Though, the mechanisms by which BAMT led to loss of STAT3/pSTAT3 or JunB/c-Jun, are currently not known, but can be attributed to the constituent phytochemicals. We earlier showed that berberine inhibits AP-1 and STAT3 (Mahata et al., 2011; Pandey et al., 2015), however, in those studies berberine targeted the activation state of these transcription factors. A number of studies showed a decline in pSTAT3(Y705) levels and STAT3 activity that were stronger than the effect on total cellular levels of STAT3 (Pandey et al., 2015; Zhu et al., 2015; Puthdee et al., 2017). These studies showed moderate effect of total STAT3 pools in the berberine-treated cells. Similar targeting of STAT3 signaling was reported with berbamine (Hu et al., 2019), and columbamine (Bao et al., 2012). The reasons underlying enhanced degradation of unphosphorylated STAT3 compared to the pSTAT3 pool that usually get translocated to the nucleus in BAMT-treated cells is not known presently. However, a calcineurin-mediated enhanced proteasomal degradation of cytoplasmic STAT3 is expected as reported earlier (Murase 2013). Berbamine was shown to increase intracellular calcium, which activates calcineurin.

Contrary to the BAMT's effect on STAT3, two key components of AP-1 signaling, JunB and c-Jun were severely affected in BAMT-treated cells. Earlier we showed berberine inhibited only c-Jun level particularly in HeLa cells (Mahatai et al., 2011). Here, BAMT also downregulated c-Jun in HeLa cells. However, in BAMT-treated SiHa cells the alteration in c-Jun was not significant. The composition of AP-1 complex varies with cell lines (Mahata et al., 2011), Under normal conditions, SiHa cell line shows AP-1 complex with c-Fos, JunB, and JunD, while in HeLa cells c-Jun participates actively in the AP1 complex making the underlying variation a cause of differential response. In contrast to berberine, magnoflorine was shown to induce AP-1 signalling by enhancing JNK activity in a different cell type (Sun et al., 2020). Nevertheless, BAMT induced loss of expression of key components of transcriptional machinery that translated into their reduced availability to promote oncogenic transcription. Further, we report here a decline in the expression of viral oncoproteins E6 and E7 following BAMT treatment. These observations are similar to our previous study on berberine

(Mahata et al., 2011). However, the extent of inhibition was relatively lesser and did not match to cytotoxic action of BAMT. Moreover, nuclear localization of the oncogenes was noted despite the loss of their gene expression. Nuclear localization of HPV16 E6 due to 3 nuclear localization signals present on this protein has been reported earlier (Tao et al., 2003). This suggests that there could be alternate mechanisms other than targeting expression of the viral oncogenes that are exerted by BAMT in inducing growth arrest and cell death in CaCx.

To address the disparity, in the next part of the investigation we examined potential interaction of BAMT constituents with HPV16 E6 by molecular docking experiments to evaluate their cooperative binding. Blind docking of these phytochemicals on two latest HPV16 E6 crystal structures available on PDB (6SJA and 4XR8) revealed a strong interaction of berberine, magnoflorine and palmatine to E6 and the binding pocket overlapped aa residues reportedly involved in E6's interaction with p53 and E6AP (Zanier et al., 2013; Martinez-Zapien et al., 2016). The results were consistent irrespective of inherent structural differences due to their co-crystallized chains, where 4XR8 showed a conformation with slightly lower resolution (2.55 Å vs. 1.50 Å) compared to 6SJA, was co-crystallized with p53 (Martinez-Zapien et al., 2016). On the other hand, 6SJA co-crystallized with IRF3 LxxLL motif and possibly represented p53 unbound form (Suarez et al. 2019). Global alignment of the two E6 sequences show 98% identity with 2 gaps and replacement of arginine at 49th position of 6SJA with phenylalanine at 47th position of 4XR8. Both the structures had mutations 4 cysteine residues that interacted with Zn. For the present investigation, we specifically used GA algorithm in AutoDoc4. GA algorithm is helps differentiate between false positives and true negatives from the clusters as it gives more pose (conformers) and hence better than LGA, which is frequently used recently by some of the investigators (Kumar et al., 2015; Kolluru et al., 2019; Nabati et al., 2020). Although the leads from docking experiments are extremely valuable to support BAMT's potential action on E6's physiological activity, detailed wet-lab experiments are essentially needed to validate these results in the experimental setup. Though it is tempting to speculate that similar inhibitions may also be observed in other HR or LR-HPV E6 due to structural similarity, the current finding cannot be used to extrapolate/predict the same for HR/LR-HPV types.

SUMMARY AND CONCLUSION

Phytochemicals and herbal preparations are globally recognized as the second line of therapy after allopathy. These medicines are economic and there are no known side effects of the therapy. Taking these aspects into consideration, present study demonstrates a multifactorial action of BAMTon CaCx cells. BAMT induced cell death and G1 growth arrest in CaCx cells irrespective of their HPV status. Molecularly, these phytochemicals targeted oncogenically-relevant transcription factors of STAT3 and AP-1 family that resulted in loss of oncoprotein expression. Among various constituents, berberine, magnoflorine and palmatine were found capable of targeting E6 functions by strong cooperative binding **Figure 7**. Collectively, these leads show that commercially-available BAMT can be an effective and economic broad-spectrum therapeutic option against cervical cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR'S CONTRIBUTION

TS: Conceptualization; Data curation; Investigation; Methodology; Software; Validation; Visualization; Roles/Writing—original draft. AC, KT, NA, PP, JY, TT, MJ, and AB: Formal analysis; helped in review—original draft. PG and AK; Formal analysis; Resources; Writing—review and editing. ACB: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing—original draft; Writing—review and editing. All authors have read and approved the final manuscript.

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CASE REPORT
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Case Report: Collapsing Focal Segmental Glomerulosclerosis After Initiation of Ado-Trastuzumab Emtansine Therapy

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Ado-trastuzumab emtansine (T-DM1) is an antibody–drug conjugate consisting of the monoclonal antibody trastuzumab linked to the maytansinoid DM1 with potential antineoplastic activity and is approved for human epidermal growth factor receptor 2 (HER2)-positive breast cancer. An analysis of the US Food and Drug Administration (FDA) Adverse Event Reporting System identified 124/1,243 (10%) renal adverse events for trastuzumab. However, there are no published case reports describing kidney biopsy findings related to nephrotoxicity of either trastuzumab or T-DM1. We report kidney biopsy findings in a case of nephrotic range proteinuria due to collapsing focal segmental glomerulosclerosis (FSGS) and tubular injury after initiation of T-DM1 therapy. After systematic exclusion of other causes, it is likely that the observed collapsing FSGS was associated with the prior initiation of T-DM1 therapy. This is further supported by the clinical course with improvement of proteinuria and kidney function 3 weeks after discontinuation of T-DM1 therapy without further specific treatment. In summary, we provide the first report of kidney biopsy findings in a case of nephrotic range proteinuria after initiation of T-DM1 therapy due to collapsing FSGS. This issue is especially relevant since T-DM1 is widely used, and nephrologists have to be aware of this potentially rare but severe complication.

Keywords: focal segmental glomerulosclerosis, collapsing FSGS, tubular injury, ado-trastuzumab emtansine, T-DM1, acute kidney injury, proteinuria, nephrotic syndrome

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INTRODUCTION

The association between kidney disease and cancer has long been recognized but has only recently received full attention as a nephrological subspecialty, called onconephrology (1). Cancer patients may develop a variety of kidney lesions that not only impair their immediate survival but also limit the adequate treatment of the underlying malignant disease. Identifying novel mediators that

regulate the growth and death of cancer cells has facilitated the development of more effective anticancer drugs that have revolutionized treatment options and clinical outcomes in cancer patients. However, many of these new drugs are often accompanied by significant side effects, including kidney injury (2). Ado-trastuzumab emtansine (T-DM1) is an antibody–drug conjugate consisting of the monoclonal antibody trastuzumab linked to the maytansinoid DM1 *via* a non-reducible thioether linkage with potential antineoplastic activity. The trastuzumab moiety of this conjugate binds to human epidermal growth factor receptor 2 (HER2) on the tumor cell surface and is subsequently internalized, therefore releasing the DM1 moiety to bind to tubulin with disruption of microtubule assembly/disassembly dynamics and inhibiting cell division and the proliferation of cancer cells that overexpress HER2 (3). T-DM1 has been approved specifically for pretreated metastatic HER2-positive breast cancer patients or for breast cancer patients with HER2-positive unresectable locally advanced or metastatic breast cancer who had previously received trastuzumab and taxanes (4). Moreover, T-DM1 has been approved for extended use to the adjuvant therapy of adult patients with HER2-positive early breast cancer who have residual invasive disease in the breast and/or lymph nodes after neoadjuvant taxane-based and HER2-targeted therapy (5). An analysis of the US Food and Drug Administration (FDA) Adverse Event Reporting System revealed that renal adverse events are observed in a considerable subset of patients treated with trastuzumab (6). However, there are no published reports describing kidney biopsy findings related to nephrotoxicity of either trastuzumab or T-DM1. We provide report kidney biopsy findings in a case presenting with deterioration of kidney function and nephrotic range proteinuria due to collapsing focal segmental glomerulosclerosis (FSGS) after initiation of T-DM1 therapy.

CASE DESCRIPTION

A 60-year-old Caucasian woman had known arterial hypertension, insulin-dependent type 2 diabetes, and chronic kidney disease (CKD) stage 1. A left axillary abscess incision identified incident breast invasive ductal carcinoma, grade 3 with positivity for estrogen receptor (ER; 80%), progesterone receptor (PR; 80%), Ki-67 (60%), and HER2. Based on diagnosis of metastatic breast cancer and reduced physical condition, the patient received neoadjuvant therapy with paclitaxel (80 mg/m^2 weekly), pertuzumab (loading dose 840 mg, 420 mg every 3 weeks thereafter), and trastuzumab (loading dose 8 mg/kg body weight, 6 mg/kg every 3 weeks thereafter) because of HER2 positivity for 2 months. Because of coronavirus disease 2019 (COVID-19) requiring hospitalization for 1 week and delayed convalescence, the chemotherapy was paused after three cycles of paclitaxel and two cycles of pertuzumab/trastuzumab and re-initiated thereafter. After a total number of 12 cycles of paclitaxel and seven cycles of pertuzumab/trastuzumab, left axillary lymph node dissection (ALND) was performed (66/102 metastatic lymph nodes; ER, 100%; PR, 90%; Ki-67, 60%; HER2, heterogeneous positive), and adjuvant T-DM1 chemotherapy (3.6 mg/kg body weight every 3 weeks, 302 mg) was initiated thereafter. Four weeks prior to admission, the patient received the first infusion with T-DM1 followed by a second infusion 1 week prior to admission (**Figure 1**). At admission, the patient presented to our emergency department with hypertensive crisis (initial blood pressure of 211/126 mmHg), acute kidney injury (AKI) stage 1, proteinuria (4.4 g/g creatinine), and albuminuria (3.1 g/g creatinine, normal range <30 mg/g, **Figure 1** and **Table 1**). A repeat nasopharyngeal swab for SARS-CoV-2 RNA testing by PCR at admission was negative. Laboratory tests excluded tumor lysis syndrome and rhabdomyolysis; serological evaluation was normal for antinuclear antibodies (ANA), anti-neutrophil cytoplasmic antibodies (ANCA), and anti-glomerular basement membrane (GBM); and no reduction in serum complement C3 and C4 was observed (**Table 1**). During the further course, the patient presented with deterioration of kidney function (serum creatinine up to 2.25 mg/dl, normal range 0.5–1 mg/dl, **Figure 1**), in addition to progressive nephrotic range proteinuria (up to >10 g/g creatinine), albuminuria (up to 6.7 g/g creatinine, normal range <30 mg/g), and hematuria. A kidney biopsy was performed showing acute tubular injury and mild interstitial fibrosis (**Figures 2A, B**). Glomeruli showed moderate mesangial expansion according to Tervaert class IIb (**Figure 2C**) (7). Notably, collapsing FSGS was present (**Figure 2D**). Severe vascular hyalinosis as previously described specifically in collapsing FSGS superimposed in diabetic nephropathy was not present (8). Moreover, no double contours of the GBM were detectable, thereby excluding collapsing FSGS due to chemotherapy-associated thrombotic microangiopathy (TMA; **Figure 2E**) (9). Furthermore, no subendothelial immune deposits (IgA, IgG, IgM, C1q, and C3c) were detectable, while IgM was entrapped in damaged glomerular capillaries (**Figures 2F–J**). After discontinuation of T-DM1 therapy, proteinuria (down to 1.1 g/g creatinine) and albuminuria (down to 0.7 g/g creatinine) improved 3 weeks after the last T-DM1 infusion without further specific treatment, associated with

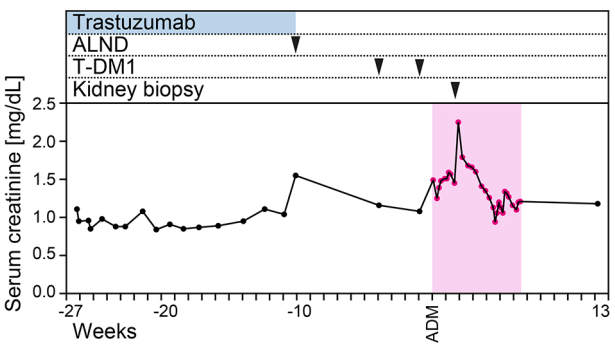

FIGURE 1 | Time course of the case. Time course of serum creatinine levels, trastuzumab and T-DM1 infusions, and kidney biopsy. Abbreviations: ADM, admission; ALND, axillary lymph node dissection; T-DM1, ado-trastuzumab emtansine.

TABLE 1 | Key clinical parameters at presentation.

Parameter	Value	Normal range
Serum creatinine, mg/dl	1.49	0.7–1.2
eGFR, ml/min/1.73 m^2	37.9	>60
BUN, mg/dl	22	8–26
CRP, mg/L	15.2	≤5.0
LDH, U/L	263	125–250
CK, U/L	92	29–168
Anti-GBM, U/ml	<0.8	<7
ANA IF	1:100	<1:100
ANCA IF	Neg	Neg
C3c, g/L	1.78	0.82–1.93
C4, g/L	0.58	0.15–0.57
uPCR, mg/g	4,381	<300
uACR, mg/g	3,069	<30
Urinary kappa, mg/L	257	<6.8
Urinary lambda, mg/L	153	<3.7
Urinary kappa/lambda, ratio	1.68	>1 or <5.2

ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; C3c, complement factor 3 conversion product; C4, complement factor 4; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate (CKD-EPI); GBM, glomerular basement membrane; Neg, negative; uACR, urinary albumin-to-creatinine ratio; uPCR, urinary protein-to-creatinine ratio; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; CK, creatine kinase.

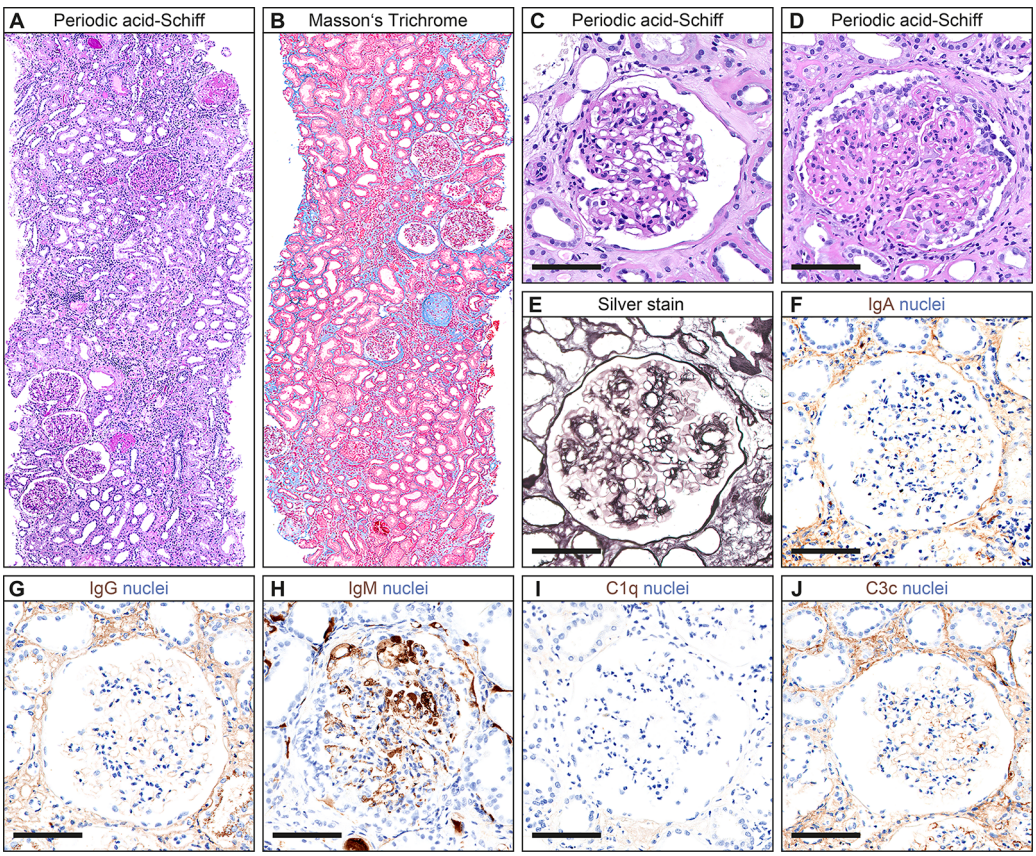

FIGURE 2 | Histopathological findings. **(A, B)** Representative kidney sections stained with periodic acid-Schiff and Masson's Trichrome showing acute tubular injury and mild interstitial fibrosis. **(C)** Glomeruli showed mild mesangial expansion according to Tervaert class IIa. **(D)** Notably, collapsing FSGS was present. **(E)** As shown by silver stain, no double contours of the basement membrane were detectable. **(F–J)** No subendothelial immune deposits (IgA, IgG, IgM, C1q, and C3c) were detectable, while IgM was entrapped in damaged glomerular capillaries. Scale bars: 100 µm. Abbreviations: C1q, complement component 1q; C3c, complement factor 3 conversion product; FSGS, focal segmental glomerulosclerosis; IgA, immunoglobulin A; IgG, immunoglobulin G.

improvement of kidney function (serum creatinine 1.13 mg/dl, normal range 0.5–1 mg/dl, **Figure 1**).

DISCUSSION

An analysis of the FDA Adverse Event Reporting System identified 124/1,243 (10%) renal adverse events (defined as proteinuria, AKI, elevated serum creatinine, and/or nephritis) for trastuzumab, reported between 2011 and 2015 (6). However, there are no published reports describing kidney biopsy findings related to nephrotoxicity of either trastuzumab or T-DM1. To our knowledge, we here provide the first report of kidney biopsy findings in a case of nephrotic range proteinuria due to collapsing FSGS after initiation of T-DM1 therapy. Collapsing glomerulopathy is a pattern of glomerular injury characterized by shriveling of the glomerular tuft in the setting of FSGS (10). Primary variants are most often observed in subjects of African ancestry with a genetic predisposition who carry an *APOL1* high-risk genotype (homozygous for G1 or G2 or compound G1/G2 heterozygotes) and triggering diseases that behave like a "second hit" leading to clinical manifestation of collapsing FSGS (11). Collapsing FSGS has also been described secondary to infection (including HIV and parvovirus), drugs (bisphosphonate and calcineurin inhibitors), severe vascular disease (TMA and cocaine use), and autoimmune diseases like systemic lupus erythematosus (SLE) (11–19). However, these conditions were excluded in our patient. Together with the fact that the kidney biopsy showed only moderate mesangial expansion (according to Tervaert class IIb) without advanced vascular hyalinosis previously described specifically in collapsing FSGS superimposed in diabetic nephropathy, it is likely that the observed collapsing FSGS was associated with the prior initiation of T-DM1 therapy (8). We also observed acute tubular injury often observed in kidney biopsies of patients presenting with nephrotic range proteinuria (20). Collapsing FSGS in association with T-DM1 is further supported by the clinical course with improvement of proteinuria and kidney function 3 weeks after discontinuation of T-DM1 therapy without further specific treatment. Since T-DM1 is an antibody–drug conjugate consisting of the monoclonal antibody trastuzumab linked to the maytansinoid DM1, we cannot conclude that observed collapsing FSGS is attributed to either drug conjugate or both.

CONCLUSIONS

In summary, we report kidney biopsy findings in a case of nephrotic range proteinuria due to collapsing FSGS and tubular injury after initiation of T-DM1 therapy. This issue is especially relevant since T-DM1 is widely used and nephrologists have to be aware of this potentially rare but severe complication.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and BT conceived the study, collected and analyzed the data, and co-wrote the first draft. SH evaluated the histopathological findings. SW and JG edited the manuscript. SW, JG, and BT were directly involved in the treatment of the patient. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cancer Chemoprevention: A Strategic Approach Using Phytochemicals

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Cancer chemoprevention approaches are aimed at preventing, delaying, or suppressing tumor incidence using synthetic or natural bioactive agents. Mechanistically, chemopreventive agents also aid in mitigating cancer development, either by impeding DNA damage or by blocking the division of premalignant cells with DNA damage. Several pre-clinical studies have substantiated the benefits of using various dietary components as chemopreventives in cancer therapy. The incessant rise in the number of cancer cases globally is an issue of major concern. The excessive toxicity and chemoresistance associated with conventional chemotherapies decrease the success rates of the existent chemotherapeutic regimen, which warrants the need for an efficient and safer alternative therapeutic approach. In this scenario, chemopreventive agents have been proven to be successful in protecting the high-risk populations from cancer, which further validates chemoprevention strategy as rational and promising. Clinical studies have shown the effectiveness of this approach in managing cancers of different origins. Phytochemicals, which constitute an appreciable proportion of currently used chemotherapeutic drugs, have been tested for their chemopreventive efficacy. This review primarily aims to highlight the efficacy of phytochemicals, currently being investigated globally as chemopreventives. The clinical relevance of chemoprevention, with special emphasis on the phytochemicals, curcumin, resveratrol, tryptanthrin, kaempferol, gingerol, emodin, quercetin genistein and epigallocatechingallate, which are potential candidates due to their ability to regulate multiple survival pathways without inducing toxicity, forms the crux of this review. The majority of these phytochemicals are polyphenols and flavanoids. We have analyzed how the key molecular targets of these chemopreventives potentially counteract the key drivers of chemoresistance, causing minimum toxicity to the body. An overview of the underlying mechanism of action of these phytochemicals in regulating the key players of cancer progression and tumor suppression is discussed in this review. A summary of the clinical trials on the important phytochemicals that emerge as chemopreventives is also incorporated. We elaborate on the pre-clinical and clinical observations, pharmacokinetics, mechanism of action, and molecular targets of some of these natural products. To summarize, the scope of this review comprises of the current status, limitations, and future directions of cancer chemoprevention, emphasizing the potency of phytochemicals as effective chemopreventives.

Keywords: chemoprevention, chemopreventives, phytochemicals, phenolic compounds, tumor suppression, chemotherapeutics

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INTRODUCTION

Cancer is one of the leading causes of mortality globally, accounting for almost 10 million deaths out of a total incidence of 19.3 million cases in 2020 (Sung et al., 2021), and is expected to increase over the next 2 decades. It is estimated that the global incidence of cancer could reach up to 28.4 million in 2040. Non-melanoma skin cancer records the highest incidence worldwide; however the mortality rate of this cancer is very low (Leiter and Garbe 2008; Lomas et al., 2012). According to GLOBOCAN 2020, breast cancer ranks at the top in terms of global incidence followed by prostate, lung and colorectal cancers (Sung et al., 2021). Lung cancer has the highest mortality rate followed by cancers of breast, prostate and liver. **Figure 1** depicts the GLOBOCAN statistics regarding worldwide cancer mortality rates.

Cancer stems from intrinsic and/or extrinsic factors that derail the cellular signalling networks that maintain a balance between cell survival, proliferation and death. Research over many decades have shown that a single genetic alteration is insufficient to drive cancer as these are often countered by alternate mechanisms that leads to genomic repair or cell death. Hence, acquiring a lethal metastatic property requires widespread genetic and epigenetic modifications that endow the cell with the potential to undergo uncontrolled proliferation, invasion, and metastasis. This transformation happens over a long time frame, and is the reason for increased incidence rate in the elderly population. Though rapid strides have been made on research focussing on the aetiology and molecular underpinnings of cancer, these have not proved to be fruitful in improving therapeutic outcome in patients having late stage cancers. In addition, chemotherapeutic agents inflict severe side effects that curtail the quality of life of patients. Hence, it is imperative to design measures to mitigate cancer incidence or to impede the progression of benign neoplasm to advanced stage cancers. The idea of chemoprevention is gaining more popularity partly due to its success in lowering the incidence of cardiovascular diseases.

CHEMOPREVENTION

Cancer chemoprevention is the use of natural and synthetic agents to suppress, prevent or delay tumorigenesis by blocking the initiation stage of carcinogenesis, or by curtailing the promotion stage wherein the initiated cells proliferate to give rise to a tumor. **Figure 2** illustrates the sequential progression of cancer and the functional stages of different classes of chemopreventives. Compounds that block the initiation stage are generally termed as blocking agents, and the ones affecting the promotion stage are termed as suppressing agents (Chen and Kong, 2005). Blocking agents act in different ways like lowering the metabolic activation of pro-carcinogens into carcinogens, decreasing the level of reactive oxygen species (ROS), and induction of genomic repair pathways. Apart from blocking DNA-damage, initiation blockers may also exert tumor preventive effect by modulating epigenetic modification like hypermethylation of tumor suppressor genes. Suppressing agents may effectuate their chemopreventive efficacy by suppressing the signalling pathways that trigger cell survival and proliferation.

Depending on the stage at which they act, chemopreventives can be classified into primary, secondary and tertiary (Rather and Bhagat, 2018). Primary chemopreventives are aimed at suppressing tumor formation in a susceptible population. Secondary chemoprevention suppresses the transition of a tumor from benign to malignant phenotype. Tertiary chemoprevention lowers the risk of tumor recurrence following a successful surgical and/or chemotherapeutic intervention.

RISK FACTORS IN CARCINOGENESIS

Factors contributing to tumorigenesis have been categorized into two *viz*., intrinsic and extrinsic. Intrinsic factor refers to the spontaneous, random mutations in the genome that occurs during DNA replication. These mutations occur at different rates in different species. Extrinsic factors are those

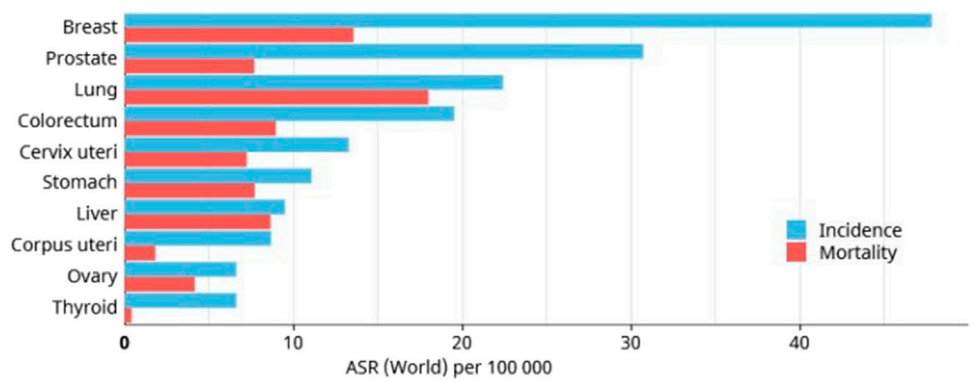

FIGURE 1 | GLOBOCAN 2020 estimate of global incidence and mortality rates of different cancers, excluding non-melanoma skin cancer.

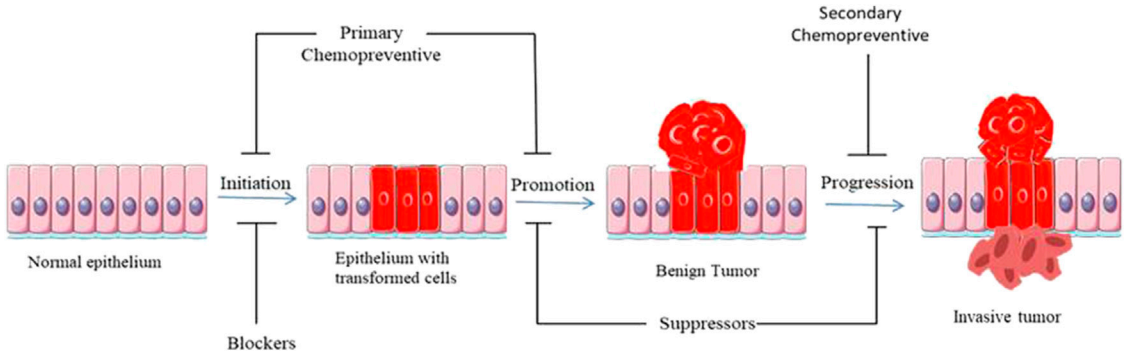

FIGURE 2 | Diagrammatic representation of sequential progression of cancer, with special reference to the stages at which different classes of chemopreventives act.

TABLE 1 | Extrinsic factors contributing to tumorigenesis.

Risk factor	Type	Cancer	References
UV and other ionizing radiation	Mutagen	skin cancer, leukemia, lung cancer	Bizzozero Jr et al. (1966), Veierød et al. (2003), Turner et al. (2011)
Cigarette smoke	Mutagen	Lung cancer	(Chapman et al., 2016); Guida et al. (2015)
Human papilloma virus	Mutagen	Head and neck cancer, Cancers of urogenital tract	(Cai et al., 2018, Sabatini and Chiocca 2020)
Hepatitis B virus	Mutagen	Liver Cancer	MacLachlan et al. (2015)
Asbestos	Mutagen	Pleural Mesothelioma	Tossavainen (2004)
Alcohol	Dietary factor	Breast cancer, liver cancer	Cancer (2002), Schütze et al. (2011)
Red Meat	Dietary factor	Breast cancer	Lo et al. (2020)

that occur due to exposure to biological and environmental agents like UV-radiation, chemicals and viruses. Aetiology of many cancers have been linked to extrinsic factors *viz.*, smoking and lung/oral cancer, viral infection and cervical cancer, exposure to asbestos and lung cancer, inflammation and colon cancer.

Table 1 summarizes the major risk factors involved in the development of various cancers.

PRECLINICAL OBSERVATIONS

Clinical trials are majorly conducted based on preclinical evidences for the efficacy of the compound of interest, or epidemiological observations that give a strong correlation between cancer incidence and physiological level of a molecule. The pipeline for identifying prospective cancer chemopreventive agents starts from *in vitro* studies on cell lines. These studies assess the potency of a compound in inducing death of transformed cell lines, lowering its potency to migrate, invade, and revert the signalling framework to that of an untransformed cell. *In vitro* assays are followed by *in vivo* testing, which measures tumor incidence rate, size, number and grade of tumors. One of the best studied models for testing chemopreventive efficacy is the multi-stage skin carcinogenesis model wherein skin tumors are induced by application of DMBA for tumor initiation and PMA for tumor promotion. DMBA is also used to induce metastatic breast cancer in rats. Other potent carcinogens like Benzopyrene, Azoxymethane and Aflatoxin are commonly being used to induce tumors of lung, colon, and liver respectively. These models recapitulate the stage-wise progression of human cancers, and hence serve as reliable experimental systems to understand the biology of tumors, and to assess the tumor suppressive effects of novel compounds. These *in vitro*/*in vivo* studies have helped in understanding the molecular mechanisms by which these compounds exert chemopreventive efficacy. Here, we discuss results of pre-clinical studies on some of the compounds that were tested in clinical settings to assess their chemopreventive potential.

Finasteride

Finasteride, a 5α-reductase inhibitor, was tested in multiple preclinical models of prostate cancer. In one study, the effect of finasteride in preventing prostate cancer induced by methyl nitrosourea/testosterone propionate was tested in Wistar rats. This study showed that rats orally administered with finasteride showed tumor incidence of only 10% while those in the control exhibited 64.3% incidence (Esmat et al., 2002). In another study the efficacy of finasteride in suppressing xenograft tumors were evaluated. Here, finasteride did not induce any change in growth of tumors from the metastatic cell line, LNCaP; however, it induced a significant increase in tumor burden in tumors induced by a combination of LNCaP and fibroblasts. Mechanistic studies

showed that c-Jun, a component of the AP-1 transcription factor, plays the key role in driving tumor growth in finasteride administered animals (Niu et al., 2016).

Dutasteride

Dutasteride is another 5α-reductase that was tested in clinical trials. In one study using LuCaP 35-generated xenograft models of prostate cancer, dutasteride was found to significantly reduce tumor growth. Dutasteride modulated the expression of genes involved in apoptotic, cytoskeletal remodeling, and cell cycle pathways (Schmidt et al., 2009). In another study, dutasteride induced apoptosis in androgen dependent prostate cancer cells but not in androgen independent cells. Dutasteride could also induce apoptosis in some but not all prostate cancer primary cultures. Here, the responsiveness of cells to dutasteride were dependent on the expression of antihuman α-methyl acyl-CoA racemase (AMACR) (Maria McCrohan et al., 2006).

Pioglitazone

Pioglitozone, an inhibitor of peroxisome proliferator-activated receptor γ (PPARγ), was tested in preclinical models of lung cancer for assessing its chemopreventive efficacy. In a mouse model of lung cancer induced by injection of vinyl carbamate, pioglitazone could reduce tumor incidence by 64% when administered after 8 weeks post injection of carcinogen in *p53* wild type mice. In mice carrying a mutant p53 ($p53^{wt/Ala135Val}$), pioglitazone could suppress tumor incidence by 50% (Wang et al., 2010). In another study, pioglitazone was found to suppress benzo(a)pyrene induced lung cancer by 63%, and a combination of pioglitazone and budesonide could achieve 90% reduction in tumor burden (Fu et al., 2011).

Epigallocatechin-3 Gallate

Epigallocatechin-3 gallate (EGCG) which is a key bioactive component in green tea extracts was found to induce reduction in tumor burden induced by 7,12-dimethylbenz(a) anthracene (DMBA) in Sprague-Dawley rats. Here, administration of EGCG delayed onset of mammary tumorigenesis, and reduced tumor invasivenesss (Kavanagh et al., 2001). In another study, EGCG was found to reduce the growth of MCF-7 xenografts in mice (Zan et al., 2019). Another study reported that physiological levels of EGCG caused growth inhibition in $P53^{WT}$MCF-7 cells while P53 mutant T47D cells were resistant to EGCG. Moreover, EGCG sensitized breast cancer cells to tamoxifen by upregulating estrogen receptor-α expression. EGCG also increased the expression of insulin-like growth factor receptor, and hence sensitized breast cancer cells to antibodies targeting these receptors (Zeng et al., 2014).

Grape Seed Procyanidine Extract

Grape seed procyanidines have been shown to exhibit potent activity against lung cancer in both *in vitro* and *in vivo* conditions. Administration of different doses of procyanidins lead to a significant decrease in the growth of A549 and H1299 xenografts. This reduction in tumor burden was accompanied by an increase in the expression of insulin-like growth factor receptor in the tumor microenvironment (Akhtar et al., 2009). Later on, a study reported that the antineoplastic effect of procyanidines on lung cancer cells and the increased expression of insulin-like growth factor receptor is mediated by down-regulation of MicroRNA-19a/b. In this study, oral administration of leucoselectphytosome could mitigate growth of A549 xenografts (Mao et al., 2016).

Celecoxib

Celecoxib, an inhibitor of COX-2, displays potent inhibitory activity against UV-induced skin carcinogenesis in murine models. Fischer et al. showed that inhibition of COX-2 could achieve dose-dependent reduction in UV-induced skin tumorigenesis. UV-induced synthesis of prostaglandins was significantly suppressed by the compound; however, tumors that constitutively expressed COX-2 did not show any growth reduction upon celecoxib treatment (Fischer et al., 1999). Another study showed that celecoxib could prevent the onset of new UV-induced tumors but the growth of pre-existing tumors remains unaffected by the inhibitor (Pentland et al., 1999).

2-phenethylisothiocynate

2-phenethylisothiocynate (PEITC), a cruciferous vegetable component, can inhibit cytochrome P450 enzymes which are involved in the conversion of carcinogens into their active forms (Nakajima et al., 2001). The compound also suppressed 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumor by 50%, and could also suppress NNK-induced DNA methylation. However, PEITC could not suppress NNK-induced tumors in the liver or oral cavity (Morse et al., 1989).

CLINICAL TRIALS

Similar to chemotherapeutic drug development, the testing of potential chemopreventive agents proceeds through many phases of studies on human subjects. Many clinical trials have been done in the past to assess the chemopreventive efficacy of compounds on the basis of epidemiological or pre-clinical data. Description of all the clinical trials is beyond the scope of the review. Hence, we detail some of the successful and failed clinical trials undertaken to assess chemopreventive potential of compounds, both natural and synthetic, on the most prevalent cancers.

Breast Cancer

The Breast Cancer Prevention Trial (BCPT) was the first trial to show a significant positive result with chemoprevention. This study included 413,000 women at risk of breast cancer and showed that tamoxifen administration for 5 years lead to ~50% reduction in breast cancer. However, tamoxifen administration caused higher incidence of endometrial cancer and thromboembolic events (Fisher et al., 1998). The efficacy of tamoxifen in reducing breast cancer incidence was further evaluated in another trial, IBIS-1, which showed similar effectiveness as seen in BCPT but the toxicity was shown to decline after 5 years post-termination of tamoxifen

administration (Cuzick et al., 2007). In another study, tamoxifen was compared to raloxifene which showed that raloxifene was as effective as tamoxifen in reducing invasive breast cancer but were devoid of toxic effects. Later on, another study with increased median follow-up of 81 months showed that raloxifene is less effective than tamoxifen though it had a better safety profile. Tamoxifen was also found to be effective in preventing contralateral tumors (Cuzick et al., 2013). The effect of exemestane, an aromatase inhibitor, on breast cancer incidence was studied in a population of high risk women without breast cancer. Aromatase inhibitors block the synthesis of oestrogens from androgens. Exemestane significantly lowered overall incidence of breast cancer by 53% and suppressed the incidence of invasive breast cancer by 65% after a median follow-up of 3 years (Goss et al., 2011). A phase-2 double-blinded placebo-controlled clinical trial assessed the effect of green tea extract on mammographic density. In the treatment group, women consumed four decaffeinated GTE capsules having 1,315 mg of catechins for 12 months. The study showed that GTE capsules could reduce percent mammographic density in younger women but did not have any effect on older women. Further investigations will be necessary to assess the efficacy of GTE as a chemopreventive against breast cancer (Samavat et al., 2017). A study was undertaken to evaluate the effect of resveratrol, a bioactive compound present in berries and grape skin, on DNA-methylation pattern in women with high risk of breast cancer. The results show that the extent of methylation of RASSF-1α, a tumor suppressor gene, is inversely proportional to serum trans-resveratrol levels. The differential expression of RASSF-1α was found to be directly proportional to change in levels of prostaglandin E2 (Zhu et al., 2012).

Prostate Cancer

The efficacy of finisteride, a 5α-reductase inhibitor, in lowering the incidence of prostate cancer was studied in a population of 18,882 men. After 7 years of administration of the drug, a reduction in incidence of prostate cancer was found though the chemopreventive efficacy was observed in low grade tumors (Thompson et al., 2003). Tumors with Gleason score of 7–10 were higher in finisteride group than in placebo group. Another trial studied the effect of Dutasteride on prostate cancer incidence. Similar to finisteride, a reduction of 22.8% was observed in prostate cancer in patients administrated with dutasteride and the effect was seen only in low grade tumors (Andriole et al., 2010). There were 12 tumors of Gleason score of 8–10 in dutasteride group compared to only one in the placebo group. In another study, the efficacy of selenium and vitamin E in preventing prostate cancer was assessed; however, the study was discontinued after an interim analysis, which indicated low chances of a positive result (Lippman et al., 2009). Another study showed that vitamin E administration could actually increase prostate cancer incidence (Klein et al., 2011). However, an independent study conducted by Heinonen et al showed that intake of α-tocopherol was correlated to reduced incidence of prostate cancer. Consumption of α-tocopherol lead to 32% decrease in prostate cancer incidence and 41% decrease in death (Heinonen et al., 1998). Two similar studies showed that selenium might exert chemopreventive efficacy against prostate cancer. Here, the effect was more pronounced in subjects with low basal selenium level in blood (Zhong et al., 2021).

Lung Cancer

Chemoprevention trials on lung cancer have met with both success and failures. A phase-2 double-blind randomized placebo-controlled clinical trial of oral pioglitazone, a thiazolidinedione, was conducted on individuals at high risk of lung cancer incidence based on the observation that diabetes patients receiving thiazolidinediones have low lung cancer rates. The study was conducted on current or former smokers with sputum cytologic atypia or endobronchial dysplasia. Former smokers treated with pioglitazone showed mild improvement in the worst biopsy scores, and recorded a decreased Ki-67 labeling index of bronchial biposies. However, slight worsening was seen in current smokers administered with pioglitazone (Keith et al., 2019). The effectiveness of 2-phenethylisothiocynate (PEITC) in detoxifying the metabolites of 1,3-butadiene, a component of cigarette smoke, was studied in subjects who were smokers. 1,3-butadiene gets metabolized by cytochrome P450 enzymes into the active metabolites like 3,4-epoxy-1-butene (EB) which subsequently gets detoxified to mercapturic acids like MHBMA and DHMBA by GST enzymes. The clinical trial showed that oral ingestion of PEITC for 1 week increased urine MHBMA levels by 58.7 and 90% in GSTM1 and GSTT1 null subjects respectively, while it had negligible effect on other subjects (Boldry et al., 2020). This shows that PEITC could be a potential primary chemopreventive against tobacco smoke-induced cancers. A phase-1, open-label clinical trial was conducted to assess the chemopreventive efficacy, safety and tolerability of leucoselect phytosome (LP), a grape seed procyanidine extract (GSE) complexed with soy phospholipids. Bronchial biopsies were taken before and after 3 months of oral administration of LP. After 3 months, LP reduced Ki-67 labelling of bronchial biopsies by 55%, lowered serum oncomiRs, miR-19a,miR-19b,miR-106b, and it was well tolerated (Mao et al., 2021). Contrary to these positive results, some clinical trials have shown that some of the proposed chemopreventive agents could actually enhance tumor incidence. One of the earliest clinical trials on lung cancer chemoprevention was a placebo-controlled trial which enrolled 29,133 men administered with α-tocopherol and β-carotene either individually or in combination. Initially, the results showed an 18% increase in incidence of lung cancer and cardiovascular disease, and an 8% increased overall mortality for those on β-carotene (Albanes et al., 1995). Further analysis of the data showed that adverse effects were stronger in smokers and in men with moderate alcohol consumption. In theβ-Carotene and Retinol Efficacy Trial, men with occupational asbestos exposure or men and women who were current/former cigarette smokers were administered with either β-carotene plus retinyl palmitate or placebo. The study was discontinued as the subjects in the intervention group had more lung cancer and cardiovascular disease mortality rates (Omenn et al., 1996). In the β-carotene and retinol efficacy trial (CARET), administration of β-carotene to a population at high risk of lung cancer has led to 28% increase in lung cancer incidence (Group 1994). The α-tocopherol and β-

carotene trial also reported a higher incidence in subjects who were administered with β-carotene. In another study that explored the effect of Non-steroidal anti-inflammatory drugs (NSAIDS) on incidence of small-cell lung cancer, it was observed that there was an increased risk of small-lung cancer incidence in subjects who had taken regular-strength aspirin (Brasky et al., 2012).

Skin Cancer

A Phase-3 double blind, placebo-controlled clinical trial showed that oral ingestion of nicotinamide at a dose of 500 mg twice daily for 12 months decreased the incidence of new non-melanoma skin cancer (NMSC) by 23% in a high-risk population. The subjects recruited for the study had at least two non-melanoma skin cancer cases within 5 years prior to the study. No adverse effects were found in the nicotinamide treatment group during the 12-months period of the study (Chen et al., 2015). In another double-blind placebo-controlled randomized trial, the efficacy of celecoxib in lowering the incidence of actinic keratoses and NMSC was studied. Actinic keratosis is a precursor of cutaneous squamous cell carcinoma. The observed rate of transition of AK lesions into cutaneous squamous cell carcinoma is between 0.025 and 16% (Marks et al., 1988). Celecoxib at 200 mg was given to participants twice daily for 9 months. The results showed no difference in actinic keratosis incidence between control and placebo at 9 months post randomization; however, a decrease in NMSC incidence was observed 11 months after randomization in the group administered with celecoxib (Elmets et al., 2010). In another study, application of an ointment of curcumin on skin of patients with external cancerous lesions could induce symptomatic relief like reduction in smell and itching. Moreover, a reduction in lesion size and pain was seen in 10% of the patients (Kuttan et al., 1987). The efficacy of dl-α-tocopherol in suppressing potential transition of AKs to NMSC was studied in subjects with sun-damaged skin. Participants with AK lesions were administered with either placebo or 12.5% dl-α-tocopherol for 6 months. The levels of PCNA, p53, polyamines were assessed following treatment with the compound. The results showed no significant decrease in the number of AKs, expression of PCNA and p53; however, the level of polyamines was relatively low in the treated group. Though the authors of the study considers reduction of polyamines as a positive indicator of its chemopreventive potential in clinic, this effect could be outweighed by the unchanged expression status of p53 and PCNA (Foote et al., 2009). Hence, this warrants further trials using incidence of NMSC as the end point. The efficacy of 5-Fluorouracil in suppressing the incidence of basal cell carcinoma and squamous cell carcinoma requiring surgery was studied in veterans who are elderly (median age: 70) and had substantial exposure to Sun. Application of 5% 5-FU twice weekly for 2–4 weeks on face and ears substantially reduced the incidence of squamous cell carcinoma while no change was observed in the incidence of basal cell carcinoma during the first year of study. However, in the entire study period, there was no difference between treatment groups in time to first incidence of the carcinoma,keratinocyte, basal cell, or squamous cell carcinoma (Weinstock et al., 2018). **Table.2** summarizes the clinical trials of some of the major chemopreventive molecules.

Table 2 provides a brief description of the clinical trials conducted using the phytochemicals as chemopreventives and the outcome of the respective studies.

PRECLINICAL EVALUATION OF NATURAL PRODUCTS AS PROSPECTIVE CHEMOPREVENTIVES

Plants synthesize an array of secondary metabolites, which aid in fulfilling physiological functions as well as help in coping with exogenous constraints. Some of the major classes of secondary metabolites include polyphenols, flavanoids, alkaloids and anthraquinones. In the context of cancer, these phytochemicals have been extensively studied for their anti-oxidant, pro-apoptotic, anti-inflammatory, anti-angiogenic, anti-carcinogenic and anti-metastatic properties (Brglez Mojzer et al., 2016). The following section encompasses a brief account of various preclinical studies on the chemopreventive efficacy of some the predominant dietary phytochemicals. The chemical structures of the phytochemicals being discussed in this report are illustrated in **Figure 3**.

CURCUMIN

Curcumin (diferuloylmethane), a polyphenol isolated from *Curcuma longa* is the most studied phytochemical as a chemopreventive. Curcumin displays a wide variety of pharmacological functions such as anti-inflammatory agent, anti-mutagenic agent, antineoplastic agent, hepatoprotective agent, nutraceutical, anti-microbial agent, anti-oxidant agent, and immunomodulator. It has been shown to have significant roles in prevention, treatment and chemo sensitization of cancer cells (Pavan et al., 2016). Studies have established the anti-proliferative, anti-angiogenic, anti-metastatic, and pro-apoptotic properties of curcumin (Anto et al., 2002; Puliyappadamba et al., 2010; Bava et al., 2011; Vinod et al., 2013).

We have reported that curcumin exhibits potential chemopreventive effect against nicotine-induced survival signalling in lung cancer cells. We found that it down-regulates multiple survival signals induced by nicotine in lung cancer cells irrespective of their p53 status (Puliyappadamba et al., 2010). Another study conducted in our laboratory revealed its chemopreventive efficacy against the lung carcinogenesis induced by B (a)P, a potential environmental carcinogen found in cigarette smoke and deep-fried food, in Swiss albino mice (Puliyappadamba et al., 2015). Curcumin treatment has a significant impact on improving the general health of colorectal cancer patients by enhancing expression of p53 molecules in tumour cells and also by promoting the apoptosis of tumour cells (He et al., 2011; Pricci et al., 2020). Curcumin intake causes the down-regulation of NF-kB, COX-2 and phosphorylated STAT3 in peripheral blood mononuclear cells from patients with pancreatic cancer (Dhillon et al., 2008). It has been reported that, daily curcumin intake leads to a significant and dose-dependent reduction in spontaneous ovarian cancer incidence and tumor growth in Hen ovarian cancer model indicating a significant role of curcumin as a chemopreventive

TABLE 2 | Outcome from clinical trials using the phytochemicals as potential chemopreventives.

Molecule	Target cancer	Outcome in treatment arm	References
Tamoxifen	Breast cancer	Reduction in breast cancer incidence but higher incidence of endometrial cancer	Fisher et al. (1998), Cuzick et al. (2013)
Raloxifene	Breast cancer	Reduction in tumor incidence	Cuzick et al. (2013)
Exemestane	Breast cancer	Reduction in tumor incidence	Goss et al. (2011)
Finisteride	Prostate cancer	Reduction in incidence of low grade tumors but increased incidence of high grade tumors	Thompson et al. (2003)
Dutasteride	Prostate cancer	Reduction in incidence of low grade tumors but increased incidence of high grade tumors	Andriole et al. (2010)
Vitamin E	Prostate cancer	Reported both reduction and increase in tumor incidence	Klein et al. (2011), Heinonen et al. (1998)
Selenium	Prostate cancer	Low tumor incidence in subjects with low basal level of selenium	Zhong et al. (2021)
β-carotene	Lung cancer	Higher incidence of lung cancer	Albanes et al. (1995)
Aspirin	Lung cancer	Higher incidence of lung cancer	Brasky et al. (2012)
Nicotinamide	Skin cancer	Lowered the incidence of skin cancer	Chen et al. (2015)
Celecoxib	Skin cancer	Lowered tumor incidence	Elmets et al. (2010)
5-FU	Skin cancer	Lowered the incidence of cutaneous squamous cell carcinoma	Weinstock et al. (2018)
Resveratrol	Breast cancer	Hypomethylation of RASSF-1α	Zhu et al. (2012)
Curcumin	Skin cancer	Minor reduction in cancerous lesions; reduced smell and itching	Kuttan et al. (1987)
Green tea extract	Breast cancer	Reduced mammographic density in younger women but not in older women	Samavat et al. (2017)
2-phenethylisothiocynate	Lung cancer	Increased detoxification of metabolites of 1,3-butadiene	Boldry et al. (2020)
Grape seed procyanidine extract	Lung cancer	Reduced Ki-67 labelling index of bronchial biopsies; lowered serum level of oncomiRs	Mao et al. (2021)

strategy for ovarian cancer (Sahin et al., 2018). The administration of curcumin to paediatric patients with relapsed brain tumours undergoing chemotherapy increased their response compared with the controls (Wolff et al., 2012).

Resistance to chemo and radiotherapy is the major reason for cancer relapse. This arises due to the presence of a subpopulation of cancer cells, having self-renewal capabilities called Cancer Stem Cells (CSCs). Studies have confirmed that curcumin could inhibit the breast cancer stem cell population by downregulating the expression of stem cell genes Oct4, Sox2 and Nanog and also the Epithelial-Mesenchymal Transition (EMT) as observed by the down-regulation of mRNA levels of Vimentin, Fibronectin and β-catenin and up-regulation of mRNA levels of E-cadherin (Hu et al., 2019).

We have demonstrated that a combination of sub-optimal dose of 5-FU and curcumin elicits synergistic antitumor potential in murine models as evaluated by a reduction in the tumor-related parameters. Mechanistically, curcumin down-regulates 5-FU induced up-regulation of Thymidylate Synthase (TS), which is responsible for 5-FU chemoresistance (Vinod et al., 2013; Haritha et al., 2021). Another study from our lab reported that cervical cancer cells can be sensitized by curcumin to paclitaxel-induced apoptosis through down-regulation of NF-κB, Akt and Bcl2 (Bava et al., 2011). The chemopreventive agent curcumin also act as a potent radiosensitizer in human cervical tumor cells. Curcumin pre-treatment increased reactive oxygen species production and overactivation of the mitogen-activated protein kinase pathway in HeLa and SiHa cells when treated with Ionising Radiation (Javvadi et al., 2008).

However, therapeutic efficacy of curcumin is hindered to a certain extent by its low bioavailability (Toden and Goel 2017). To overcome this limitation, numerous curcumin analogues were developed, which include asymmetric curcuminoid analogues, curcumin analogue P1, curcumin analogue 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31), C-5 curcumin analogs etc. Asymmetric natural analogue of curcuminoid is shown to have anti-cancer activity by the presence of 4-hydroxy-3-methoxy benzene unit in it (Qiu et al., 2017). In Chronic Myeloid Leukemia and colon cancer cell line HCT116, C-5 Analog of curcumin shows the more cytotoxic effect by inhibiting the TNF-α-induced NF-κB activation than the curcumin (Allegra et al., 2017). Studies have reported that, purely aqueous PLGA nanoparticulate formulations of curcumin exhibit enhanced anticancer activity against human epithelial cervical cancer cells, HeLa (Nair et al., 2012). Our laboratory has demonstrated that, chitosan encapsulation enhances the bioavailability and tissue retention of curcumin and hence improves its efficacy in preventing Benzopyrene-induced lung carcinogenesis in Swiss albino mice (Vijayakurup et al., 2019).

Mechanism of action of curcumin mainly involves down-regulation of transcription factor NF-κB by inhibition of Notch signalling, which is involved in cell proliferation, apoptosis, maintenance of stem cell and their renewal. This results in a reduction in expression of NF-κB regulated genes, which includes Bcl-2, cyclin D1 and VEGF (O'riordan et al., 2005). Curcumin is a strong inhibitor of Protein Kinase C (PKC) and several oncogenes such as c-jun, c-fos, c-myc, NIK, MAPKs, ERK, ELK, PI3K, Akt and CDKs. Curcumin also inhibits of the Notch-1 downstream target Hes-1 in esophageal cancer cells. Hes-1 is an important notch signalling target and mediator (Subramaniam et al., 2012). The curcumin analog, 2-pyridylcyclohexanone has also been shown to decreases basal STAT3 phosphorylation and promotes apoptosis in esophageal cancer cell, ESCC cells (Wang et al., 2018).

Curcumin quenches free radicals, induces antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase), and up-regulates antioxidative protein markers, Nrf2 and HO-1 that led to the suppression of cellular oxidative stress. In cancer cells, curcumin aggressively increases ROS that results in DNA damage and subsequently cancer cell death (Ak and Gülçin 2008).

Curcumin was found to suppress inflammatory cytokines such as IL-6, IL-8, granulocyte macrophage colony stimulating factor and TNF-α as well as IKKβ kinase in the saliva of HNSCC patients. Kim SG., et al., also suggested that IKKβ kinase could be a plausible biomarker for the detection of the effect of curcumin in head and neck cancer as curcumin inhibited IKKβ kinase activity and this resulted in the reduced expression of a number of cytokines (Kim et al., 2011).

Molecular docking studies further aids in identifying the role of curcumin in numerous signalling cascades involved in carcinogenesis and confirms the already suggested molecular mechansims responsible for the chemopreventive efficacy of curcumin. Using inverse molecular docking several proteins associated with cell proliferation and tumor formation namely, macrophage colony stimulating factor 1 receptor, aldo-keto reductase family 1 member C3, amiloride-sensitive amine oxidase and tyrosine-protein phosphatase non-receptor type 11 were identified as potential targets of curcumin. Curcumin was previously reported to inhibit the NFkB mediated activation of genes linked to cell survival and proliferation (Divya and Pillai 2006). Proteins such as MMP-2, NAD-dependent protein deacetylase sirtuin-2, core histone macro-H2A.1, NAD-dependent protein deacetylase sirtuin-1 and epidermal growth factor receptor were also revealed to be targets of curcumin, the binding of which regulates the activity of NF-kB (Furlan et al., 2018). These results provide a mechanistic explanation for the anticancer effects of curcumin. Targeting Phosphodiesterase 4 (PDE4) has been reported to be a potential therapeutic strategy against inflammatory disorders (I Sakkas et al., 2017). Studies suggest that curcumin may exhibit its anti-cancer property through the inhibition of PDE2 and PDE4 (Abusnina et al., 2015). Furlan et al. also gives evidences for the inhibitory effect of curcumin on PDE4 (Furlan and Bren 2021).

RESVERATROL

Resveratrol (3, 4′, 5-trihydroxystilbene), is a natural polyphenolic stilbene and common phytoalexin which is present mainly in grape skin, red wine, mulberries, blueberries, pistachio and peanuts. The abundant biological and pharmacological properties of resveratrol pave way to the wide-ranging therapeutic spectrum offered by the compound. Resveratrol has been shown to regulate oxidative stress, inflammation, apoptosis, and is also known to possess neuroprotective effects. Furthermore, it potentially regulates various cellular signaling events including immune cell regulation, cytokines/chemokines secretion, and also controls the expression of several immune-related genes. Resveratrol is capable of modulating various cellular events such as apoptosis, autophagy, cell cycle, inflammation, invasion and metastasis, which collectively contribute to its chemopreventive efficacy.

As a chemopreventive agent, resveratrol influences all the major stages of carcinogenesis. Being polyphenolic in nature, it displays strong antioxidant activity and exercises control over multiple molecular events. Resveratrol causes the activation of tumor suppressor genes and inhibition of oncogenes that are crucial to carcinogenesis. Resveratrol has been shown to possess pro-apoptotic role and is known to decrease the viability and mitotic index of a number of cancer cell lines. Several studies have documented the excellent chemopreventive effects of resveratrol in various cancer types including oral, colorectal, prostate and breast cancer. Previous studies have demonstrated that resveratrol potentially suppressed the transcription and translation of E6 and E7, through induction of apoptosis and by causing G1/S phase transition arrest (Sun et al., 2021), thereby inhibiting cervical cancer under both *in vitro* and *in vivo* conditions. Resveratrol operates through various signalling pathways such as STAT3, AKT/mTOR AMPK, IGFR and Wnt pathways (Lohse et al., 2018). Amin et al., investigated the *in vitro* and *in vivo* antitumor effects of a combination of epigallocatechin gallate (EGCG) and resveratrol, and they found that their combination at low doses exhibited a synergistic growth inhibition in head and neck cancer, both *in vitro and in vivo*. Furthermore, the mechanistic studies unravelled that the combination inhibited AKT-mTOR signaling and the over-expression of constitutively active AKT protected cells from apoptosis induced by the combination of EGCG and resveratrol (Amin et al., 2021). Numerous studies have established SIRT1 to be one of the key targets of resveratrol. An up-regulation of SIRT1 mandates the chemopreventive effects of resveratrol in colorectal cancer cells (Buhrmann et al., 2016). Resveratrol potentially down-regulates NF-κB phosphorylation which consequently restricts tumor invasion and metastasis. Previous reports have mentioned the significant role of NF-κB in manifesting SIRT1-dependent anticancer activity of resveratrol (Bourguignon et al., 2009). MALAT1 is a key driver in the progression of multiple cancers including renal, cervical, liver, osteosarcoma, etc. Resveratrol hinders the invasion and metastasis of colorectal cell lines via MALAT-1-mediated Wnt/β-catenin signaling pathway (Ji et al., 2013). Studies involving mouse mammary organ culture model have revealed that resveratrol prevents the transcriptional activation of cytochrome P-450 1A1. Resveratrol is also known to cause blockade of G1/S phase transition in a number of cell lines. Resveratrol has been proven to inhibit the expression of COX-1/2. Furthermore, it downregulates TPA-induced activation of protein kinase C and AP-1-mediated gene expression in mammary epithelial cells (Bhat and Pezzuto, 2002). Resveratrol targets a wide array of molecules including ERK 1, PI3K, ERα/β, AMPK, AKT1, STAT3, FAS, COX1/2, p53, NF-κB, IKKB, p38, JNK 1. Studies on the effect of resveratrol on three mutagens namely, aflatoxin B1, 2-amino-3-methylimidazo (4,5-f) quinoline and N-nitroso-N-methyl urea have established that resveratrol significantly decreased the mutagenicity of all three mutagens (Langová et al., 2005). In spite of its remarkable cancer chemoprevention properties, the low oral bioavailability of

resveratrol has often impeded its translation to *in vivo* effects. Earlier reports indicate that while the oral administration of resveratrol effectively inhibited colorectal carcinogenesis, it failed to protect mice from chemical-induced lung carcinogenesis. Therefore, Monteillier et al., attempted intranasal administration of resveratrol and they observed that this method successfully prevented lung cancer in A/J mice. This proves to be an effective solution to overcome the issue of low oral bioavailability of the compound (Monteillier et al., 2018).

Besides being an excellent chemopreventive, reseveratrol also functions as an effective chemosensitizing agent. Our studies have established that docetaxel and resveratrol elicit a synergistic response against Her-2 over-expressing breast cancer cells. We have also documented that HER-2–Akt signaling axis plays a significant role in regulating the synergistic effect of docetaxel and resveratrol (Vinod et al., 2015).

Multiple studies have elucidated the strong anti-oxidant potential of resveratrol. The compound modulates NF-κB pathway and confers protection against myocardial ischemic injury and aflatoxin induced hepatocellular carcinoma (Rawat et al., 2021; Ro et al., 2021).

Molecular docking studies identified the proteins NTMT1, LSD1 and BIRC4 as potential targets of resveratrol, the inhibition of which could contribute to the chemopreventive effects of resveratrol (Kores et al., 2019).

TRYPTANTHRIN

Tryptanthrin, an indoloqinazoline alkaloid, was isolated initially by sublimation of natural indigo. Following this, the compound was isolated from other natural sources like *Isatis, polygonim* and *Wrightia* species. The compound exhibits antimicrobial, anti-inflammatory, antiprotozoan and antiparasitic activity. Tryptanthrin was also found to be effective against intestinal disorders and allergy.

Multiple studies have demonstrated the chemopreventive efficacy of tryptanthrin. The efficacy of tryptanthrin as a primary chemopreventive was first studied in animal models of intestinal tumorigenesis. In this study, the effect of tryptanthrin in suppressing azoxymethane-induced intestinal tumors in F344 rats was compared to that of a crude ethyl acetate extract of *Polygonumtinctorium Lour*. Animals were administered with either 15 mg/kg of Azoxymethane for 3 weeks to induce atypical crypt foci or 7.5 mg/kg of Azoxymethane for 10 weeks to induce intestinal tumors. It was observed that in the short-term experiment, the incidence of atypical crypts was significantly lower in both the treatment groups when compared to the control. Similarly, in the long term study, the treatment groups had low intestinal tumor burden when compared to the control (Koya-Miyata et al., 2001).

We have demonstrated the potency of tryptranthrin in suppressing the promotion stage of skin carcinogenesis. Here, tryptanthrin at a dose of 1 mg when applied before each application of PMA on DMBA-initiated skin could suppress tumor burden in terms of both tumor size and number. Though we have observed 100% incidence in the treatment group at the end of the experiment, there was significant delay in tumor incidence and this could be many years when extrapolated in a clinical setting. Mechanistic evaluation showed its ability to suppress the proliferation of hair-follicle cells and downregulated key pro tumorigenic signaling pathways *viz*., MAPK, β-catenin. Tryptanthrin could also accomplish reduction in PMA-induced immune cell infiltration into the epidermis. Further, we demonstrated that the compound could exert anti-cancer activity comparable to 5-FU against the epidermoid cancer cell line, A431 (Shankar G et al., 2020).

The efficacy of tryptanthrin as an anticancer compound has also been studied. Tryptanthrin exhibits appreciable activity against cancers of different origins like blood, lung, colon etc. Tryptanthrin at low concentrations induced the differentiation of monocytic and promyelocyticleukemia cells while it induces caspase-mediated apoptosis at higher concentrations (Kimoto et al., 2001). Tryptanthrin was found to exhibit moderate anti-cancer effect against lung cancer cells (Yang et al., 2013). The compound also exerts significant anticancer effect against the neuroblastoma cell line, LA-N-1 by attenuating the expression of N-myc (Liao and Leung 2013).

Tryptanthrin affects a wide variety of cellular processes that are implicated in many diseases. Tryptanthrin is known to exert a protective effect against hepatocyte stress by affecting ERK2 and Nrf2 pathways (Moon et al., 2014). Similarly, *Indigo naturalis*, in which tryptanthrin is one of the bioactive compounds, is known to protect keratinocytes from oxidative stress, by abrogating intracellular ROS formation (Lin et al., 2013).

Tryptanthrin is known to inhibit the activity of COX-2 with the inhibitory potential comparable to standard COX-2 inhibitors like NS-398 and nimesulide (Danz et al., 2001). In concordance to this observation, another study reported that tryptanthrin can inhibit inflammation by suppressing the formation of prostaglandins and leukotrienes (Danz et al., 2002). Tryptanthrin was also found to exert inhibitory activity against 5-LOX, another mediator of inflammation (Danz et al., 2002). In another study, tryptanthrin was found to exert anti-inflammatory activity in murine macrophage-like RAW 264.7 cells by lowering the expression of iNOS through down-regulation of NF-κB (Ishihara et al., 2000).

Tryptanthrin is reported to be an inhibitor of angiogenesis, a vital process in cancer progression. In this study, tryptanthrin was found to inhibit the proliferation, migration and tube formation of human microvascular endothelial cells (HMEC-1) in a concentration dependent manner. The compound was found to suppress angiogenesis by inhibitingVEGFR2-ERK1/2 signaling (Liao et al., 2013). The study showed that VEGFR2 is a direct molecular target of tryptanthrin.

DERIVATIVES OF TRYPTANTHRIN

Multiple structural derivatives of tryptanthrin and their nanoformulations have been synthesized and their activities against cancer were assessed by many researchers. These studies reveal the enhancement of the activity of tryptanthrin after structural modification.

A bromo analogue of tryptanthrin (TBr) was found to induce apoptosis in leukemia cell lines. Treatment of the leukemia cell line, HL-60 with TBr caused the inactivation of STAT-3 through a ubiquitin dependent mechanism (Pathania et al., 2014). In another study the copper derivative of tryptanthrin (Try-Cu) and bromotryptanthrin (BrTry-Cu) was tested for their cytotoxic activity against four cancer cell lines, BEL-7402, T-24, MGC80-3 and Hep-G2 (Qin et al., 2018). It was observed that Try-Cu exhibited appreciable anti-cancer activity against all for cancer cells while being non-toxic to the normal cell line, HL-7702. Another study revealed the potential of platinum complexes of tryptanthrin in exerting anti-cancer activity against the human bladder cancer cell line, T-24 without affecting the normal cells. Benzo(b)tryptanthrin, the benzo-annulated derivative of tryptanthrin exerts cytotoxicity in several human cancer cells. Benzo(b)tryptanthrin induced apoptosis through the activation of caspase-3 in the colon cancer cell line, HCT15. Benzo(b) tryptanthrin also reversed adriamycin resistance in breast cancer cells by down-regulation of multidrug resistance protein 1 (MDR1). Interestingly, this effect was much better than that displayed by tryptanthrin (Jun et al., 2015).

A study on the pharmacokinetic properties of tryptanthrin showed that oral administration of 80 mg/kg of the compound to mice models yielded maximum plasma concentration of 3.13 μg/ml, and the maximum concentration was reached within 2.5 h (Zhang et al., 2017). Attempts have been made for improving the therapeutic efficiency of tryptanthrin by improving its pharmacokinetic properties. Fang et al., encapsulated tryptanthrin in various nanoparticles like solid lipid nanoparticles, nanostructured lipid carriers and lipid emulsions. Among the three systems, the release rate of tryptanthrin was found to be maximum in nanostructured lipid carriers. The cytotoxicity against MCF-7 cells were significantly increased upon treatment with nanoparticle encapsulated tryptanthrin, suggesting that encapsulation of the compound in nanoparticles can improve drug delivery and facilitate the sustained release of the compound to the cells (Fang et al., 2011).

KAEMPFEROL

Kaempferol [3,4′,5,7-tetrahydroxyflavone-(MW: 286.2 g/mol)] is a major flavonoid aglycone found in many natural products. Kaempferol displays several pharmacological properties, such as antimicrobial, anti-inflammatory, antioxidant, antitumor, cardioprotective, neuroprotective, and antidiabetic activities. We have done an overview of its major applications in the field of cancer therapy.

Kaempferol has been shown to effectively inhibits the growth of breast cancer cell lines (VM7Luc4E2, MDA- MB-231, MCF-7) (Azevedo et al., 2015). In the highly invasive breast cancer cell line, MDA-MB-231, kaempferol did significantly inhibit MMP-3 protein activity in a dose-dependent manner, which accounts for its anti-metastasis property (Ferraz da Costa et al., 2020). It has been reported that kaempferol inhibits both growth and migration of glioma cells, *in vitro* (Jeong et al., 2009). Kaempferol activates the IRE1-JNK-CHOP signaling from cytosol to nucleus, and G9a inhibition, which activates autophagic cell death in gastric cancer cells (Kim et al., 2018). Our laboratory has characterized kaempferide, a methyl derivative of kaempferol, isolated from the plant, *Chromoleana odorata*. Among the cancer cell lines that were screened against the compound, the cervical cancer cell line, HeLa was the most sensitive. Kaempferide is pharmacologically safe in murine models and exhibits excellent anti-tumor efficacy *in vivo* (Nath et al., 2015).

Kaempferol significantly inhibits the proliferation of human hepatic cancer cell lines (HepG2, SK-Hep-1, Huh7) (Mylonis et al., 2010) and human colorectal cancer cell lines (HCT116, HT-29, HCT-15, LS174-R colon, and SW480). Kaempferol treatment increases membrane-bound FAS ligand levels, decreases intact caspase-8 and Bid, and increases cleavage of caspase-8 in human colon cancer cells indicating that kaempferol-induced apoptosis is associated with the activation of cell surface death receptors and the mitochondrial pathway (Lee et al., 2014). Experimental studies combining kaempferol with 5-Fluorouracil in LS174-R cells has revealed interesting antiproliferative effects (Riahi-Chebbi et al., 2019). Experiments using human ovarian cancer cell lines (A2780/CP70, A2780/wt, SKOV-3, OVCAR-3) have shown that kaempferol could inhibit tumor growth, proliferation, and angiogenesis by decreasing vascular endothelial growth factor (VEGF) expression (Luo et al., 2009).

Kaempferol has the capacity to decrease the production of free radicals and reactive oxygen species (ROS). ROS production inhibition can reverse malignant cancer cell phenotype. Kaempferol can control the cancer through its antioxidative property by inhibiting the NF-κB pathway and up-regulating the Nrf2 transcriptional pathway (Saw et al., 2014).

Kaempferol appears to inhibit VEGF expression and angiogenesis through an ERK-NFκB-cMyc-p21 pathway. Kaempferol administration has been shown to discourage ERK phosphorylation as well as NFkB and c-Myc expression, the reduction of which promotes p21 expression. p21 is a tumor suppressor protein known to antagonize VEGF secretion (Luo et al., 2012). A diet high in flavanols, especially kaempferol, has been found to correlate with reduced serum interleukin-6 levels, an inflammatory cytokine. In HEK 293 cells, kaempferol blocked both TNF-induced IL-8 promoter activation, but also IL-8 gene expression. IL-8 has been found to be a potent enhancer of angiogenesis (Bobe et al., 2010).

Studies on the *in vitro* and *in vivo* pharmacokinetics of kaempferol commonly ingested as high polarity glycosides has revealed that this polyphenol is poorly absorbed compared to the aglycones with intermediate polarity (Boadi et al., 2020). Kaempferol shows very low bioavailability of approximately 2%. Nano research had also been conducted focusing on enhancing the bioavailability of kaempferol specifically. The PEO-PPO-PEO (Poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) formulation and the PLGA (Poly (DL-lactic acid co-glycolic acid) encapsulated kaempferol have been shown to improve efficacy of the compound in preferentially killing malignant cells (Chen and Chen, 2013).

Moreover, the inhibitory effect of kaempferol on NF-κB was revealed in a molecular docking study where kaempferol was compared with MG-132, a known inhibitor of NF-κB. In silico calculations suggest that kaempferol inhibits the DNA binding pf NF-κB by intercalating into DNA thus accounting for its anti-inflammatory and anticancer activities (Fiedler et al., 1998; Kadioglu et al., 2015).

GINGEROL

Gingerol [5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decan-3-one], is an aromatic ketone, and the most abundant constituent of the fresh roots and rhizomes of ginger plant, Zingiber officinale (Zhang et al., 2021). Gingerol is responsible for the strong pungency of ginger and is one of the major active components of the plant. Though several derivatives of gingerol are present in ginger, 6-gingerol is the most abundant among them. Gingerol has a wide array of pharmacologic effects. It is highly effective against chemotherapy related nausea and vomiting. Gingerol possesses anti-cancer, antioxidant, anti-angiogenic, anti-atherosclerotic anti-spasmodic and hepatoprotective potentials. 6-Gingerol possesses remarkable anticancer potential and it affects a variety of biological pathways involved in apoptosis, cell cycle regulation, cytotoxic activity, and inhibition of angiogenesis.

Studies conducted in our laboratory have revealed that (6)-gingerol induces caspase-dependent apoptosis in colon cancer cells and prevents PMA-induced proliferation through inhibition of MAPK/AP-1 signaling. The underlying mechanism was found to be the down-regulation of PMA- induced phosphorylation of ERK1/2 and JNK MAP kinases and activation of AP-1 transcription factor. However, it showed only little effects on phosphorylation of p38 MAP kinase and activation of NF-κB (Radhakrishnan et al., 2014). Nigam et al., have reported that (6)-gingerol mediated induction of apoptosis is associated with the modulation of p53 and involvement of mitochondrial signaling pathway in B [a]P-induced mouse skin tumorigenesis (Nigam et al., 2010). (6)-gingerol has also been proven to impede hamster buccal pouch carcinogenesis associated with chemically-induced inflammation and cell proliferation via the modulation of Nrf2 signaling (Sun et al., 2021). Gingerol elicits its protective activity through different mechanisms and cell signaling pathways, of which, MAPK, NF-κB, Wnt/β-catenin, Nrf2/ARE, TGF-β1/Smad3, and ERK/CREB are prominent (Yahyazadeh et al., 2021).

(10)-Gingerol, a derivative of gingerol has been shown to improve the anti-cancer efficacy of doxorubicin and ameliorate the side effects caused by the drug in triple negative breast cancer models (Martin et al., 2020).

Gingerol and shogaol, and other structurally-related substances inhibit the biosynthesis of prostaglandin and leukotriene. They are also capable of inhibiting the synthesis of pro-inflammatory cytokines such as IL-1, TNF-α, and IL-8 (Shukla and Singh 2007; Ray et al., 2015).

Molecular docking studies between four ginger ligands, namely, 6-gingerol, 8-gingerol, 10- gingerol, 6-shogaol and identified cancer targets such as EGFR, C-Met, PI3K, COX-2, NF-kB, and AP-1 suggests that 6-gingerol is more effective as an anticancer phytocompound among ginger ligands (Kumara et al., 2017).

It is well known that (6)-gingerol efficiently scavenges chemical carcinogens, especially those belonging to the epoxy type. The pharmacokinetics underlying adduct-formation by (6)-gingerol has been investigated (Furlan and Bren 2020). The study focused on the changes in the activation free energy of the rate-limiting step of the alkylation reactions of (6)-gingerol with nine epoxy type chemical carcinogens. The activation barrier i.e., $\Delta G^{\square}$, for the reaction between natural scavengers and chemical carcinogen is much lower than that of the competing reaction between the chemical carcinogen and nucleophilic DNA base, guanine. Hence, (6)-gingerol confers protection against carcinogen-mediated DNA alkylation and prevents initiation of cancer by virtue of its lower activation barrier and the resultant faster reaction rate *via SN2* reaction mechanism. An independent study has reported the pharmacokinetics of various derivatives of gingerol using HRMS analytical method which was followed by oral administration of ginger extract in rats to assess the distribution of gingerol derivatives in tissues. Furthermore, they quantified the concentration of 6-gingerol, 6-shogaol, 8-gingerol, 8-shogaol, 10-gingerol and 10-shogaol in the plasma and tissues of rats. The results illustrated that 6-gingerol, 6-shogaol, 8-gingerol, 8-shogaol, and 10-gingerol are rapidly absorbed into the circulatory system, but, 10-shogaol is poorly absorbed in comparison to the other compounds upon administration of ginger extract orally. It was also observed that 6-shogaol failed to penetrate the blood–brain barrier and enter the brain (Li et al., 2019). However, the pharmacokinetics of gingerol and its derivatives needs to be explored in further detail.

EMODIN

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone present in the roots and barks of plants such as *Cassia obtusifolia, Fallopia japonica, Polygonum cuspidatum*, and *Rheum palmatum* (Dong et al., 2016). It is also present in certain species of moulds and lichens. Emodin is an active constituent of numerous Chinese medicinal herbs. Emodin is a well- known tyrosine kinase inhibitor and displays an inhibitory effect on mammalian cell cycle modulation in specific oncogene over-expressed cells (Sakalli-Tecim et al., 2021). Emodin is known to inhibit angiogenesis and metastasis processes which make it a promising candidate for chemoprevention.

Several studies have demonstrated the chemopreventive potential of emodin. The anti-tumor promoting effect of emodin was elucidated using two-stage chemically induced carcinogenesis models of skin tumor in mice (Koyama et al., 2002). Huang et al., have demonstrated emodin-mediated inhibition of HSC5 and MDA-MB-231 cell invasion by inhibiting AP-1 and NF-κB signaling pathways (Huang et al., 2004). Previous reports illustrate the ability of emodin to directly target androgen receptor and in turn suppress prostate cancer cell growth *in vitro* and prolong the survival of C3 (1)/SV40

transgenic mice *in vivo*. It is speculated that emodin treatment represses the androgen-dependent transactivation of androgen receptor (AR) by inhibiting nuclear translocation of AR (Cha et al., 2005). Studies by Shimpo *et al.*, have revealed that dietary administration of low dose of aloe emodin, a derivative of emodin exerts chemopreventive effects against development of colorectal tumor in mice by reducing cell proliferation in colorectal mucosa (Shimpo et al., 2014). Emodin at concentrations of 10–20 μM has been reported to trigger apoptosis of IMR-32 cells *via* an apoptotic signaling cascade which sequentially involves ROS, Ca^{2+}, NO, p53, caspase-9 and caspase-3 (Huang et al., 2013). Epstein-Barr virus (EBV) lytic replication plays an important role in the pathogenesis of nasopharyngeal carcinoma. Emodin inhibits the tumorigenic properties induced by repeated EBV reactivation, which encompasses micronucleus formation, cell proliferation, migration, and matrigel invasiveness and repression of tumor growth in mice which is induced *via* EBV activation (Wu et al., 2019). Previous studies illustrate that emodin potentiates apoptosis in a p53-dependent manner in SK-HEP-1, PLC/PRF/5, and HepG2/C3A cells (Shieh et al., 2004). Emodin is believed to function as a Janus-activated kinase 2 inhibitor, which accounts for its cytotoxic effects against multiple myeloma (Muto et al., 2007). Emodin has been reported to prevent lipid raft coalescence in HepG2 cells, impede the gathering of integrin in HeLa cells and restrict the formation of focal adhesion complex (FAC) in MDA-MB-231 cell lines (Huang et al., 2006). There is documented evidence that emodin treatment stimulates Cyt- c release and activates caspase-2, -3, and -9. Emodin treatment in A549 cells resulted in the inactivation of AKT and ERK and formation of ROS, Further, it disrupted the mitochondrial membrane potential and reduced the levels of mitochondrial Bcl-2 and increased the mitochondrial Bax levels (Akkol et al., 2021). Studies conducted in our laboratory has proved that emodin-induces caspase-dependent apoptosis in human cervical cancer cells presumably through the mitochondrial pathway (Srinivas et al., 2003).

Emodin chemosensitizes a wide spectrum of chemotherapeutic drugs including paclitaxel, platinum drugs, 5-FU and As2O3 (Vinod et al., 2013). Several reports have elucidated the chemosensitizing efficacy of emodin in various types of cancer which include melanoma, pancreatic, ovarian, renal, cervical, colorectal, prostrate and lung cancer.

Reversal of multidrug resistance, induction of autophagy and apoptosis are the major pharmacological roles of anthraquinones in cancer cells (Liu et al., 2020). Guo et al., have documented that emodin mediated inhibition of MDR1/P-glycoprotein and expression of MRPs alleviates gemcitabine resistance in pancreatic cancer (Guo et al., 2020).

Previous studies have speculated that the immunosuppressive effect of emodin is mediated through hydrogen peroxide and regulated by the by products of arachidonic acid metabolism (Huang et al., 1992).

Emodin treatment decreased the mutagenicity of benzo (a) pyrene [B (a)P], 2-amino-3-methylimidazo (4,5-f)quinoline (IQ) and 3-amino-1-methyl-5H-pyrido (4,3-b) indole (Trp-P-2). in a dose-dependent manner in *Salmonella typhimurium* TA98. This was achieved through emodin-mediated direct inhibition of the hepatic microsomal activation (Lee and Tsai, 1991). Previous studies have attributed the poor oral bioavailability of emodin to its glucuronidation metabolism. Shia et al., have studied the *in vivo* levels of emodin after intagastric administration in rats and they observed that the detected *in vivo* emodin levels remained extremely low (Shia et al., 2010). Another study revealed that piperine could considerably increase the Cmax and area under concentration-time curve (AUC) of emodin and cause a simultaneous decrease in the AUC and Cmax of emodin glucuronide (Di et al., 2015).

QUERCETIN

Quercetin, also known as 3, 3, 4, 5, 7—pentahydroxyflavone, is a flavonoid generally present in several fruits, vegetables, leaves, seeds and grains, where it is conjugated with residual sugars such as glucose, rutinose, or xylose to form quercetin glycosides. Several reports suggest that quercetin can induce cell cycle arrest and apoptosis by virtue of its antioxidant (Robaszkiewicz et al., 2007), anti-inflammatory and immune protective effects (Nair et al., 2002). Quercetin and its derivatives being naturally occurring phytochemicals with promising bioactive effects, its intake via diet or food supplements might ensure a protective effect.

In vitro studies conducted by Senthilkumar et al., presents evidences of an inhibitory effect of quercetin on androgen independent prostate cancer, where quercetin was found to modulate the expression of the components of IGF system leading to apoptosis (Senthilkumar et al., 2010). Quercetin was also reported to significantly reduce the level of VEGF-3 in PC-3 cells, indicating its antiangiogenic properties (Pratheeshkumar et al., 2012).

Chemopreventive effect of quercetin in *in vivo* model of prostate cancer has been demonstrated for the first time by Sharmila et al., in Sprague Dawley rats. Animals were given a periodical administration of the carcinogen N-nitroso-N-methyl urea (MNU) and hormone testosterone with a simultaneous supplementation of quercetin (Sharmila et al., 2014). The study revealed that quercetin supplementation decreases the expression of IGF-1R, by reducing pAkt, Raf-1 and pMEK protein expressions in comparison with the cancer induced rats. Over expression of IGF-1/IGF-1R has been found to result in the initiation of prostate cancer (Cox et al., 2009) with the PI3/Akt and Ras/Raf/MEK/MAPK being the major pathways associated with the activation of the same (Ozkan, 2011).

Quercetin has previously been reported to affect the signal transduction pathways involved in the process of carcinogenesis which eventually result in the induction of apoptosis and inhibition of cell proliferation (Sun et al., 2010). Similar studies were conducted by other groups in DMBA -treated hamsters with the aim of investigating the chemopreventive efficacy of quercetin on oral squamous cell carcinoma (OSCC) and its mechanism of action. The study has reported that the animals in the groups that received medium (25 mg/kg) to high (50 mg/kg) doses of quercetin showed no tumor development

(Zhang et al., 2017). The study suggests that the chemopreventive effect displayed by quercetin in the DMBA-induced carcinogenesis model could be on account of suppression of NF-κB pathway by quercetin, followed by the modulation in the expression of NF-κB target genes Bax and Bcl-2, which led to apoptosis and tumor regression.

Oxidative stress plays a central role in cancer development and progression as it promotes damage to proteins, lipids, membranes and DNA alike (Khandrika et al., 2009). The anticancer potential of quercetin can be attributed to various mechanisms, such as the induction of cell cycle arrest and/or apoptosis, as well as its antioxidant properties. Quercetin has been shown to exhibit its antioxidant activity through its regulatory effect on glutathione (GSH), enzymatic activity, signal transduction pathways and reactive oxygen species (ROS) (Xu et al., 2019).

Interaction of quercetin with cell cycle regulatory proteins triggers a G2/M phase cell cycle arrest through the activation of the transcription factor p53, that has been suggested as a potential target for cancer therapy (Haupt et al., 2003). Quercetin is reported to induce a p53-p21 mediated cell cycle arrest at the G2/M phase and to suppress the NF-κB pathway, thus inhibiting the proliferation of HeLa cells (Priyadarsini et al., 2010). In another study, quercetin has been shown to induce apoptosis and antioxidant activity by two-fold in colon cancer cells (Atashpour et al., 2015).

Along with the antioxidant, anti-inflammatory and immunoprotective effects, quercetin has also been reported to show antimutagenic properties which may account for its role in chemoprevention. Shivakumar et al., have demonstrated the protective role of quercetin against a set of mutagens using a series of tests including Ames test, mice bone marrow micronucleus test, cell gene mutation test and chromosomal aberration test. According to this study Quercetin displays significant antimutagenicity against several mutagens such as sodium azide, benzo(a)pyrene, cyclophosphamide monohydrate, methyl methane sulphonate and etoposide (Shivakumar et al., 2017).

GENISTEIN

Genistein, (4′, 5, 7-trihydroxyisoflavone), an isoflavone with a heterocyclic diphenolic structure found in soy-based foods and legumes, has been extensively investigated to determine its chemopreventive and therapeutic activities (El-Rayes et al., 2011).

Several antitumor studies have shown that genistein inhibits the process of carcinogenesis through cell cycle regulation, induction of apoptosis, modulations in the signal transduction pathways and inhibition of angiogenesis.

Studies have demonstrated the chemoprotective effect of genistein against breast cancer, irrespective of the receptor status of the human breast cancer cell lines (Shon et al., 2006). COX-2 overexpression and increased CYP1A1 and ornithine decarboxylase (ODC) activity are frequently observed patterns in human breast cancer (Half et al., 2002; Deng et al., 2008; Androutsopoulos et al., 2009). Reports suggest that genistein inhibits the expression and activity of COX-2, CYP1A1 and ODC indicating the potential of genistein to be used as a chemopreventive.

A clear dose dependent antimutagenic effect of genistein has been reported against the mutagens, Aflatoxin B1 (AFB_1), 3-methylimidazo (4, 5-f) quinoline (IQ) and N-nitroso-N-methyl urea (MNU) (Polivkova et al., 2006). Genistein also exhibited dose dependent inhibition of mutagenicity of PhIP 2-amino-1-methyl-6-phenylimidazol (4, 5-b) pyridine, a heterocyclic amine (Weisburger et al., 1998).

Another study has found that genistein induces apoptosis in colon cancer cells by up-regulating caspase-3 gene expression and inhibiting the proliferation and migration of the cancer cells The study also reports a down-regulation of p38 MAPK gene expression and a decrease in the level of p38 MAPK protein by genistein in colon cancer cells (Shafiee et al., 2016).

Anticancer efficacy of genistein has been demonstrated in preclinical models of gastric cancer. The study reveals that genistein mediates the down-regulation of the expression of the antiapoptotic protein B cell lymphoma 2 (Bcl-2) and up-regulation of the expression of proapoptotic Bcl-2 associated X protein (Bax) (Zhou et al., 2004). In another study, human gastric cancer cells (SGC7901) were injected subcutaneously in nude mice followed by direct administration of different doses of genistein at a site adjacent to the tumor. A decrease in tumor size was observed in all groups administered with genistein. The study revealed that genistein induces apoptosis by decreasing the Bcl-2/Bax ratio, suggesting its efficacy against preventing gastric carcinogenesis (Zhou et al., 2008). Another study has demonstrated the pro-apoptotic and antiproliferative effect of genistein against gastric carcinogenesis. Here genistein suppressed the NF-κB pathway, consequently reducing the levels of COX-2 (Li et al., 2011).

Genistein has been reported to exhibit its anti-inflammatory effect by inhibiting the expression of inflammatory cytokines (Jeong et al., 2014). More recently, the anti-inflammatory and anticancer effect of long-term genistein treatment was reported in diethyl nitrosamine-induced liver carcinogenesis model. A consistent increase in the levels of phospho-AMPK has been reported along with a down-regulation of the pro-inflammatory cytokines, TNF and IL-6. Genistein was also found to increase the level of p53, leading to the induction of apoptotic markers. Altogether, these results indicate that long-term dietary intake of genistein would aid in the prevention of hepatocellular carcinogenesis (Lee et al., 2019).

The inhibitory effect of genistein on NF-κB was further explored by molecular docking analysis, where the binding interaction of genistein with the active sites of NF-κB proteins was studied. The findings from the in silico analysis suggested that the amino acids (Lys52, Ser243, Asp274, Lys275) might play a pivotal role in anti- breast cancer activity (Mukund 2020).

Research findings also revealed an antimetastatic role of genistein in colon cancer cells and salivary adenoid cystic carcinoma cells. Genistein inhibited COX-2, MMP9, Ang-1, vasodilator-stimulated phosphoprotein and VEGF in HCT116 (Kang et al., 2018). Similarly, a decrease in the expression of VEGF and MMP-9 was observed in salivary adenoid cystic carcinoma, following treatment with genistein (Liu and Yu, 2004).

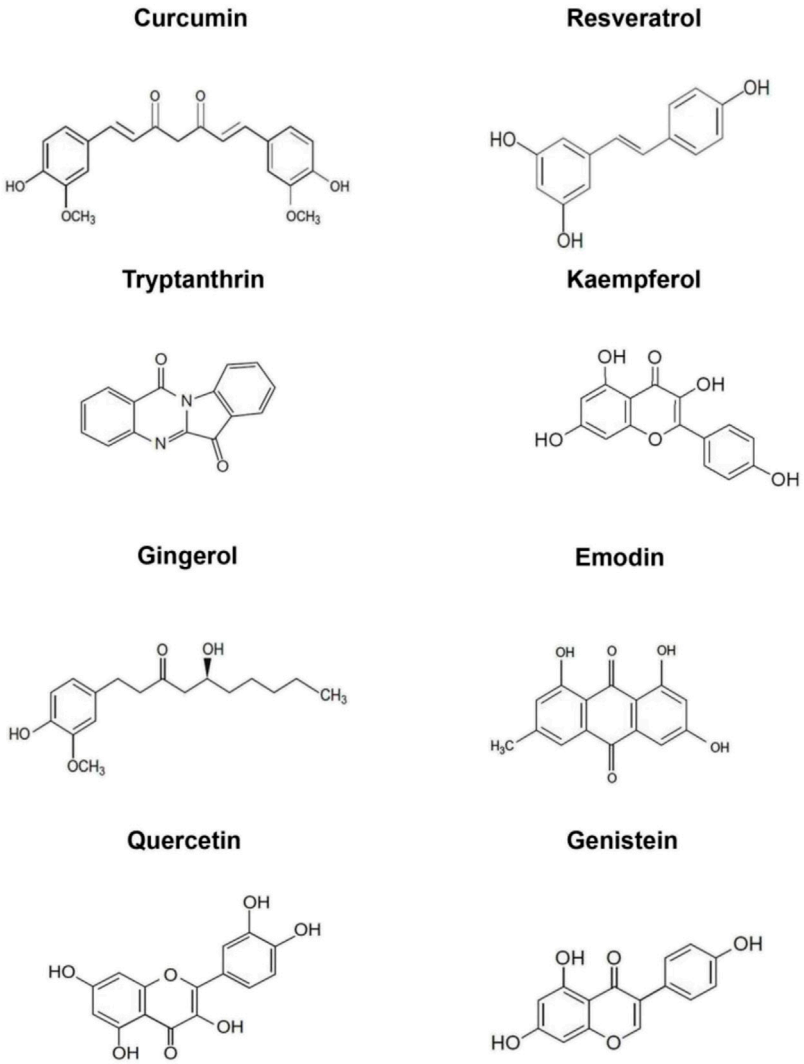

FIGURE 3 | Chemical structures of the phytochemicals analysed as chemopreventives in the current study. i) Curcumin ii) Resveratrol (Nair et al., 2021) iii) Tryptanthrin (Shankar et al., 2015) iv) Kaempferol (Silva dos Santos et al., 2021) v) Gingerol vi) Emodin vii) Quercetin viii) Genistein (Nair et al., 2021).

Figure 4 is a graphical representation of the inhibition of major tumorigenic factors by the phytochemicals.

Figure 5 is a graphical account of the major signalling events modulated by the chemopreventives discussed in this report.

Table 3 summarizes the important signaling pathways and key target molecules modulating the chemopreventive potential of phytochemicals.

Table 4 provides a brief description of the important studies conducted for improving the pharmacokinetics of the chemoprevention strategies of the prospective phytochemicals.

CHALLENGES AND FUTURE DIRECTIONS

The idea of chemoprevention, especially primary chemoprevention, requires identification of a population susceptible to a particular type of cancer. Depending on genetic, epigenetic, dietary habits and medical history, individuals vary in their susceptibility towards developing cancer when exposed to a carcinogen. Hence, a more accurate identification of susceptible individuals based on these parameters is essential for initiating a chemoprevention intervention. In addition, narrowing down the timeframe to estimate the time point at which the intervention has to be commenced presents a significant impediment to the whole idea. Moreover, the appropriate dose of the chemopreventive agent must also be estimated. This is important as inappropriate usage might result in highly deleterious side effects. For example, chronic use or high doses of aspirin may result in gastric haemorrhages (Harker et al., 1999); other chemopreventives like tamoxifen also has side effects (Yang et al., 2013). Considering this, discovery of biomarkers to more accurately identify and stratify individuals according to risk of cancer

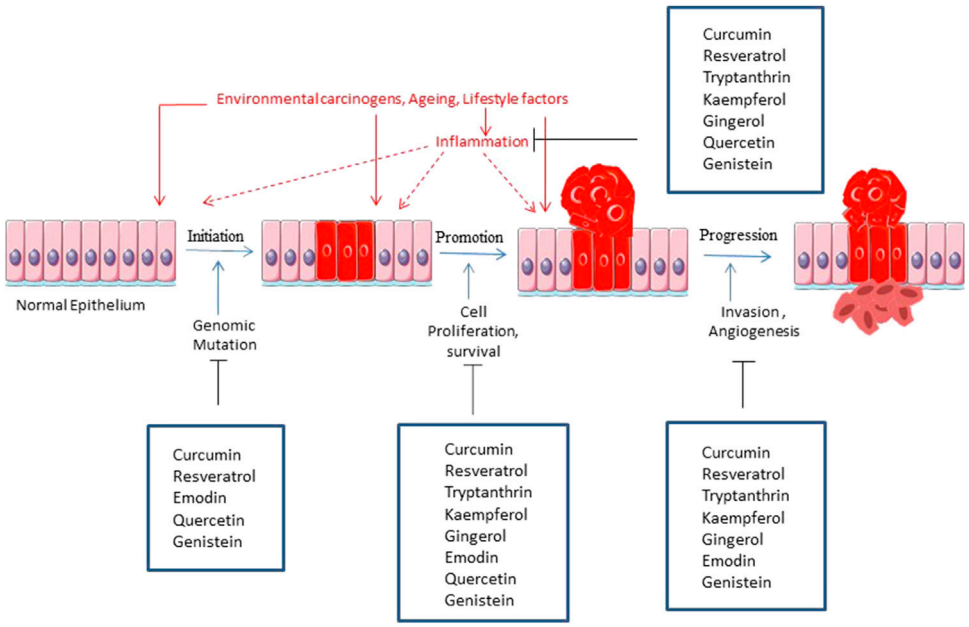

FIGURE 4 | Illustration of various steps of carcinogenesis inhibited by the phytochemicals.

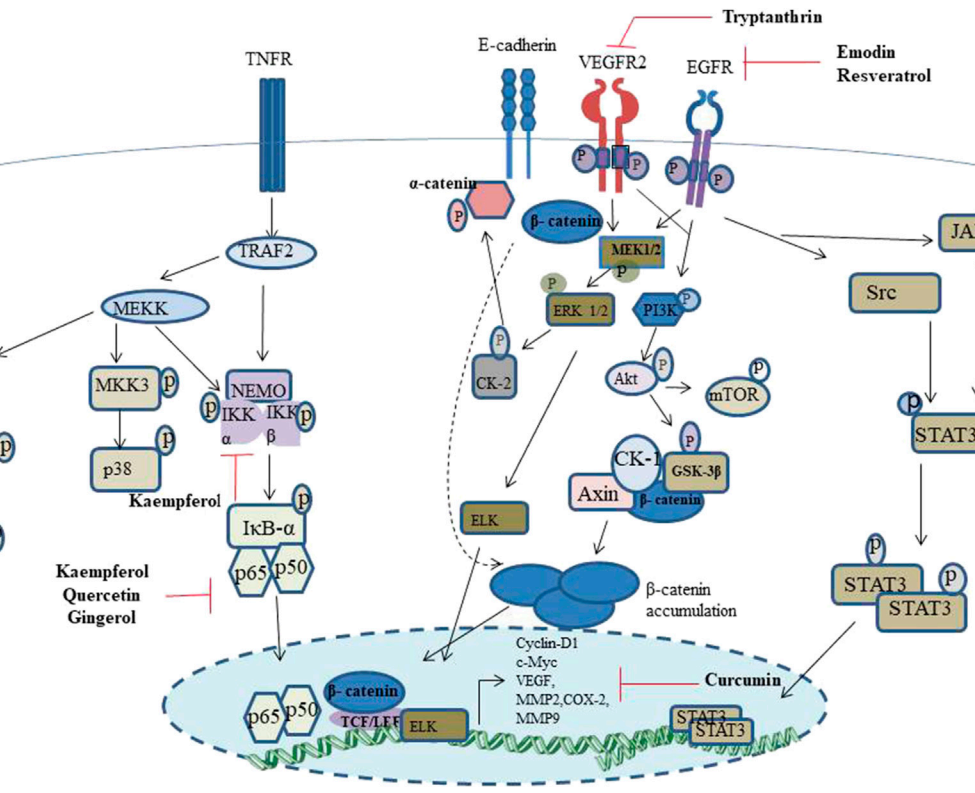

FIGURE 5 | Schematic representation of the major signalling events regulated by the phytochemicals discussed in the current report.

TABLE 3 | Signaling pathways modulated by the phytochemicals explored as chemopreventives and their molecular targets in different cancers.

Compound	Molecular targets	Cancer	Pathway	References
Tryptanthrin	VEGFR2 Liao et al. (2013)	Chronic myeloid leukemia	Bax, Bcl2, Cytochrome C, Caspase 3	Miao et al. (2011)
		Neuroblastoma	N-myc	Liao and Leung (2013)
		Breast cancer	MDR1, p53, P-glycoprotein, GST II, JNK	Yu et al. (2007), Yu et al. (2009)
		Monocytic and Promyelocytic leukemia	Caspase 3/FAS Antigen pathway	Kimoto et al. (2001)
		Epithelial colorectal adenocarcinoma	P-gp, MRP-2	Zhu et al. (2011)
		Skin cancer	MAPK, β-catenin	Shankar G et al. (2020)
Curcumin	M-CSF 1 receptor, aldo-keto reductase family 1 member C3, amiloride-sensitive amine oxidase and tyrosine-protein phosphatase non-receptor type 11 MMP-2, NAD-dependent protein deacetylase sirtuin-2, core histone macro-H2A.1, NAD-dependent protein deacetylase sirtuin-1 and epidermal growth factor receptor Furlan et al. (2018)	Myeloid leukemia	Cytochrome C-PARP-Caspase 9 cleavage	Mutlu Altundağ et al. (2021)
		Melanoma	ROS-GSH-MMP	Liao et al. (2017)
		Gastric cancer	ROS-ASK1-JNK	Liang et al. (2014)
		Breast cancer	Cyclin B1, CDC2, NF-κB	Akkoç et al. (2016)
	Phosphodiesterase 4 (PDE4) Furlan and Bren (2021)	Glioblastoma	MAPK, NF-κB, JAK/STAT3-IAP	Gersey et al. (2017)
		Liver cancer	MAPK, NF-κB, JAK/STAT3-IAP	Marquardt et al. (2015)
Kaempferol	NF-κBIKK Kadioglu et al. (2015)	Breast cancer	MAPK-ERK-MEK1&ELK1 AP-1-Cathepsin B&D, MMP-2, MMP-9	Kim et al. (2008)
		Brain cancer	Bcl2-Cleaved caspase 3,8, XIAP, Cleaved PARP	Sharma et al. (2007)
		Liver cancer	STAT3-CDK1, Cyclin B, PI3K/AKT/mTOR	Huang et al. (2013)
		Gastric cancer	IRE1-JNK-CHOP	Kim et al. (2018)
		Lung cancer	AKT/PI3K&ERK PTEN, Bax, miR-340, Fas, cleaved caspase3,8,9 and cleaved PARP	Nguyen et al. (2003), Han et al. (2018)
		Pancreatic cancer	EGFR, AKT, Src, ERK1	Lee and Kim (2016)
		Stomach cancer	COX2, Bcl2, p-ERK,p-AKT, Bax, Cleaved caspase 3,9	Song et al. (2015)
		Oral cancer	MMP2, TIMP2, C-jun, ERK1/2	Lin et al. (2013)
Resveratrol	LSD1 NTMT1 BIRC4 Kores et al. (2019) EGF Kumara et al. (2017)	Breast cancer	p53, AKT, NF-κB	Athar et al. (2009)
		Prostate cancer	PI3K/AKT, AMPK	Rashid et al. (2011)
		Colon cancer	AMPK, p53	Liu et al. (2019)
		Pancreatic cancer	NF-κB	Mouria et al. (2002)
		Ovarian cancer	ERK, NF-κB	Tino et al. (2016)
		Epidermoid carcinoma	MEK-1, AP-1	Kim et al. (2006)
		Osteosarcoma	pERK1/2	Alkhalaf and Jaffal (2006)
		Squamous cell carcinoma	MEK, VEGF, AKT	Athar et al. (2009)
		Leukemia	mTOR and p38 MAPK	Jiao et al. (2013)
		Lung cancer	mTOR and p38 MAPK	Wang et al. (2018)
Gingerol	PI3K NF-κB C-Met COX2 Kumara et al. (2017) LTA_4H (El-Naggar et al., 2017)	Cervical cancer	PI3K/AKT AMPK mTOR	Zhang et al. (2021)
		Skin cancer	COX-2, NF-κB and p38 MAPK	Kim et al. (2005)
		Breast cancer	PI3K/AKT and p38 MAPK	Joo et al. (2016)
		Colon cancer	LTA_4H	Jeong et al. (2009)
Emodin	FGFR2 Chen et al. (2013)	Colorectal cancer	VEGF, Wnt	Dai et al. (2019), Gu et al. (2019)
		Breast cancer	ERα-MAPK, AKT-Cyclin D1/Bcl-2 Her2	Zhang et al. (1995), Sui et al. (2014)
		Cervical cancer	PI3K/AKT, TGF-β	Olsen et al. (2007)
		Lung cancer	PKC	Lee (2001)
		Pancreatic cancer	EGFR, STAT3 NF-κB	Wang et al. (2019), Tong et al. (2020)
		Head and neck squamous cell carcinoma	β-catenin, AKT	Way et al. (2014)
		Hepatocellular carcinoma	STAT3, PI3K/AKT/mTOR	Subramaniam et al. (2013)
Quercetin	NF-κB Mukund et al. (2019)	Prostate cancer	PI3/AKT Ras/Raf/MEK/MAPK	Sharmila et al. (2014)
		Oral squamous cell carcinoma	NF-κB	Sun et al. (2010)

(Continued on following page)

TABLE 3 | (*Continued*) Signaling pathways modulated by the phytochemicals explored as chemopreventives and their molecular targets in different cancers.

Compound	Molecular targets	Cancer	Pathway	References
		Gastric cancer	PI3/AKT	Shen et al. (2016)
		Brain cancer	JAK 2/STAT 3	Wang et al. (2013)
		Skin cancer	MEK, ERK, PI3/AKT	Rafiq et al. (2015)
		Mesothelioma cancer	JNK, p38, MAPK/ERK	Demiroglu-Zergeroglu et al. (2016)
Genistein	ERα Pang et al. (2018)	Breast cancer	(a) NF-κB (b) Notch 1-NF-κB	Pan et al. (2012), Mukund (2020)
		Prostate cancer	PI3/AKT	Li and Sarkar (2002)
		Colon Cancer	(a) Notch 1/NF-κB Slug/E-cadherin (b) Wnt pathway	Zhang and Chen (2011), Zhou et al. (2017)
		Endometrial cancer	AKT/mTOR, MAPK	Malloy et al. (2018)
		Esophageal cancer	JAK1/2, STAT3 and AKT/MDM2/p53	Gao et al. (2020)

TABLE 4 | Strategic approaches aimed at improving the pharmacokinetics of prospective chemopreventives.

Compound	Approach	References
Curcumin	Chitosan encapsulation	Vijayakurup et al. (2019)
	PLGA encapsulation	Nair et al. (2012)
Resveratrol	Co-administration with piperine	Johnson et al. (2011)
	Combination with Magnesium dihydroxide based formulation	—
Kaempferol	Poly (ethyleneoxide)-poly (propyleneoxide)-poly (ethylene oxide) encapsulation	Luo et al. (2012)
	PLGA encapsulation	Luo et al. (2012)
Emodin	Co-administration with piperine	Di et al. (2015)

incidence might prove to be advantageous while designing a chemoprevention regimen.

Another challenge in cancer chemoprevention is to identify the individuals who might show a positive/negative outcome following a chemoprevention intervention. For example, a study was conducted to assess the influence of genotypic variation of *NKX3.1* on prostate cancer chemoprevention in the SELECT trial. The study assessed the influence of two prostate cancer-related polymorphisms, rs11781886 and rs2228013, in the *NKX3.1* on prostate cancer incidence following selenium or vitamin E administration. Their results showed a significant influence of NKX3.1 genotypes on increased tumor incidence on subjects who took selenium or vitamin E (Martinez et al., 2014). Likewise, another study identified that common single-nucleotide polymorphisms (SNP) in proximity or in the *ZNF423* or *CTSO* genes are associated with the effectiveness of chemoprevention approaches using selective estrogen receptor modulators (SERM). Mechanistic studies showed that these genes are associated with *BRCA1* expression in a SNP-dependent manner (Ingle et al., 2013). Some studies show that vitamin intake by subjects with vitamin deficiencies leads to positive chemopreventive effects while intake by those without deficiency did not show a positive outcome.

Discovery of candidates for chemoprevention trials in the clinic is based on epidemiological observations or data from preclinical studies. However, clinical trials based on epidemiological clues have often resulted in increased tumorigenesis. This could be due to the fact that molecules often act in multiple combinations to exert a protective effect, and hence a similar effect may not be observed with single molecules. Rather than relying on epidemiological data to design clinical trials, the mechanisms that drive the progression of these tumors and the anti-cancer activity of the compounds must be studied. A caveat in cancer drug discovery is the testing of the compound of interest in an appropriate model. Choosing a model that bears appreciable similarity with human tumors is critical as tumorigenesis is a multifactorial process and hence, individual factors that may be crucial for the outcome of the result may not be easily predictable. An example for this is the unexpected observation from a preclinical study on the effectiveness of finasteride against prostate cancer. Here, while the compound did not have effect on the growth of LNCaP cells, it increased the growth of tumors that developed from a combination of LNCaP cells and fibroblasts. Considering the significance of fibroblasts in prostate cancer, combination of fibroblasts and cancer cells is a better model for prostate cancer growth than a tumor originating from cancer cells alone. Interestingly, finasteride administration increased the incidence of high grade tumors in clinical trial.

CONCLUSION

Cancer chemoprevention is a good strategy to mitigate the morbidity/mortality associated with tumor incidence. The success of chemoprevention in bringing down the mortality associated with cardiovascular disease has further reinforced the idea of implementing this strategy in lowering cancer incidence and associated mortality. Identification of major risk factors such as inherited mutations and exposure to environmental carcinogens is essential for designing appropriate chemoprevention intervention. Multiple clinical trials have shown success in lowering tumor incidence in

susceptible population; however, multiple factors like the identification of susceptible individuals, difficulty in fixing time frame for chemoprevention intervention, and risk factors associated with chemoprevention could deter the adoption of this strategy on a larger scale. For example, less than 10% of women in the high-risk group who are offered an anti-estrogen medication as a primary chemopreventive, agree to take it (Crew et al., 2017). Discovery of novel, safe and effective chemopreventives is essential for this idea to gain more acceptability. The use of appropriate preclinical models that, to certain extend, mimics human tumorigenesis could lead to more success of candidate compounds in clinical trials. This is important considering the duration of clinical trials involved in discovering cancer chemopreventives. Moreover, clinical trials for primary chemopreventives are done on an at-risk population which doesn't have tumor incidence at the time of the trial. Hence, clinical trial design must be based on sufficient experimental data regarding its safety, efficacy and mechanism of action so as to avert or minimize incidences of increased tumor burden in the treatment group.

AUTHOR CONTRIBUTIONS

GM, MS, CK, and TR collected, analyzed, and interpreted the relevant literatures; GM and MS drew all the figures and tables; GM, MS, CK, and TR wrote the manuscript; RA supervised the study and revised the manuscript. The final version of the manuscript was read and approved by all authors.

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Safranal Prevents Liver Cancer Through Inhibiting Oxidative Stress and Alleviating Inflammation

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Despite all efforts, an effective and safe treatment for liver cancer remains elusive. Natural products and their derived biomolecules are potential resources to mine for novel anti-cancer drugs. Chemopreventive effects of safranal, a major bioactive ingredient of the golden spice "saffron", were evaluated in this study against diethylnitrosamine (DEN)–induced liver cancer in rats. Safranal's mechanisms of action were also investigated in the human liver cancer line "HepG2". When administered to DEN-treated rats, safranal significantly inhibited proliferation (Ki-67) and also induced apoptosis (TUNEL and M30 CytoDeath). It also exhibited anti-inflammatory properties where inflammatory markers such as NF-kB, COX2, iNOS, TNF-alpha, and its receptor were significantly inhibited. Safranal's *in vivo* effects were further supported in HepG2 cells where apoptosis was induced and inflammation was downregulated. In summary, safranal is reported here as a potent chemopreventive agent against hepatocellular carcinoma that may soon be an important ingredient of a broad-spectrum cancer therapy.

Keywords: liver cancer, prevention, safranal, oxidative stress, inflammation

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BACKGROUND

Cancer is one of the most common causes of illness and death worldwide. In 2015, there were 17.5 million recorded cases of cancer and 9.5 million deaths from the disease, making it the second highest cause of death globally after heart disease (Zhou et al., 2016; Bray et al., 2018). Liver cancer is considered the fifth cause of death from cancer worldwide and the death rate from liver cancer represents 70% of deaths in African and Asian countries (Chessum et al., 2015; Plummer et al., 2016; Bray et al., 2018). In Egypt, for example, hepatocellular carcinoma (HCC) is responsible for about 14.8% of all cancer fatalities (Aglan et al., 2017). There are a number of factors that lead to the spread of HCC in Asia and Africa, for instance, chronic hepatitis (B and C) infection, chronic alcohol consumption, exposure to environmental pollutants such as aflatoxin, and environmental carcinogens including nitrosamines (Plummer et al., 2016; Zhou et al., 2016). Diethylnitrosamine (DEN) is considered an environmental carcinogen that we are exposed to daily since it is a component in processed food, cosmetics, gasoline, and tobacco (Park et al., 2009; Hayden and Ghosh, 2014; Tolba et al., 2015; Santos et al., 2017). Moreover, thanks to the human resemblance of lesions it induces in rats, DEN is often used to study different types of benign and malignant tumors in humans (Li et al., 2005). In fact, the initiation-promoting cancer development model, used in this study, mimics the early events of the latent period of human carcinogenesis

(Tolba et al., 2015; Santos et al., 2017). The initiation of HCC can be produced by the administration of a single dose of DEN, a carcinogen that causes DNA ethylation and mutagenesis, and the biotransformation of normal hepatocytes into initiated cells (Santos et al., 2017). To expedite HCC development, exposure to a tumor promotor such as such as 2-acetyl aminofluorene (2-AAF) helps developing altered hepatocytes foci (AHF) and hyperplastic nodules and the final progression into HCC (Tolba et al., 2015; Santos et al., 2017).

Considering their great efficacy and low toxicity, natural substances and plants have been extensively studied and proposed as a chemoprotective therapy for many diseases (Amin and Mahmoud-Ghoneim, 2011; Koh et al., 2020; Hamza et al., 2021). Anti-cancer medicinal plants are used for several reasons; they contain nutritional and anti-tumor compounds, are able to delay or prevent cancer onset, can boost physiological status and the immune system (Block et al., 2015; Zhou et al., 2016; Koh et al., 2020), and most importantly, they represent a great alternative to conventional cancer treatments by decreasing or even eliminating side effects (Zhou et al., 2016). Consequently, anti-oxidative, anti-inflammatory, and hepatoprotective properties possessed by some natural compounds qualify them as potential candidates to protect against tumor initiation and growth (Zhou et al., 2016). The use of natural-based materials (Baig et al., 2019, Benassi et al., 2021) and phytochemicals that are low in toxicity and effective at the beginning of exposure to carcinogens is considered the most successful strategy available to protect against the development of liver cancer (DiMarco-Crook and Xiao, 2015; Maru et al., 2016).

Saffron is one of the oldest spices that was used since ancient times in Egypt and Rome as both a remedy and a culinary spice (Rios et al., 1996; Winterhalter and Straubinger, 2000). It consists of the dried stigmas of *Crocus sativus* flowers and is also one of the most commonly used species in folklore medicine to treat depression, asthma, and smallpox (Siddique et al., 2020). Saffron and its active substances have been reported to have anti-cancer, anti-oxygenic, and anti-inflammatory effects (Abdullaev, 2002; Abdullaev and Espinosa-Aguirre, 2004; Das et al., 2010; Amin et al., 2011; Amin et al., 2016; Ashktorab et al., 2019). For example, the presence of saffron and all its major constituents, such as crocin, crocetin, and safranal, were found to have significant anticancer activity in various tumors including prostate cancer, cervical cancer, leukemia, lung cancer, and liver cancer (Amin et al., 2011; Amin et al., 2016; Milajerdi et al., 2016; Mollaei et al., 2017; Khorasanchi et al., 2018). Similarly, saffron and the active substance crocin reduced the incidence of HCC induced by DEN and promoted by 2-acetyl aminofluorene (2-AAF) in rats (Amin et al., 2011; Amin et al., 2016; Amin and Awad, 2021) as well as reducing the incidence of stomach cancer induced by methyl-3-nitro-1-nitrosoguanidine in rats (Bathaie et al., 2013). Safranal, which gives saffron its aroma (Bathaie and Mousavi, 2010; Khorasanchi et al., 2018), was found to have dose-dependent antitumor effects against Hela and MCF7 cells (Malaekeh-Nikouei et al., 2013), prostate cancer cells (Samarghandian and Shabestari, 2013), and liver HepG2 cells (Al-hrout et al., 2018). In addition to its anti-cancer effects, this monoterpene also has antioxidant and anti-inflammatory properties in different animal models (Nanda and Madan, 2021). Despite all of its anti-tumor activities *in vitro*, the effects of safranal alone on an HCC model is yet to be determined and the mechanism that mediates its anti-cancer effect has yet to be fully understood.

The present experiments were designed to study the potential chemopreventive effects of safranal in a well-described model of HCC in rats, which was induced by DEN and promoted by 2-acetyl aminofluorene (2-AAF) during the early stages of hepatocellular tumor promotion. When promoting carcinogenesis experimentally, the AHF serve as pre-neoplastic indicators of HCC, weeks or months prior to its emergence. This strongly resembles the progression of human hepatocarcinogenesis (Li et al., 2005). In this animal model, the mechanisms of safranal's different effects, such as its antioxidant, pro-apoptotic, anti-proliferative, and anti-inflammatory effects, were investigated and key regulators of different pathways were assessed. HepG2 cells were also used to assess safranal's affects in human liver cancer cells.

MATERIALS AND METHODS

Reagents and Materials

DEN, 2-AAF, 5,5′-dithiobis-(2-nitrobenzoic acid), thiobarbituric acid, Folin's reagent, pyrogallol, SOD enzyme, H_2O_2, and bovine albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Primary antibodies of Ki-67, COX-2 (Clone SP 21), iNOS (Ab-1), and NF-kB-P65 (Rel A, Ab-1) were purchased from Thermo Fisher Scientific, Anatomical Pathology, Fremont, USA (1:100 dilutions). GST-p form was obtained from Medical and Biological laboratories Co., Tokyo, Japan (1:1,000 dilution). M30 CytoDeath was purchased from Enzo life Science, USA. Anti-CD68 (ED1), -CD163 (ED-2) (1:300 dilution), and -phosphorylated form of tumor necrosis factor alpha receptor 1 (p-TNFR) (1:200 dilution) antibodies were obtained from Santa Cruz, CA, USA. Safranal (W338907 Aldrich) was obtained from Sigma-Aldrich, USA. CellTiter-Glo luminescent Cell viability assay kit and Caspase-Glo 3/7 luminescent assay kit were obtained from Promega (Woods Hollow. Rd., Madison Wisconsin, USA). HDAC Colorimetric Assay Kit and Human IL-8 ELISA Kit (EZHIL8) were obtained from the Millipore Corporation (28820 Single Oak Drive, Temecula, CA 92590, USA) and the TNF-α ELISA kit came from R&D Systems (Minnesota, USA).

Animals

The experiment was conducted on 30 healthy albino Wistar rats, weighing 180–200 g, and aged 10–12 weeks. They were obtained from the animal house of the College of Medicine, Emirates University, after the protocol was approved by the Animal Research Ethics Committee, UAE University. All efforts have been made to reduce the suffering of animals and the number of animals used. The animals were placed in an air-conditioned room and were randomly distributed across five polycarbonate cages lined with wood chip bedding that was changed daily. The animals were placed under controlled conditions with a

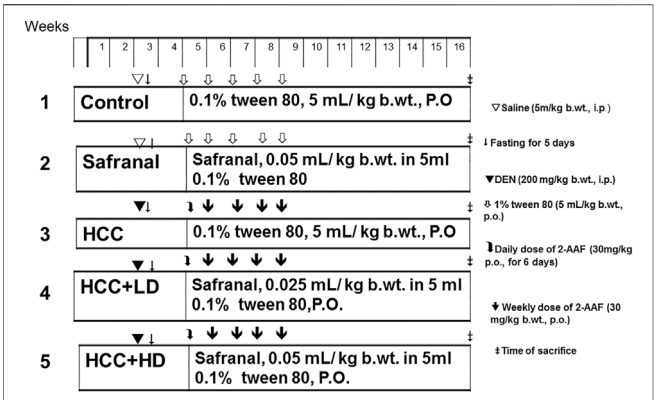

FIGURE 1 | Schematic diagram showing the experimental design to induce and treat liver cancer *in vivo* study.

temperature of 22–24°C and a 12-h light/dark cycle throughout the experiment. Rats were provided with free access to standard pellet diet and tap water *ad libitum* and were acclimated to the environmental conditions for 2 weeks before the experimental procedure.

Hepatocarcinogenesis Model

The development of the experimental hepatocarcinogenesis was carried out according to the protocol used by Espandiari et al. (2005) and Santos et al. (2015). In this protocol, the formation of cancer cells is initiated by an intraperitoneal injection of a single dose of the carcinogenic DEN (200 mg/kg b wt.) dissolved in saline. The neoplastic cell division was promoted with 5 days fasting-refeeding followed by the use of the promoting agent, 2-acetylaminofluorene (2-AAF). Fasting-refeeding and employing 2-AAF after 2 weeks of using DEN are reported as mitotic proliferative stimuli (Hayden and Ghosh, 2014). 2-AAF was introduced in the form of intra-gastric doses given daily for 6 days and then weekly for 4 weeks (30 mg/kg in 1% Tween 80).

Experimental Design

Safranal suspended in 0.1% (w/v) Tween 80 was administered orally at doses of 0.025 ml and 0.05 ml/kg b wt. to rats. The two doses used in this experiment are non-toxic and were shown to have anti-inflammatory and anti-oxidant effects in previous chemically induced inflammation and oxidative stress in rats (Hosseinzadeh and Sadeghnia, 2007; Hariri et al., 2011; Hosseinzadeh et al., 2013; Xue et al., 2020). A total of 30 adult male albino Wistar rats were randomly divided into five groups (n = 6) and subjected to different treatments. Group 1 (control) was orally administered the daily dose of distilled water containing 0.1% Tween 80 (5 ml/kg b wt.) (Safranal vehicle) throughout the experimental duration and were also injected with a single dose of saline (DEN vehicle). Group 2 (Safranal only) was subjected to a daily dose of safranal (0.05 ml/kg) through oral administration for the duration of the experimental period. Hepatocarcinogenesis was induced by DEN and promoted by 2-AAF, as reported previously, in group 3 (HCC). Rats in protective groups (groups 4–5) were treated with daily low/high doses of safranal at the beginning of promotion periods and continued for 12 weeks. The low dosage treatment with safranal consisted of 0.025 ml/kg and the high dosage treatment consisted of 0.05 ml/kg. The experimental design is illustrated in **Figure 1**.

Sample Preparation

After 14 weeks of treatment with DEN and safranal, all rats were anesthetized and blood was collected via retro-orbital puncture under the influence of diethyl ether 24 h after last treatment. Then the rats are sacrificed under the influence of the diethyl ether by a cervical dislocation. Rat livers were rapidly dehydrated, washed with ice-cold normal saline solution, and then dried with blotting paper. Liver slices from different lobes were immediately fixed in 10% buffered formalin for histological and immunohistochemical analyses, and the other parts were rapidly frozen in liquid nitrogen and kept at −80°C for bio experiments. The serum was separated from the blood samples by centrifugation at 3,000 rpm for 20 min (4°C). Frozen liver samples are ground in ice-cold 150 mM Tris–HCl buffer (pH 7.4) with a 1:10 wt/v ratio (Hamza et al., 2018). Aliquots were prepared for the purpose of biochemical marker determination. Fixed tissue samples were processed and embedded in paraffin before being sectioned off into 5-μm sections. The fixed sections were placed onto glass slides and a routine staining by H&E was performed prior to examination under light microscope (Olympus DP71) (Hamza et al., 2018).

FAH Formation and GST-p Expression

Histological examinations of the livers such as the classical foci of altered hepatocytes (FAH) were performed using an Olympus DP71-light microscope. FAH are characterized by the presence of a group of pale hepatocytes that are irregular in shape and contain large vacuoles in the cytoplasm with large hyperchromatic nuclei (Jiang et al., 2018). The presence of these precancerous changes was confirmed by immune histochemical detection of GST-p, which is a true indicator for quantitation of FAH (Satoh, 2018). The number and areas (mm^2) of foci /cm^2 of liver sections were estimated using a magnification of ×100. GST-p foci larger than 15 cells were considered and measured by using color image analysis software (NIS Elements Basic Research, version 3; Nikon, USA).

Antioxidant Status in Liver

For determination of catalase activity CAT, a method by Aebi was followed (Aebi, 1984). CAT decomposes hydrogen peroxide (H_2O_2) to oxygen and water; therefore, the activity of CAT was evaluated according to the exponential decomposition of H_2O_2 at 240 nm. Results are expressed in terms of units per milligram of protein. A method by Nandi and Chatterjee was followed to assay superoxide dismutase (SOD) levels in liver homogenates (Nandi and Chatterjee, 1988). This method utilizes the inhibitive ability of SOD on autooxidation of pyrogallol (1,2,3-benzentriol) at an alkaline pH. A method by Hillegass et al. (1990) was used to determine myeloperoxidase (MPO) activity by measuring peroxidase activity of MPO that catalyzes the oxidation of peroxide. The amount of MPO required to

degrade 1 μM of peroxide/min describes one unit of MPO. Peterson modified-Lowry's method was used to evaluate total protein content in liver homogenates (Peterson, 1977). UV-160-Shimadzu recording spectrophotometer was used to record absorbances. Malondialdehyde (MDA) level was assayed spectrophotometrically by measuring the product of MDA reaction with thiobarbituric acid (TBA), a pink complex, at 535 nm (Uchiyama and Mihara, 1978). To determine liver homogenate content of protein carbonyl (P. carbonyl), a method by Reznick and Packer (1994) was followed. This method is based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) to form a spectrophotometrically detectable hydrazone product at 370 nm. The results are expressed as nanomoles of carbonyl group per milligram of protein, with a molar extinction coefficient of 22,000 M/cm.

TUNEL Assay

TUNEL assay was performed for the purpose of assessing apoptosis. Liver sections (4 μm) were deparaffinized and subjected to subsequent gradual hydration prior to staining. ApopTag peroxidase *In Situ* Apoptosis Detection kit was used according to the manufacturer's instructions (Serological Corporation, Norcross, USA). DNA fragmentation, a key indicator of apoptosis, is detected using this kit. Cell death was confirmed using M30 CytoDeath monoclonal antibodies by detecting the caspase-cleaved fragment of cytokeratin18.

Immunohistochemical Staining

Mounted sections were immersed in sodium citrate buffer (0.1 M, pH 6) and placed in a water bath for 15 min to unmask antigen epitopes. Afterwards, sections were incubated with 0.3% H_2O_2 in methanol to block nonspecific binding to endogenous peroxidase. Sections were incubated overnight at 4°C with rabbit anti-rat primary antibodies, anti-COX-2, anti-iNOS, anti-NF-$_k$B-P65, and anti-Ki-67; in addition to M30 CytoDeath, monoclonal ED-2 anti-rat antibody, and polyclonal anti-rabbit antibodies, anti- GST-p and anti-p-TNFR. After incubation, slides were washed with PBS and incubated with polyvalent biotinylated goat-anti-rabbit, a secondary antibody, for 10 min at room temperature (1:200 dilution). Universal LSAB kit and DAB plus substrate kit were both used to perform a standard staining protocol. Hematoxylin was used in additional counter-staining. Slides were observed under an Olympus DP71 optical microscope, and tissue images were obtained.

Histone Deacetylase Activity Assay

HDAC Colorimetric Assay Kit (Millipore Corporation, 28,820 Single Oak Drive, Temecula, CA 92590, Catalog number: 17-374) was used to measure HDAC activity in liver homogenate.

Determination of Tumor Necrosis Factor-α

TNF-α level in serum was quantitatively measured using ELISA (Chessum et al., 2015), according to the manufacturer instructions (ELISA kits; R&D Systems, Minnesota, USA). Results are presented in picograms per milligram.

Cell Culture

Human liver carcinoma cell lines (HepG2) were obtained frozen in liquid nitrogen from the American Type Culture Collection (ATCC). HepG2 were grown as "monolayer culture" in RPMI 1640 medium (HyClone, USA) and 1% of 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, USA) supplemented with 10% fetal bovine serum (Sigma) at 37°C in a humidified 5% CO_2 atmosphere. Cells were sub-cultured each 4–6 days using trypsin 0.25% (HyClone).

Cell Viability Assay

HepG2 cells were seeded in 96-well plates at the density of 10,000 cells/well and grown in 100 μl of complete growth medium. Complete growth medium was replaced by serum-free medium after cells were allowed to attach for 24 h, after which cells were incubated for at least 12 h. Cells were incubated for 24 h after treatment with various concentrations of safranal (1, 0.3, 0.1, 0.03, 0.01 mM) prepared from 10 mM stock solution. After the incubation period, the viability of HepG2 cells was assessed using Cell Titer-Glo luminescent cell viability assay kit according to manufacturer's instruction (Promega, 2800 Woods Hollow Rd., Madison, Wisconsin, USA).

Caspase-3 and -7 Assay

HepG2 cells were seeded in 96-well plates at the density of 10,000 cells/well and grown in 100 μl of complete growth medium. Complete growth medium was replaced by serum-free medium after cells were allowed to attach for 24 h, after which cells were incubated for at least 12 h. Cells were incubated for 48 h after treatment with various concentrations of safranal (1, 0.7, and 0.5 mM) prepared from 20 mM stock solution. After the incubation period, caspase-3 and -7 activities were measured using Caspase-Glo 3/7 luminescent assay kit according to manufacturer's instruction (Promega G8091). Luminescent signal was detected using GloMax Discover System (Promega). The induction of DNA double-strand breaks (DSBs) was assessed by measuring phosphorylation of the histone H2X using western blotting.

ELISA

Supernatant of safranal-treated cells were used to investigate the effect of safranal on IL-8 (CXCL8) secretion level. Human IL-8 ELISA Kit (EZHIL8, Millipore, USA) was used according to manufacturer's instructions. Absorbance was recorded at 450 nm with background subtraction at 570 nm using a microplate reader (Biotek, Winooski, VT, USA).

Statistical Analysis

SPSS (version 20) statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and plotting graphs. Values are expressed as mean ± SEM of six rats per group. A one-way study of variance (Benassi et al., 2021), followed by post hoc Dunnett's test, was used to compare the differences among the groups. All p-values less than 0.05 ($p < 0.05$) were considered as statistically significant.

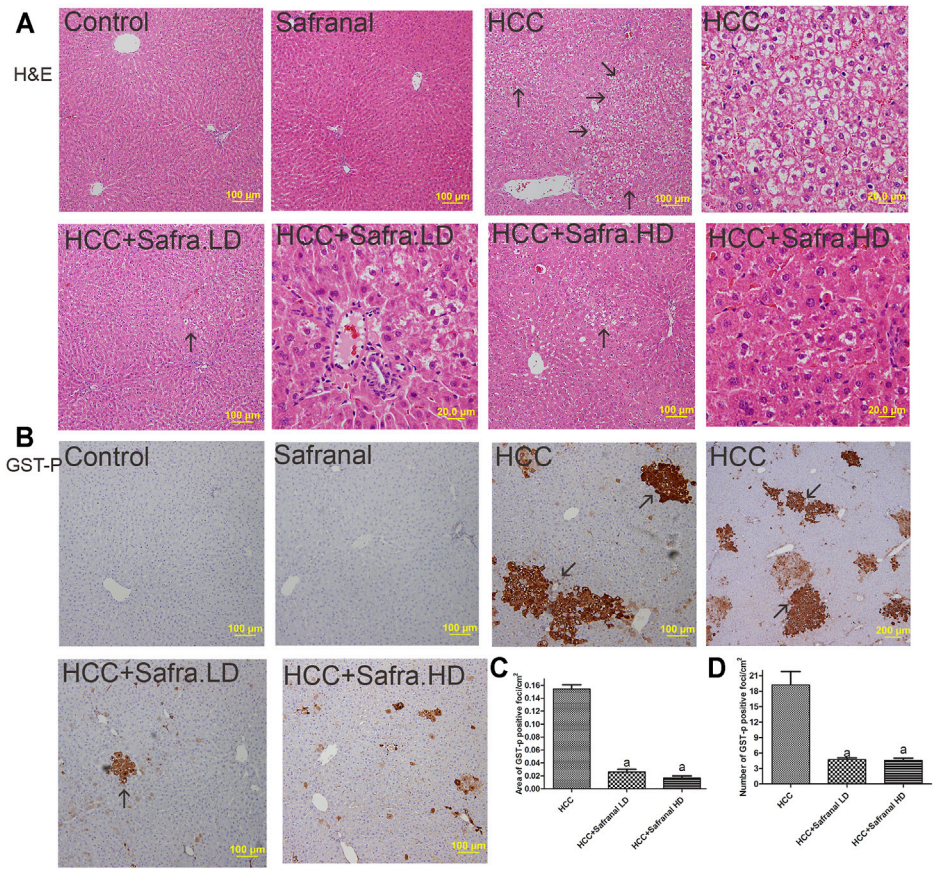

FIGURE 2 | Safranal inhibits DEN/2-AAF–induced induction of AHF **(A)** and of GST-p expression **(B–D)**. **(A)** Figures representing the livers of all groups with a magnification of ×100 and ×400 (scale bars = 100 and 20 μm) using H&E staining. AHF is indicated by arrows at ×100 magnification (scale bar = 100 μm). **(B)** Representative Images of immunohistochemical stains with GST-p of in all groups studied. The brown color represented those cells and is indicated by arrows. **(C, D)** Present quantitative analyses in 10 fields of each section of the GST-p–positive foci and quantitative region analysis of the GST-p–positive foci ×100 magnifications. Treatment of HCC rats with safranal decreased the number and area (mm^2) of GST-positive foci. The value was evaluated by one-way ANOVA followed by Dunnett's *t*-test: $^{a}p < 0.05$ vs. HCC group. Data are represented as mean ± SEM of six animals per group.

RESULTS

Safranal Inhibits DEN/2-AAF–Induced FAH Formation and GST-p Expression

Figure 2 shows the classical FAH in the livers of the cancer group (**Figure 2A**), which are represented by the presence of large, irregular, and pale hepatocytes as well as extensive cytoplasmic vacuums with large hyperchromatic nuclei. The number and size of these foci were remarkably decreased in the protective groups treated with low/high doses of safranal (**Figure 2A**).

An increase in the number and area of placental glutathione S-transferase (GST-p) in the liver is a reliable marker of HCC induced with carcinogens in the liver. **Figure 2B** shows that, compared with the control group, the GST-p protein expression is significantly increased as reflected by the area per square centimeter and the number of foci in the livers of the HCC group. The number of GST-p positive foci and area per square centimeter increased significantly in animals treated with DEN/2-AAF. However, safranal treatment alone did not induce formation of such foci. GST-p was reduced in the livers of rats treated with safranal before cancer development compared with rats that were given only carcinogens.

Safranal Induces Apoptosis, Inhibits Proliferation, and Decreases HDAC Activity in Rats With HCC

The nuclear Ki-67 is a marker of cell division and its overexpression is an indicator of tumorigenesis (Scholzen and Gerdes, 2000). DEN-2AAF treatment caused a significant increase in the number of Ki-6–expressing cells in the livers of HCC rats compared with those of the control group (**Figure 3A,B**). Treatment with safranal alone did not induce a significant change in the number of Ki-67–expressing

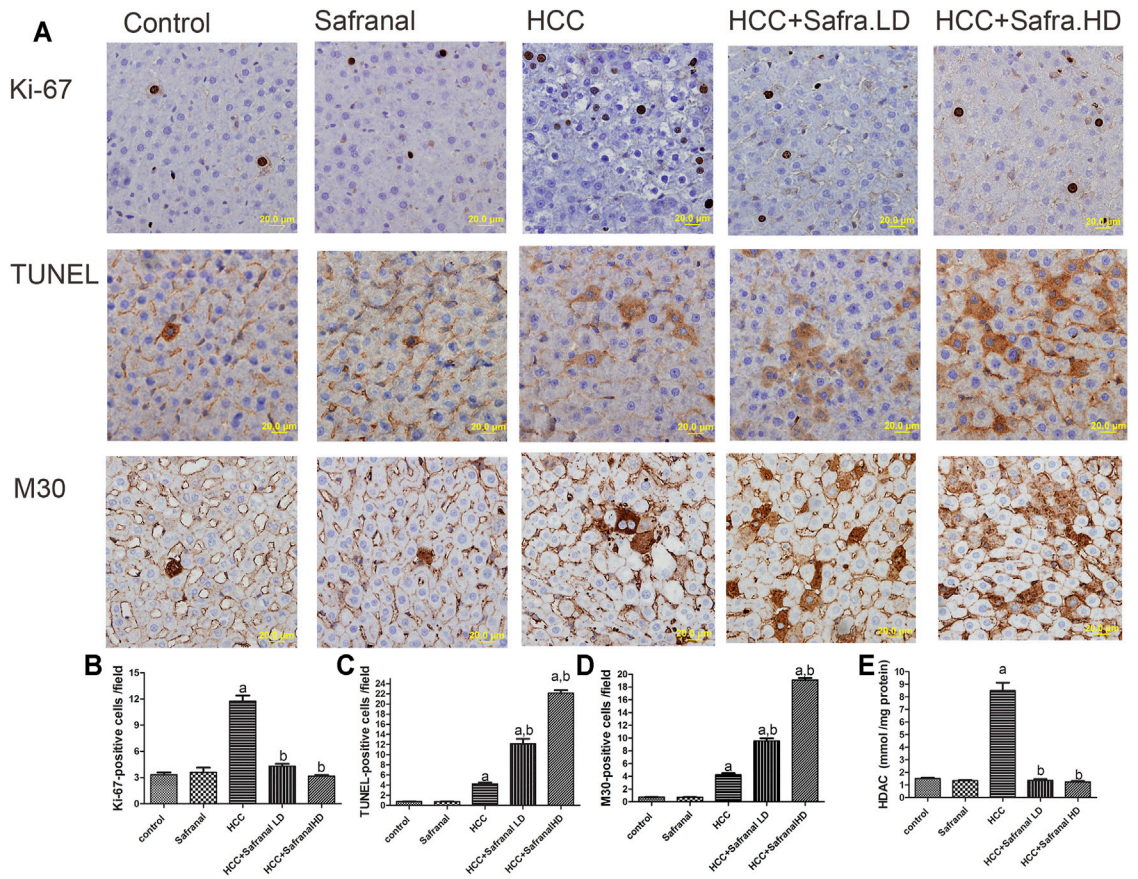

FIGURE 3 | Effects of safranal on proliferation (Ki-67) and apoptotic cell death (TUNEL and with M30) and HDAC activity in rat livers. **(A)** The upper panel includes representative images of immunohistochemical staining with Ki-67, TUNEL, and M30 in liver sections from all the groups (scale bar = 20 μm). The bottom panel represents quantitative analysis of Ki-67 **(B)**, TUNEL **(C)**, and M30 **(D)** positive cells as well as HDAC activity **(E)**, which is expressed in mmol/mg protein. The positive expression in each section was calculated by counting the number of brown staining in 10 fields at ×400 magnifications, then the number of positive cells/field. Values expressed as mean ± SEM for six animals in each group. Significance was determined by one-way ANOVA followed by Dunnett's *t*-test: $^{a}p < 0.05$ vs. control group, $^{b}p < 0.05$ vs. HCC group.

hepatocytes. However, safranal treatment reduced the protein expression of this protein in the livers of rats treated with DEN-2AAF after the administration of safranal.

Given the importance of programmed cell death (apoptosis) in the prevention and treatment of liver cancer, apoptosis was assessed here by estimating numbers of TUNEL-positive cells and M30 CytoDeath-positive cells in animal livers. Interestingly, cell death represented by the number of TUNEL and M30 CytoDeath-positive cells increased significantly in the livers of HCC rats compared with the control group. The percentage of apoptotic cells increased in the HCC rats that were pre-treated with safranal. Such increase in TUNEL-positive cells and M30 CytoDeath-positive cells was significant compared with that of HCC group and the control group. However, there was no increase in these markers in normal rats treated with a high dose of safranal in comparison with the control group.

It is equally interesting that the livers of rats treated with DEN-2AAF had a significant increase in the activity of histone deacetylase (HDAC) enzyme compared with the control group (**Figure 3E**). Although when administered alone did not affect the HDAC activity in the livers of normal rats, safranal had the ability to reduce HDAC activity in the livers of HCC group in a non–dose-dependent manner (**Figure 3E**).

Safranal Reduces Oxidative Stress and Enhances Antioxidant Capacity in Livers of DEN-2-AAF–Treated Rats

As shown in **Table 1**, levels of MDA, and P. carbonyl and MPO activity increased significantly in the cancer group (HCC) and were accompanied by a decrease in the activity of CAT and SOD enzymes. In contrast to these dramatic changes in oxidative stress markers in the cancer model, there were improvements in those indicators in the livers of rats treated with low/high doses of safranal compared with the results of the HCC and the control groups. Interestingly, these effects were dose dependent, as rats of a high safranal dose alone did not affect any of these results in normal rats and stayed at control treated with high dose showed results that were very close to those of the control group.

TABLE 1 | Effect of safranal on oxidative stress markers

Groups	MDA	P. carbonyl	CAT	SOD	MPO
Control	0.66 ± 0.02	1.51 ± 0.04	1.51 ± 0.04	4.07 ± 0.04	33.55 ± 0.31
Safranal (Safra)	0.64 ± 0.02	1.52 ± 0.03	1.52 ± 0.03	4.05 ± 0.09	32.61 ± 1.61
HCC	0.87 ± 0.02[a]	2.16 ± 0.06[a]	2.16 ± 0.06[a]	3.38 ± 0.06[a]	49.92 ± 4.5[a]
HCC + Safra LD	0.61 ± 0.02[b]	1.61 ± 0.06[b]	1.61 ± 0.06[b]	3.96 ± 0.13[b]	27.47 ± 1.77[b]
HCC + Safra HD	0.63 ± 0·03[b]	1.45 ± 0.06[b]	1.45 ± 0.06[b]	4.02 ± 0.8[b]	31.54 ± 2.4[b]

Values are expressed as mean ± SEM of six rats per group. Concentration is expressed as nmol/mg protein for MDA, P. carbonyl activity is expressed as unit/mg protein for CAT, and SOD activity is expressed as m unit/mg protein for MPO. Significance was determined by one-way ANOVA followed by Dunnett's t test:

[a]$p < 0.05$ *vs. normal group.*

[b]$p < 0.05$ *vs. HCC.*

Safranal Reduces the Upregulation of Liver Tissue Expressions of ED-1, ED-2, and p-TNF-R1 and Increases the Serum TNF-α Concentration in DEN/2-AAF–Treated Rats

Expression of both ED-1 and ED2 is considered a cytometric marker to assess the activity of macrophages and resident macrophages (Kupffer cells) (Atretkhany et al., 2016; Taniguchi and Karin, 2018). **Figure 4** shows that there was a significant increase in the number of microphages in the livers of rats treated with DEN-2-AAF compared with the control group. This increase in the number of microphages decreased significantly in the rats treated with safranal at low/high doses (**Figure 4A–C**). Once again, safranal alone had no effect on the expression of ED1 and ED2 compared with control. There was a significant increase in serum TNF-α concentration and number of p-TNF-R1–positive cells in the livers of HCC group compared with the results of the control group (**Figure 4D,E**). Safranal alone had no significant impact on TNF-α and p-TNF-R1 expressions in normal rats. However, when administered to rats with cancer, safranal reduced both TNF-α level in serum and p-TNF-R1 expression in liver.

Safranal Reduces the Upregulated Expressions of NF-κB p65, COX-2, and iNOS in Livers of DEN/2-AAF–Treated Rats

Figure 5 shows that DEN-2-AAF treatment caused a significant increase in the number of both of NF-kB-p65-, COX-2-, and iNOS-positive cells mostly in hepatocytes around the central vein and in Kupffer cells. Pre-treatment with low/high doses of safranal almost completely abolished the effects of DEN-2-AAF in comparison with HCC group (**Figure 5**).

In Vitro Analyses

In vitro analysis was performed to highlight the anticancer effects of safranal on HepG2 cells. Various concentrations of safranal (0.01, 0.03, 0.1, 0.3, 1 mM) were used to treat the cells for 24 h. Cell viability was assessed using Cell Titer-Glo kit. Safranal exhibited a significant dose-dependent reduction of HepG2 cell viability. At a concentration of 1 mM, safranal was able to reduce cell viability by almost 70% (**Figure 5A**). Following the Post treatment with various concentrations of safranal for 48 h, a significant increase in caspase-3 and -7 activities was noted at a concentration of 1 mM (**Figure 5B**). A dramatic decrease in IL-8 secretion as early as 6 h was also reported when HepG2 cells were treated with various concentrations of safranal (**Figure 6C**).

DISCUSSION

In the continuation of our previous studies to evaluate the anticancer effect of safranal both *in vivo* and *in vitro* (Al-hrout et al., 2018; Amin, 2020; Amin, 2021; Amin et al., 2021), here we investigate safranal potential to prevent liver cancer in a drug-induced HCC animal model. The DEN's two-step HCC animal model represents the onset of tumor cell formation by DEN and its stimulation by 2-AAF causes pathophysiological changes that mimic human liver cancer (Tolba et al., 2015; Santos et al., 2017). This model enabled us to investigate mechanisms that safranal may utilize during the early stage of stimulating liver cancer and their relationship to oxidative stress and inflammation in livers of HCC-bearing rats.

As reported here, using DEN and 2-AAF caused paraneoplastic changes in livers of rats. Those histopathological changes included the development of FAH. Paraneoplastic cells in these FAH are characterized by cell irregularity, multinucleation, lack of cytoplasm, and large size of the nucleus relative to the cytoplasm. Administering safranal to HCC-induced rats decreased the incidence of FAH. HCC-induced rats also showed an increase in the area and number of GST-p positive foci, a reliable and sensitive marker of pre-neoplastic foci and nodules in liver cancer (Tew et al., 2011; Satoh, 2018). Safranal's inhibition of pre-neoplastic lesions and FAH was accompanied by a significant decrease in both the number and area of GST-p–positive foci in HCC groups. These results confirm that safranal is a promising candidate in cancer chemoprevention by acting against early, pre-neoplastic liver events that promote HCC progression.

One of the important changes induced by DEN and 2-AAF in the liver of rats is the presence of a dramatic increase in cell division represented by the Ki-67 marker (Scholzen and Gerdes, 2000). In this study, the safranal caused inhibition of the number of Ki-67–positive cells in DEN-treated animals, which confirms further inhibition of cell proliferation in the livers of carcinogenic rats under the influence of the safranal treatment. In HCC-induced animals, a significant increase in apoptotic cell death was shown and was represented by an increased number of

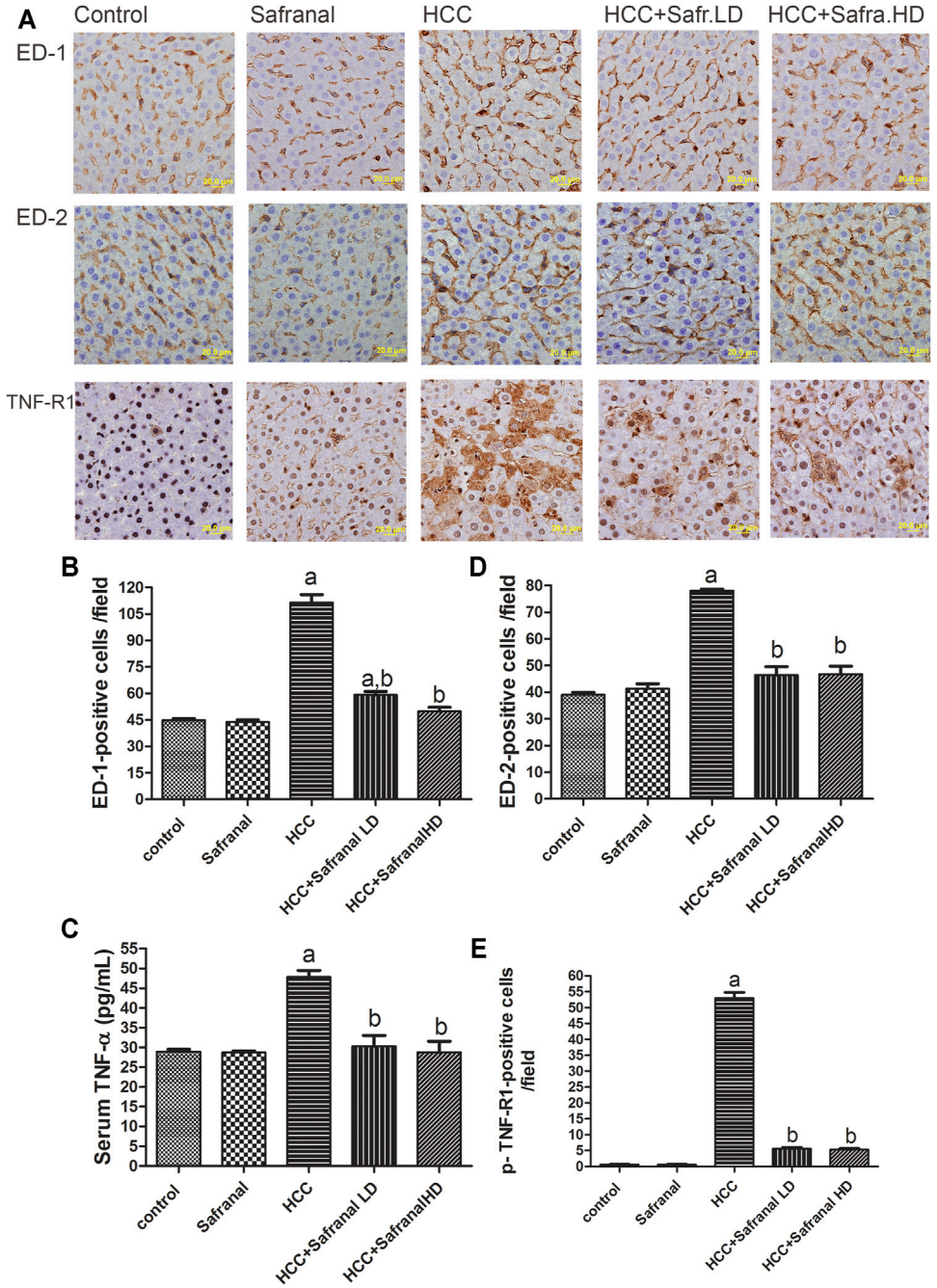

FIGURE 4 | Safranal reduces the upregulation of ED-1, ED-2, and p-TNF-R1 in liver and TNF-α in serum of HCC rats. **(A)** The upper panel are representative images of immunohistochemical staining with ED-1, ED-2, and p-TNF-R1 in the liver section from all the groups. **(B–D)** The positive expression of cells in each section was calculated by counting the number of brown staining in 10 fields at ×400 magnifications then the number of positive cells/fields. **(E)** Shows quantitative analyses of TNF-α in serum. Data are represented as mean ± SEM for six animals in each group. Significance was determined by one-way ANOVA followed by Dunnett's *t*-test: $^{a}p < 0.05$ vs. control group, $^{b}p < 0.05$ vs. HCC group.

TUNEL- and M30 CytoDeath-positive cells, both of which are indicators of DNA fragmentation and early apoptosis, respectively. Induced apoptosis could compensate for the increased cell proliferation in HCC-induced groups. Continued development as a result of loss of apoptotic mechanisms in association with the increased cell proliferation are main physiological changes in developing cancer (Maru et al., 2016; Zhou et al., 2016). Inhibiting cell proliferation and inducing apoptotic activity is consistent with what has been reported in alveolar human cancer lung cell line, human prostate cancer cell line, and in human liver cancer (HepG2) and colorectal cancer cells (Samarghandian and Boskabady, 2012; Samarghandian and

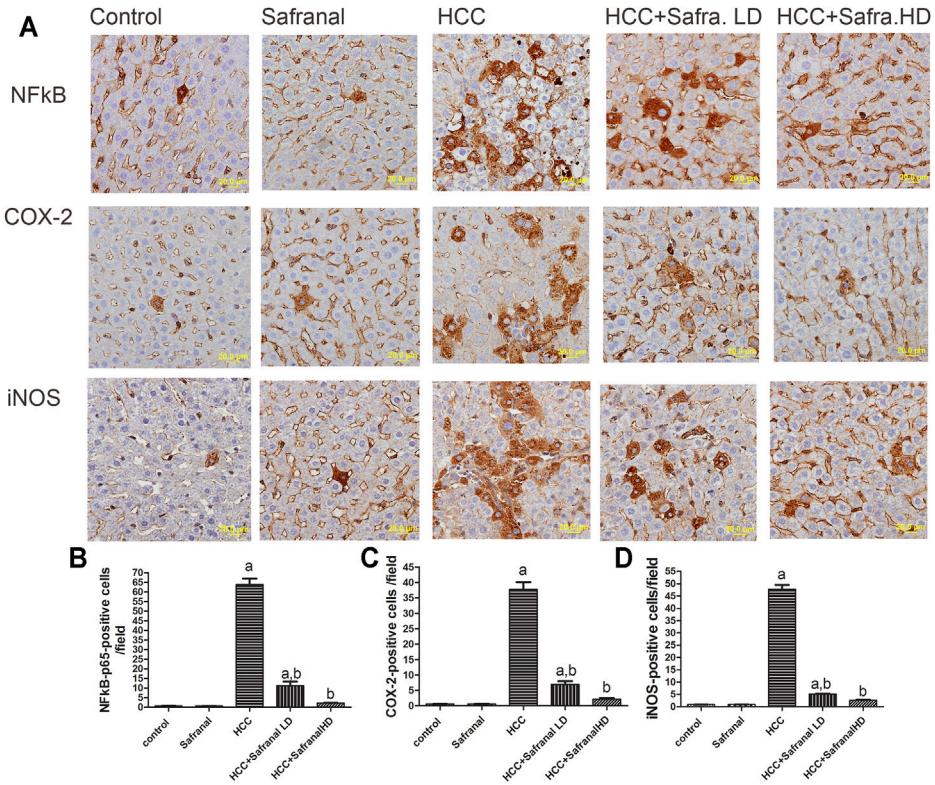

FIGURE 5 | Safranal inhibits DEN/2-AAF–induced upregulation of NF-kB-p65 and COX-2 and iNOS-positive cells expressions. **(A)** The upper panel are representative images of immunohistochemical staining with NF-kB-p65, COX-2, and iNOS in the liver section from all the groups (scale bar = 20 μm). **(B, C, D)** show quantitative analyses of NF-kB-p65, COX-2, and iNOS-positive cells. The positive expression of cells in each section was calculated by counting the number of brown staining in 10 fields at ×400 magnifications then the number of positive cells/fields. Data are represented as mean ± SEM of six rats per group. Significance was determined by one-way ANOVA followed by Dunnett's *t*-test: $^{a}p < 0.05$ vs. control group, $^{b}p < 0.05$ vs. HCC group.

Shabestari, 2013; Al-hrout et al., 2018). In these experiments, safranal activated DNA double strand breakage damage and activated pro-apoptotic effects through the activation of both intrinsic and extrinsic initiator caspases, indicating endoplasmic reticulum (ER) stress-mediated apoptosis. Similar results were confirmed here in the hepatocyte cell model HepG2, where the ability of safranal to increase apoptosis was mediated by caspase-dependent increased abundancy (caspase-3 and -7 activities) and mediated by activated DNA double strand damage (upregulated p-H2AX protein level as assayed by immunoblotting). Thus, the present results indicate that the safranal-induced inhibition of hepatic neoplasia was mediated by both upregulation of apoptosis and downregulation of cellular proliferation.

A wide range of cellular changes resulting from oxygen stress, such as cellular DNA damage and inflammation, are strongly associated with the occurrence of cancer. In addition, genetic instability and higher cell proliferation contribute to the development of FAH and its progression to adenomas and HCC (Reuter et al., 2010; Marra et al., 2011). In this study, DEN/2-AAF stimulated an increase of several indicators of hepatic oxidative stress such as MDA and P. carbonyl. They also increased the hepatic MPO, which is an indicator of oxidative stress and inflammation (Loria et al., 2008). In addition and in agreement with previous observations in HCC model, the livers of HCC rats in this study showed that the depletion of endogenous antioxidants such as SOD and CAT were responsible for reversing the ROS-induced oxidative damage (Marra et al., 2011; Hamza et al., 2018; Hamza et al., 2021). On the contrary and in HCC-induced animals, the administered saffron attenuated the changes of antioxidants in the livers of rats given nitrosamines, and this improvement was accompanied by reduction of oxidized lipid (MDA) and protein (P. carbonyl). It also upregulated H2AX protein level, a sensor for DNA double strand breaks *in vitro* hepatocyte cell model (HpG2), as markers of oxidative stress in DNA (Al-hrout et al., 2018). Recent studies reported an anti-oxidant effect of safranal as represented by decreasing the oxidative stress, assessed by MDA, and by restoring normal levels of anti-oxidant enzymes including SOD and CAT (Hamza et al., 2015; Cerdá-Bernad et al., 2020).

Chronic inflammation plays an important role in the onset and progression of cancer (Taniguchi and Karin, 2018). This cross-talk between chronic inflammation and cancer includes activation of immune cells such as macrophages, Kupffer cells, and neutrophils, and the production of pre-inflammatory mediators including COX-2 and iNOS, and of transcription factors, like the nuclear transcription factor kappa B (NF-κB) (Reuter et al., 2010; Atretkhany et al., 2016; Taniguchi and Karin,

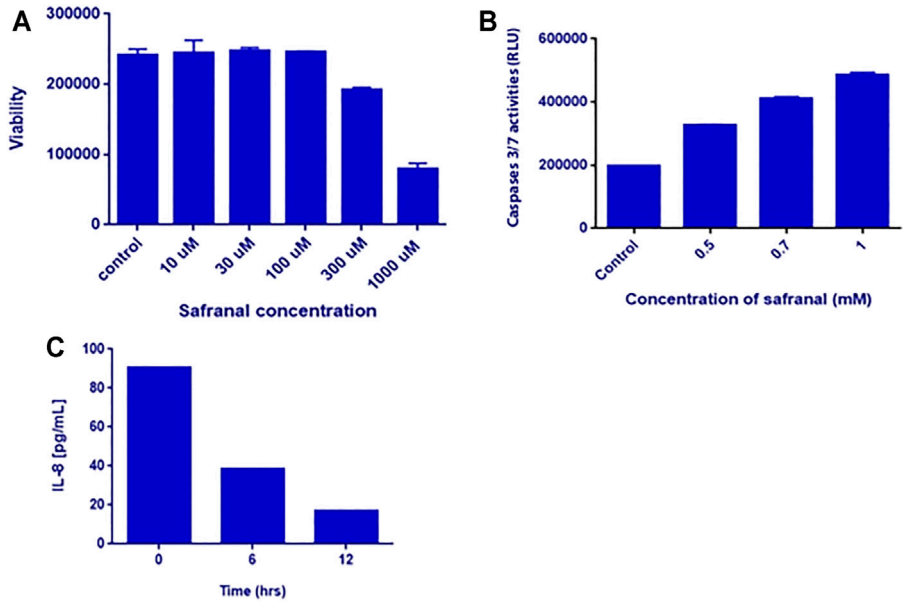

FIGURE 6 | *In vitro* analysis. **(A)** Viability of HepG2 cells after safranal treatment for 24 h. HepG2 cells were treated with 0.01, 0.03, 0.1, 0.3, and 1 mM of safranal. **(B)** Caspase-3/7 activities after safranal treatment for 48 h. HepG2 cells were treated with 0.5, 0.7, and 1 mM of safranal. **(C)** IL-8 secretion after safranal treatment. HepG2 cells were treated with 2 mM for 6 and 12 h, and subsequently, the supernatants were analyzed by IL-8 ELISA.

2018). In many human cancer tissues and in cancer induced by chemicals such as DEN, the development of cancer has been associated with an increase in macrophage cells and neutrophils, as well as an increase in the expression of TNF-α and its receptor TNFR-1 (Roberts et al., 2007; Hayden and Ghosh, 2014; Amin et al., 2016; Hamza et al., 2021). TNF-α is mainly produced by activated immune cells such as macrophages and neutrophils, and then triggers other pro-inflammatory cytokines (Balkwill, 2009; Atretkhany et al., 2016). TNFR1 activation by TNF-α has tumor-promoting action, which is associated with proliferation and activation of NF-κB (Roberts et al., 2007). In the present work, treating HCC-induced rats with safranal alleviated cancer-associated inflammation by reducing the number of both hepatic ED1- and ED2-stained microphages and restoring normal hepatic MPO levels, a marker of neutrophil infiltration (Loria et al., 2008), as well as inhibiting the TNF-mediated inflammatory pathway via reducing the content of TNF-α and the number of p-TNF-R1–positive cells.

IL-8 is a chemokine produced by macrophages that induces a series of physiological responses required for migration and phagocytosis. In HepG2 cells, safranal reduced the concentration of IL8 in a time-dependent manner. This decrease in inflammatory cell infiltration and TNF-α was associated with the inhibition of the protein expression of COX-2, a pro-inflammatory enzyme involved in PG production, and iNOS, the key enzyme in NO production. This indicates that the protective effects of safranal against carcinogenesis could be mediated by decreasing inflammation through downregulation of COX-2 and iNOS. In this regard, the anti-inflammatory effect of safranal was studied in a recent study in which saffron dose-dependently decreased iNOS and COX-2 levels in lipopolysaccharide-stimulated RAW264.7 cells and bone marrow–derived macrophages (Lertnimitphun et al., 2019). Safranal also reduced the production of IL-6 and TNF-α in RAW264.7 cells, and this was accompanied by a reduction in the phosphorylation and expression of NF-κB signaling pathway proteins (Lertnimitphun et al., 2019).

NF-kB is considered as one of the most powerful players involved in the development of many types of cancer, and it is characterized by linking chronic inflammation and oxidative stress to the tumorigenesis and inflammation associated with cancer (Hayden and Ghosh, 2014; Taniguchi and Karin, 2018). Inflammatory-associated cancer has been found to be active by increasing oxidative stress and upregulation of inflammatory markers including NF-κB and iNOS (Xia et al., 2014). NF-κB plays a major role in promoting cancer by modulating the expression of many genes via nuclear oxidative stimuli. This modulation in gene expression by NF-kB is responsible for altered inflammatory responses, upregulation of COX-2 and iNOS, promoting cell proliferation, and inhibiting cell death (Hayden and Ghosh, 2014; Taniguchi and Karin, 2018). In the present work, safranal treatment decreased oxidative stress, which was accompanied with inhibition of inflammatory markers including COX-2, iNOS, and NF-κB. The decrease of Kupffer cells and neutrophils reported here seems to be associated with an early inactivation of the NF-kB signaling pathway, as reflected in the early *in vitro* inhibition of p-IkB and IL-8. The findings reported here suggest that safranal's anti-cancer properties could be attributed to its anti-inflammatory activities through downregulation of NF-κB, COX-2, and iNOS expression levels as well as to the reduction of both TNF-α and its receptor TNFR1.

The process of regulating gene expression by epigenetic modulators has been introduced as a novel mean of targeted therapy in the fight against cancer (Chessum et al., 2015). One of the target enzymes is histone deacetylase (HDAC), which is responsible for packing DNA tightly around histones by removing acetyl groups from an ε-N-acetyl lysine amino acid on histones, minimizing the chance of RNA polymerases contacting DNA and resulting in decreased gene expression (Park and Kim, 2020). HDACs play a major role in the onset and development of cancer by removing acetyl groups from histones that are involved in the regulation of the cell cycle, apoptosis, the DNA-damage response, metastasis, angiogenesis, and autophagy (Li and Seto, 2016). Results presented here showed a significant effect of safranal pre-treatment on inhibiting the increased HDAC expression in a DEN-treated HCC model and restoring it to control levels. Taken together, these findings suggest that anti-proliferative and pro-apoptotic properties of safranal could be attributed, at least in part, to its inhibitory ability of HDAC overexpression in cancer.

CONCLUSION

Findings reported in this study showed the potent efficacy of safranal against drug-induced HCC *in vivo*. Safranal treatment was efficient in inhibiting FAH formation in DEN-induced HCC models, restoring the antioxidant normal levels, and reducing all tested oxidative stress markers. In addition, significant decreases in the activity of inflammatory markers, COX-2, iNOS, NF-$_k$B, TNF-α, and its receptor p-TNF-R1 were observed in DEN-induced HCC model pre-treated with safranal. Moreover, pre-treatment with safranal induced a reduction in the number of Kupffer cells and macrophages. These findings were also confirmed *in vitro* by utilizing the human hepatoma cell line "HepG2" where safranal has consistently demonstrated pro-apoptotic and anti-inflammatory properties.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the UAEU institutional Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

AAH, YA, AAb, and AAm designed the study. AAH, YA, AAb, and AAm performed the experiments and did the statistical analysis. AAH, YA, AAb, and AAm assisted with methodology and contributed resources. AAH, YA, AAb, and AAm wrote the first draft of the article, and all authors contributed to the editing of the revised article and approved the article.

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Safranal Inhibits Angiogenesis *via* Targeting HIF-1α/VEGF Machinery: *In Vitro* and *Ex Vivo* Insights

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Nature has a nearly infinite inventory of unexplored phytochemicals and biomolecules that have the potential to treat a variety of diseases. Safranal exhibits anti-cancer property and the present study explores its antiangiogenic property. Hepatocellular carcinoma (HCC) ranks as the sixth deadliest among all cancer types. Targeting the non-tumor vasculature supporting system is very promising as it has less plasticity, unlike malignant cells that are often associated with issues like drug resistance, poor prognosis, and relapse. In this study, we successfully inhibited the proliferation of primary human umbilical vein endothelial cells (HUVEC) with an IC50 of 300μM and blocked VEGF secretion in HepG2 cells. Furthermore, safranal inhibited VEGF-induced angiogenesis *in vitro* and *ex vivo via* scratch wound assay, tube formation assay, transmembrane assay, and aortic ring assay. In addition, safranal downregulated the *in vitro* expression of HIF-1α, VEGF, VEGFR2, p-AKT, p-ERK1/2, MMP9, p-FAK, and p-STAT3. The present study is the first to reveal the antiangiogenic potential of safranal and propose its possible underlying mechanism in HCC.

Keywords: safranal, VEGF, HIF-1α, angiogenesis, cancer

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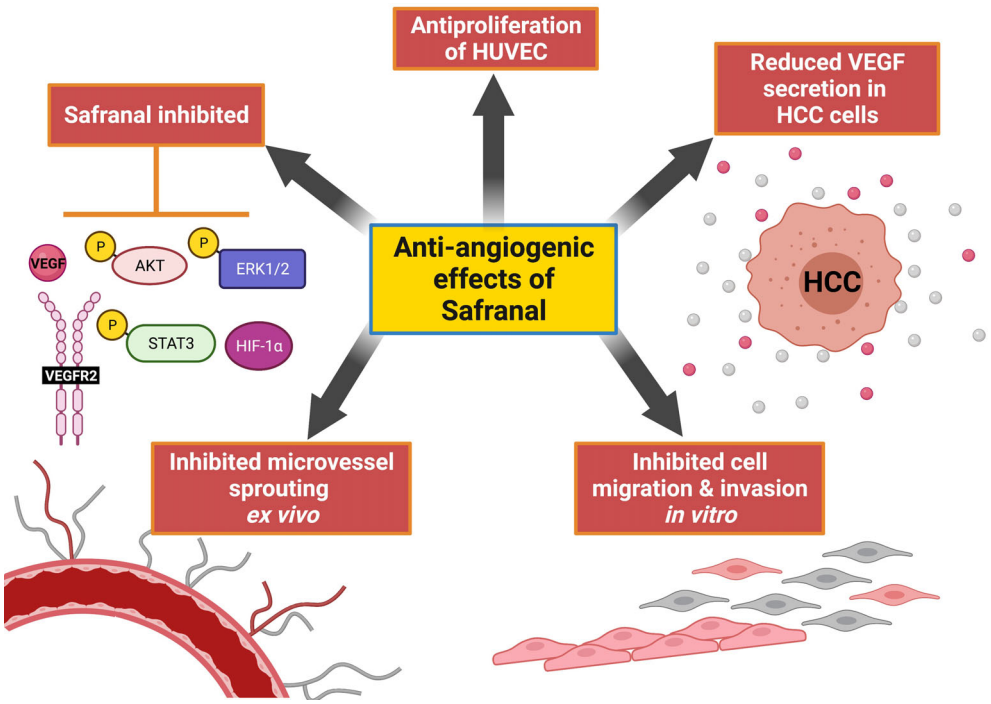

GRAPHICAL ABSTRACT | Safranal exhibited antiangiogenic properties in this study. Safranal shows pleiotropic effect by repressing HIF-1α which is a strong activator of VEGF. This could be due to the inhibition of ERK and AKT phosphorylation which tightly regulates HIF-1α synthesis. Finally preventing the activation of VEGF/VEGFR2 axis. Hence, aalteration of the key vascular endothelial signaling molecules are in favor of inhibiting the *in vitro* and *ex vivo* angiogenic assays. This figure was created with BioRender.com.

INTRODUCTION

Globally, liver cancer is one of the most fatal cancers (1). Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, ranks sixth deadliest among all cancer types (2). The high frequency of HCC can be traced back to a host of risk factors that often lead to the development of HCC. Viral hepatitis infections, specifically with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcoholism, smoking, nonalcoholic fatty liver disease, chronic liver disease, and cirrhosis represent some of the major risk factors (3). Many forms of cancer, including HCC, cause the deregulation of multiple signaling pathways that manage cell proliferation, metastasis, and angiogenesis (4). Neovascularization is a crucial event in tumor progression from the sprouting phase to more aggressive metastasis (5). Once fully developed, a solid tumor can remain latent if deprived of its blood supply (6). A thorough study of new blood vessels establishing in the tumor microenvironment is a promising prognostic marker both for grading the tumor and determining proper therapy for cancer patients (7). Inhibition of angiogenesis as an anti-cancer therapy was first hypothesized by Folkman in 1971 (8). Under normal conditions, angiogenesis is a tightly regulated physiological process. It is essential in wound healing, embryogenesis, and other vital processes in growth and development (9). Epidermal growth factor (EGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) are the most frequently studied signaling molecules in angiogenesis. Overexpression of these growth factors, particularly VEGF and its receptors, has been widely reported in HCC patients (10).

Typically, surgery would be the first treatment option for HCC. However, as the majority of patients are not eligible candidates for surgery at the time of their diagnosis, HCC therapeutics have significantly developed over recent years. Along with a growing list of novel curative agents and molecular targets, liver-directed, systemic, and immunotherapy treatments have been the center of attention in HCC treatment (11). Many of these novel agents are natural-product based compounds that possess potent anticancer properties that can overcome chemoresistance and offer effective therapeutic and preventive alternatives with higher safety margins and minimal adverse effects (12). The use of these biomolecules in conjunction with other therapies has the potential to bring cancer to heel. With the ability to block fibrogenesis, suppress tumorigenesis, and inhibit oxidative stress in the liver, medicinal biomolecules have gained a great deal of momentum as an effective and affordable modality to treat chronic liver diseases across the globe (13).

Nutraceuticals are food components responsible for physiological and metabolic functions and they have been known to protect against a variety of chronic diseases. Their active biomolecules are known to promote health and can be

used to prevent or treat a variety of ailments (14). Interestingly, a vast spectrum of therapeutic properties that include antidiabetic, anti-inflammatory, antioxidant, cardioprotective, antidepressant, antitussive, antitumor, and anticonvulsants have been attributed to special types of nutraceuticals derived from saffron - the stigma of *Crocus sativus* L (15). Crocus sativus L. is a perennial valuable medicinal food herb (Iridaceae family) that has been used in folk medicine and has a great exporting importance in Iran and India (16). Saffron and its fundamental components have been shown to have no cytotoxic effects on normal cells while still proving to be lethal to cancer (17–21). Studies have proved that saffron and its constituents suitably act against cancer development and show selective toxicity against tumors (22, 23). The precise mechanism of saffron's anti-cancer properties remains elusive, but a few hypotheses have been drawn in these studies.

Due to the hypervascular nature of HCC, angiogenesis plays a key role in its progression. In this study we investigate the anti-cancer potential of safranal with a special interest in its anti-angiogenic capacity.

MATERIALS AND METHODS

Cell Culture

Liver cancer cells, HepG2 (ATCC HB-8065), were cultured in RPMI 1640 medium (Hyclone, USA) and 1% of 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, USA) supplemented with 10% FBS (Sigma, USA). HUVEC (CLS, CRL-1730) cells were cultured in endothelial cell growth medium (ECGM) containing 20% FBS, at 37 °C in a humidified 5% CO_2 atmosphere. Cells were sub-cultured for 2-4 days using trypsin 0.25%-EDTA (Hyclone, USA).

Cell Viability

Cell proliferation assay on HepG2 and HUVEC cells were done as described in Al-Hrout et al. (17). Briefly, cells were seeded in triplicate at a density of 5000 cells/well in 96-well plates and grown in 100µl of complete growth medium and allowed to grow for 24 hours. Cells were then treated with various concentrations of safranal (Sigma-Aldrich, USA) (300, 500, 700 µM) and incubated for 24 hrs with or without recombinant 30 ng/ml human VEGF (rhVEGF) (Abcam). After the incubation period, cell viability was assessed using the CellTiter-Glo luminescent cell viability assay kit according to manufacturer instructions (Promega, WI).

VEGF ELISA Assay

The presence of VEGF in cell culture media with and without safranal treatment was assessed by the ELISA kit (SIGMA) according to the manufacturer's instructions. Each sample was analysed in triplicate.

HUVEC Cell Wound Scratch Assay

HUVEC cells were seeded in a six-well plate in complete medium (ECGM, Sigma) and allowed to grow into a 70-80% confluence monolayer. The monolayer was then scratched with a new 10µl pipette tip across the centre of the well. After scratching, the detached cells were removed by gently washing the well with culture medium. Media containing 0.5% FBS was added with 30 ng/ml rhVEGF along with, or without, different concentrations of safranal. The area of the wound was photographed randomly at 0 h, 8 h, and 12 h and the wound area was measured, considering rhVEGF-induced untreated control wells as 100%.

Western Blotting

HepG2 cells were seeded at a density of $1X10^6$ cells/100 mm plate and allowed to attach. Cells were then treated with increasing concentrations of safranal (300, 500, 700µM) for 24 hours. Whole cell lysates were separated using 10-15% SDS polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membranes prior to incubation with various primary antibodies; MMP9, AKT, p-AKT, p-FAK, p-ERK1/2, ERK1/2, p-PLCγ, p-STAT3, STAT3 (Cellsignalling technologies), and GAPDH (Abcam) were used as loading controls. As secondary

Antibodies, anti-mouse IgG (FC) peroxidase antibody (Cellsignalling technologies, 1:2000) and anti-rabbit IgG peroxidase antibody (Cellsignalling technologies, 1:2000) were used. Protein bands were detected using WesternSure Chemiluminescent Substrate (LI-COR) and C-DiGit blot scanner (LI-COR).

Immunocytochemistry and Fluorescent Staining

HepG2 cells were seeded at a density of $3 X 10^4$ cells/well in an 8-chambered glass plate and allowed to attach before being treated with the most effective concentrations of safranal for 24 hours. Cells were then fixed with 4% paraformaldehyde followed by incubation with primary antibody for VEGFR2 (Cellsignalling technologies) and with secondary antibodies tagged with FITC (Alexa Fluor, Molecular Probes). Finally, the nuclei were stained using 4, 6-diamidino-2-phenylindole (DAPI; 0.5 µg/mL in PBS; for 5 min at room temperature). Cells were imaged using an Inverted Phase Contrast Microscope, model IX53, with a fluorescent attachment complete with the Olympus microscope high resolution digital camera, and model PD73.

Transwell Migration Assay

Transwell invasion assay was done as previously described (24). Briefly, to the bottom chambers of the transwell plate (Corning), serum-free medium containing rhVEGF (30 ng/ml) was added. HUVECs were trypsinised and suspended with serum-free medium and 1×10^5 cells per well were seeded into the top chambers of the transwell plate coated with, or without, extracellular matrix (ECM) in the presence, or absence, of safranal at stated concentrations. The transwell plate was incubated in a 5% CO_2 incubator at 37°C for about 8-10 hrs. After the incubation, non-migrated cells on the surface of the membrane were wiped with a cotton swab and the invasive cells located on the bottom membrane were fixed with cold 4% paraformaldehyde for 30 min and stained with crystal violet solution or the nuclear stain DAPI. Images were taken using the Inverted Phase Contrast Microscope, model IX53, with a

fluorescent attachment complete with the Olympus microscope high resolution digital camera model PD73.

Rat Aorta Ring Assay

The present study was approved by the institutional (UAE University) Animal Ethics Committee (approval Reference number: A 8-15). This assay was carried out on rat aortic explants as previously described in Al-Salahi et al. and Al-Dabbagh et al. (25, 26). Thoracic aortas were removed from 3% sodium pentobarbital -euthanized male rats, rinsed with serum free medium, and cleaned from fibro adipose tissues. In total, 10 rats were used in this assay and the aortas were cross sectioned into small rings (each ring is about 1 mm thickness). The rings were seeded individually in 48-wells plate in 300µL serum free M199 media containing 3 mg/ml fibrinogen and 5 mg/ml aprotinin. Ten microliters of thrombin (50 NIH U/ml in 1% bovine serum albumin in 0.15 M NaCl) was added into each well and incubated at 37°C for 90 min to solidify. A second layer (M 199 medium supplemented with 20% HIFBS, 0.1% ε-aminocaproic acid, 1% L-Glutamine, 2.5µg/ml amphotericin B, and 60µg/ml gentamicin) was added into each well (300µL/well). All the extracts were added at final concentrations of 100µg/ml. On day two, the medium was replaced with a fresh one containing safranal at 500 µm. Aortic rings were photographed on day 2, 4, 6, and 8 using an Inverted Phase Contrast Microscope, model IX53, with the Olympus microscope high resolution digital camera model PD73. Subsequently, the length of the blood vessels outgrowth from the primary tissue explants was measured using Leica Quin software.

The inhibition of blood vessels formation was calculated using the formula:

$$\%\ blood\ vesselsinhibition = [1 - (A0/A)] \times 100$$

Where;

A0 = distance of blood vessels growth in treated rings in µm and A = distance of blood vessel growth in the control in µm.

Tube Formation Assay

96-well plate was coated with 50µl of growth factor reduced Corning Matrigel matrix (Corning Lifesciences, USA) according to manufacturer's protocol. The plate was then incubated at 37°C for 45 min to solidify the Matrigel. HUVEC cells were seeded (2×10^4) on the top of the Matrigel in 100µl serum free culture medium with, or without Safranal under the stimulation of rhVEGF (30 ng/ml). 6-8 hrs later, tubular structures of endothelial cells and extend of network formation mimicking angiogenesis were examined using the Inverted Phase Contrast Microscope, model IX53, with the Olympus microscope high resolution digital camera model PD73. The number of the tubes was quantified from three random fields.

Quantitative Real-Time PCR (qPCR)

For qPCR, cDNA corresponding to 50 ng of total RNA was used per transcript to be quantified. Quantitative PCR reactions were performed on an Applied Biosystems instrument system using the GoTaq® qPCR Kit (PROMEGA, USA) with gene-specific primers according to the manufacturer's instructions. Data was normalized using housekeeping gene averages for the same time point and condition (ΔCt). Values are shown as fold change relative to the untreated control (RQ). The primers for the qPCR reactions are listed in **Supplementary Table 1**.

Statistical Analysis

All experiments were conducted in replicates. The quantitative data were shown as Mean ± SD and the statistical differences between two groups was examined by a two-tailed Student's t test. $p< 0.05$ indicated the significant difference.

RESULTS

Safranal Inhibits VEGF-Induced Angiogenesis

Based on our earlier study on the HCC cells (17), and in order to further assess its antiangiogenic potential, we examined the inhibitory effect of safranal on cell viability in HUVEC cells. Interestingly, safranal inhibited cell growth at a dose of 300 µM attaining IC50 on rhVEGF induced HUVECs compared to the non-induced cells (**Figure 1A**). The pro-angiogenic tumor derived factor, vascular endothelial growth factor (VEGF), was assessed after safranal treatment. HepG2 cells were treated with various concentrations of safranal for 24 hrs and the supernatant of the cell culture medium was collected and then secreted VEGF was examined using ELISA. As shown in **Figure 1B**, safranal reduced the levels of VEGF secreted by HepG2 cells in a dose dependent manner. After 24 hrs, there was nearly a 70% reduction in VEGF secretion from treated cells as compared to untreated control cells. We then assessed the effect of safranal on the highly expressed VEGF receptor in HCC, VEGFR2. Immunofluorescence analysis showed that the expression of VEGFR2 was reduced in HepG2 cells upon safranal treatment at higher doses of 500µM and 700µM (**Figure 1C**). There was not much difference in VEGFR2 expression at 300µM compared to control (data not shown). These results encouraged us to further investigate the effect of safranal on the VEGF/VEGFR2 signalling pathway.

Safranal Inhibits the Migration and Tube Formation of Endothelial Cells

In order to study the effect of safranal on cellular migration and angiogenesis *in vitro*, wound healing assay and matrigel tube formation assay were performed in HUVECs. As shown in **Figure 2A**, a scratch wound was made in HUVEC cells which was followed by safranal treatment with rhVEGF induction. The wound area at 0 h was considered as 100% during the quantitative analysis (**Figure 2B**) and safranal inhibited the rhVEGF induced HUVEC migration thereby preventing the wound from healing in a dose dependent manner. After 12 hrs, the untreated, rhVEGF induced, HUVEC cells migrated and closed up the wound, yielding a nearly 0% wound area, while safranal proved effective, especially at 700 µM, where the wound area remained almost more than 60% open. In the matrigel tube

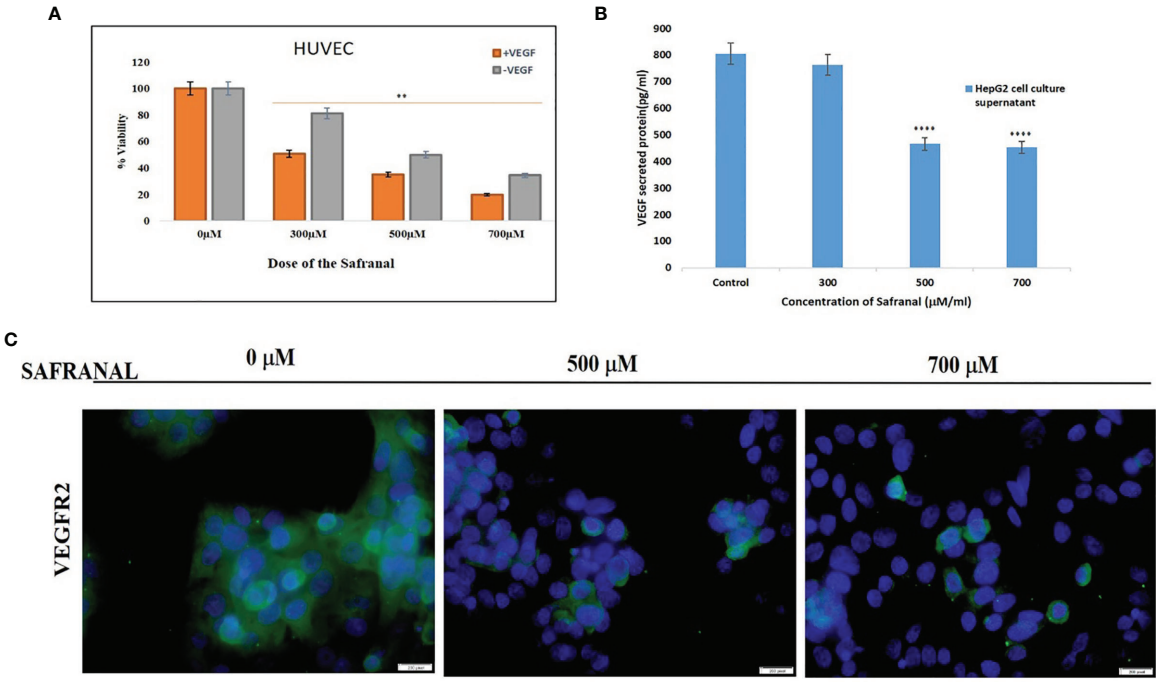

FIGURE 1 | Safranal supressed growth by antagonising the tumor angiogenic proteins: **(A)** Safranal inhibited VEGF-induced HUVECs proliferation. Assessed viability of HUVEC cells that were serum starved overnight and then incubated with or without VEGF (30 ng/ml) and various concentrations of safranal for 24 hrs. **(B)** Safranal suppressed VEGF secretion in HepG2 cells. HepG2 cells were treated with various concentrations of safranal for 24 hrs, the supernatant of cell culture medium was collected, and the content of VEGF was examined using ELISA. **(C)** Immunofluorescence staining of HepG2 treated with various concentrations of safranal for 24 hrs, with rhVEGF (30 ng/ml) and then staining with VEGF (green), nuclei labelled by DAPI (blue). Scale bars: 100μm. Statistical analysis was carried out in all experiments by student's t-test using GraphPad Prism software and $p < 0.05$ was considered as statistically significant. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

formation assay, untreated endothelial cells formed tubes when induced with rhVEGF, while those in the presence of safranal failed to sprout despite rhVEGF induction. As shown in **Figure 2C**, the tube-like structures affect decreased at the doses of 500 μM and 700 μM of safranal and the significance was determined by counting the number of junctions and segment lengths of the modelled neovascularization (**Figures 2D, E**). There was no significant difference in 300 μM treated cells compared to control (data not shown)

Safranal Suppresses VEGF Induced Cell Invasion

As safranal showed a significant effect in blocking the wound healing and tube formation in HUVEC, we proceeded to examine the antiangiogenic effect of safranal *via* transwell invasion assay on non-endothelial, HCC cells, HepG2. HepG2 cells were seeded in the upper chamber of transwell coated with (invasion), or without (migration), matrigel and incubated with various concentrations of safranal. The bottom chamber was added with culture medium containing 30ng/ml of rhVEGF. After 24 hrs, the nuclei of the invaded (**Figure 3B**) and migrated (**Figure 3A**) cells through the membrane were stained with DAPI/crystal violet respectively. Images of these cells were captured using a fluorescence microscopy in five random fields. The number of migrating and invading cells evidently decreased at a dose of 500 μM and 700 μM of safranal. The relative migration and invasion were quantified and analysed compared to untreated control cells (**Figure 3C**). There was no significant difference in 300 μM treated cells compared to control (data not shown)

Safranal Blocks Angiogenesis in *Ex Vivo* Setting

The present study shows the inhibitory effect of safranal on angiogenesis in rat aortic explants. The antiangiogenic effect of safranal was measured in the presence of VEGF in a time dependent manner (500μm). As displayed in **Figure 4**, aortic rings showed reduction in the number of sprouts upon safranal treatment as compared to the control. Day 8 showed an average of 227 micro vessels in rhVEGF induced untreated control whereas the safranal treated aortic ring sprouts only averaged 170 micro vessels despite rhVEGF induction.

Angiogenic-Related Gene Expression Profiling Upon Safranal Treatment

Similar to the antagonistic effect on VEGF/VEGFR2 signalling, safranal affected the proangiogenic factors in HepG2 cells after 24 hrs of treatment in a dose dependent manner. The expression of p-AKT (Ser473), p-ERK1/2, p-FAK, and p-STAT3 were decreased by safranal under VEGF stimulus (**Figures 5A, B**). Further, matrix metalloproteinase-9 (MMP9) expressions were

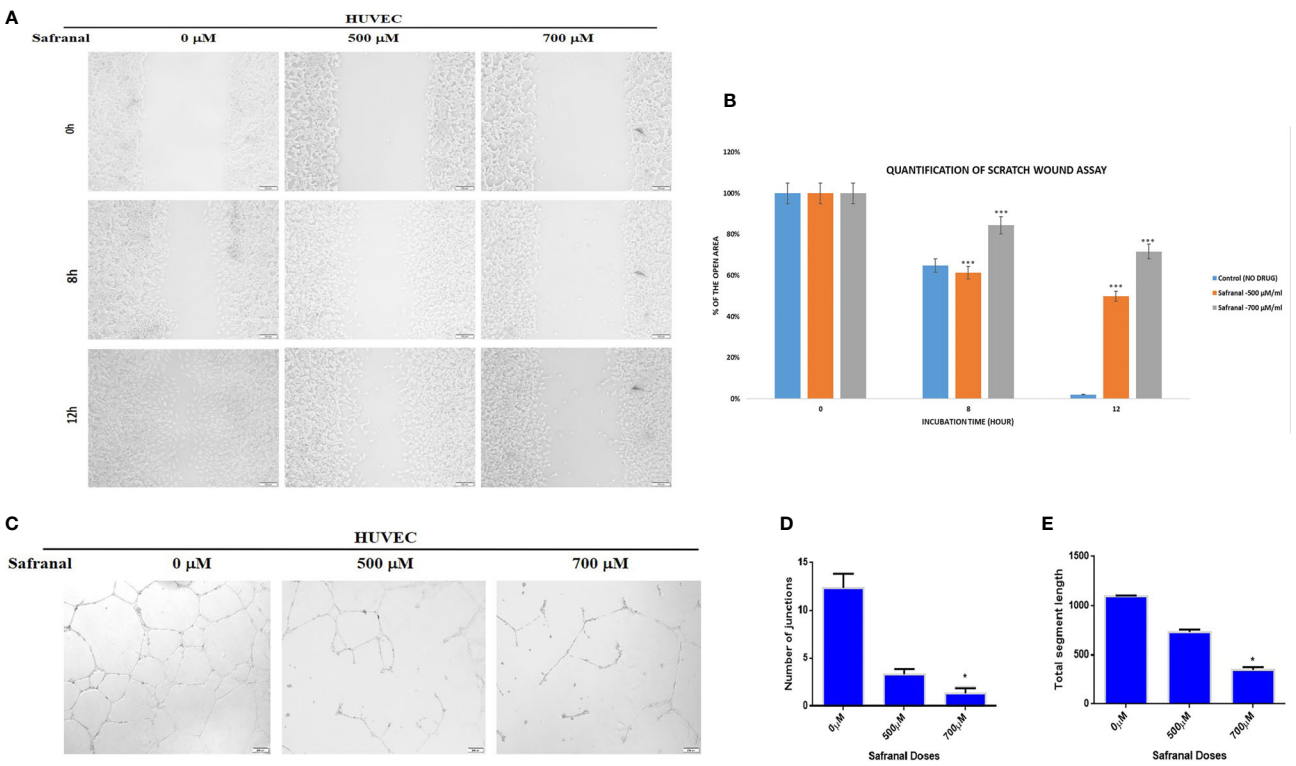

FIGURE 2 | Safranal inhibited HUVECs migration and tube formation. **(A)** HUVECs were grown into full confluence in six-well plate, then cells were wounded with pipette. Further treated with 30 ng/ml rhVEGF as well as various concentrations of safranal. **(B)** The area of the wound was measured at 0 hr, 8 hrs and12 hrs. **(C)** HUVECs incubated with different concentrations of safranal were seeded into 96-well plate pre-coated with Matrigel. After 6 to 8 hrs, tubular structures were photographed, and the number of the tubes was quantified **(D, E)**. Statistical analysis was carried out in all experiments by student's t-test using GraphPad Prism software and $p < 0.05$ was considered as statistically significant. *$p < 0.05$ and ***$p < 0.001$.

downregulated upon 24 hrs of safranal treatment, agreeing with the results of the invasive assays described earlier. Furthermore, we checked the effect of safranal on the transcription of VEGF and its functional cohorts, VEGFR2, and HIF-1α. Gene expression analysis using real time PCR showed a significant decrease of up to 80-90% of mRNA levels of VEGF, VEGFR2, and HIF-1α expression at a 300 µM dose of safranal treatment for 24 hours (**Figure 5C**). Using higher doses showed an irregular, but significant, decrease in the expression of these genes.

DISCUSSION

Safranal, the volatile component extracted from the stigma of the plant, *Crocus sativus* L; saffron, has been reported as potent anticancer and anti-inflammatory agent (27). This study attempts to provide an insight on safranal's role in exerting antiangiogenic properties that could contribute to its anticancer potential. Safranal inhibited the growth of human endothelial cells, HUVEC, at the two tested doses with, and without, VEGF induction suggesting an antiproliferative capacity that may be mediated through VEGF and its receptors. VEGF and its receptors are key regulators of angiogenesis and vascular permeability that contribute heavily to the various stages of tumorigenesis (28). VEGF/VEGFR2 interaction acts as a key switch in the formation of new blood vessels that supply nutrients and oxygen to tumours (29). Here, safranal attenuated both VEGF secretion and VEGFR2 expression in HepG2 cells (**Figures 1B, C**). VEGFR2 is the central receptor for VEGF-induced endothelial cell migration (30). We also successfully demonstrated the impact of safranal in cell migration *in vitro via* a wound healing assay. The wound area was completely closed by migrating untreated HUVEC cells (100%) whereas 50% and 72% of the wound area remained open using safranal doses of 500 µM and 700 µM respectively after 12 hours (**Figures 2A, B**). As HUVEC endothelial cells are reported to migrate to extracellular matrix creating scaffolds aiding the formation of new blood vessels (31), this study, employs the Matrigel tube formation assay to mimic that sprouting of blood vessels in angiogenesis. In this assay safranal significantly ($p<0.01$) reduced the quantity (number of junctions) and quality (length of segments) (**Figures 2C–E**) of capillary-tube like structures formed after 8 hrs of safranal exposure and supported the anti-proliferative effect on HUVEC cells as discussed earlier. Tumor angiogenesis and metastasis requires many signalling circuits which involves

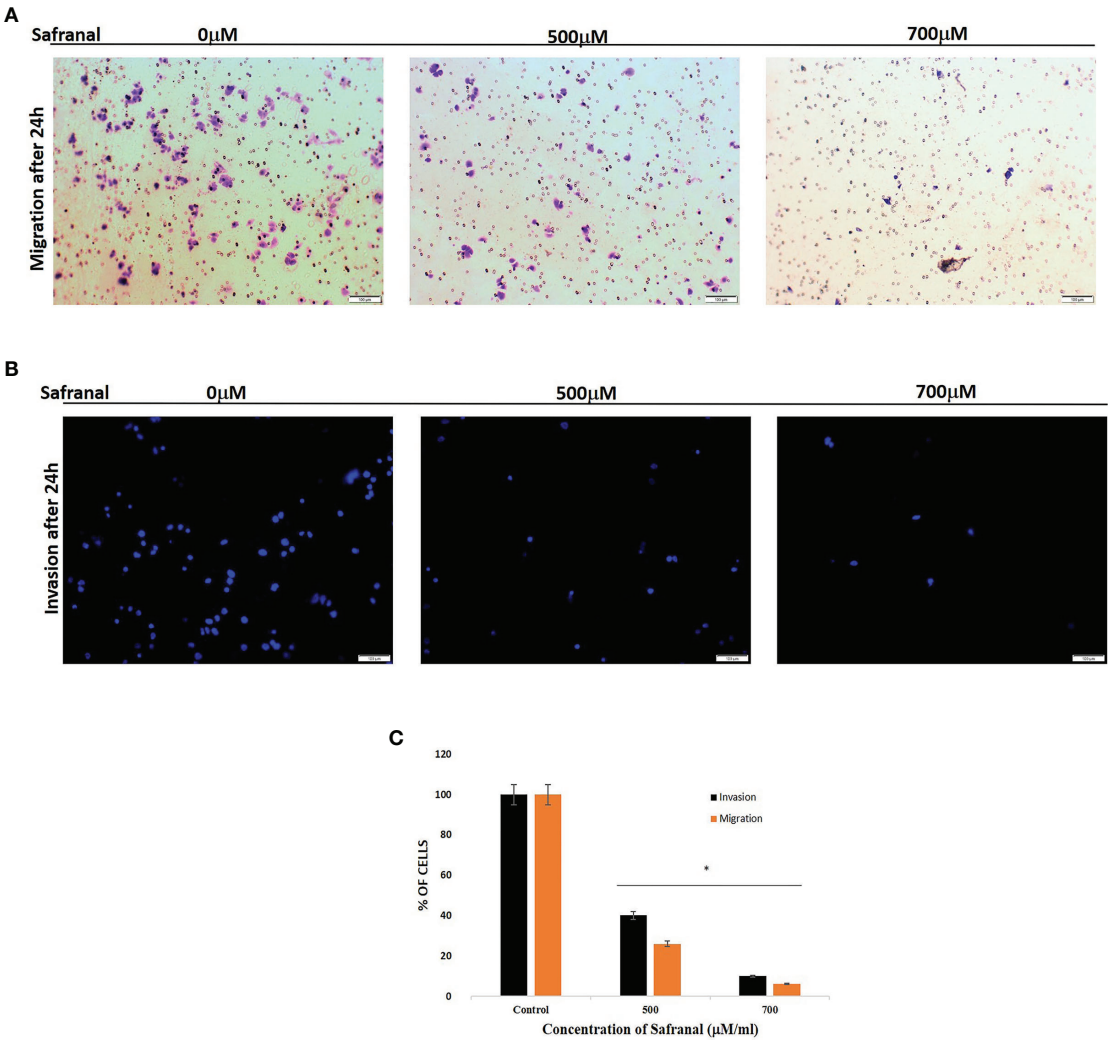

FIGURE 3 | Safranal suppressed HUVECs invasion. Cells were seeded in the upper chamber of Transwell coated with matrigel and incubated with various concentrations of Safranal. The bottom chamber was added with culture medium with 30ng/ml rhVEGF. 24 hrs later, the nucleus of the migrated or invaded cells were stained with crystal violet **(A)** or DAPI **(B)**. The cells were quantified **(C)** through manual counting and presented as the mean ± standard deviation of three independent experiments performed in triplicate. Statistical analysis was carried out by student's t-test using GraphPad Prism software and $p < 0.05$ was considered as statistically significant. *$p < 0.05$.

invasion and the crosstalk between the environment of tumor and host cells (32). As shown in **Figure 3**, safranal inhibited the movement of HepG2 cells through the Boyden chamber without (crystal violet) and with (DAPI blue) extracellular matrix, thereby attesting to its anti-migratory and anti-invasive response in the presence of a chemoattractant. Sprouting of micro vessels *ex vivo* using the rat aortic ring angiogenesis assay can be used as a model for VEGF induced biological event (33). Morphological alterations were detected in aortic ring assays where treatment with safranal reduced the number of sprouting micro vessels in a time-dependent manner. These alterations were then quantitatively substantiated (**Figure 4**). Thanks to its inhibitory effects on angiogenesis, saffron was insinuated as a promising chemotherapeutic agent in breast cancer treatment (34). Collectively, such *ex vivo* results provide preliminary evidence of safranal's possible chemotherapeutic, preventive, and adjunctive applications.

Tumor angiogenesis is associated with altered gene expression of angiogenic factors that are highly irregular compared to normal cells, hence forming vulnerable targets for cancer therapy (35). There was a remarkable reduction in various signalling molecules downstream of the VEGF autocrine pathway upon safranal treatment (**Figures 5A, B**). Typically, anti-angiogenesis treatments focus on central events like wound healing, migration, ECM interaction, infiltration, and invasion fuelling tumor growth (36). ERK and Akt activation by VEGF is a proven signalling pathway that enables cell migration, thereby facilitating vascular homeostasis and angiogenesis (37–39). Here, the expressions of

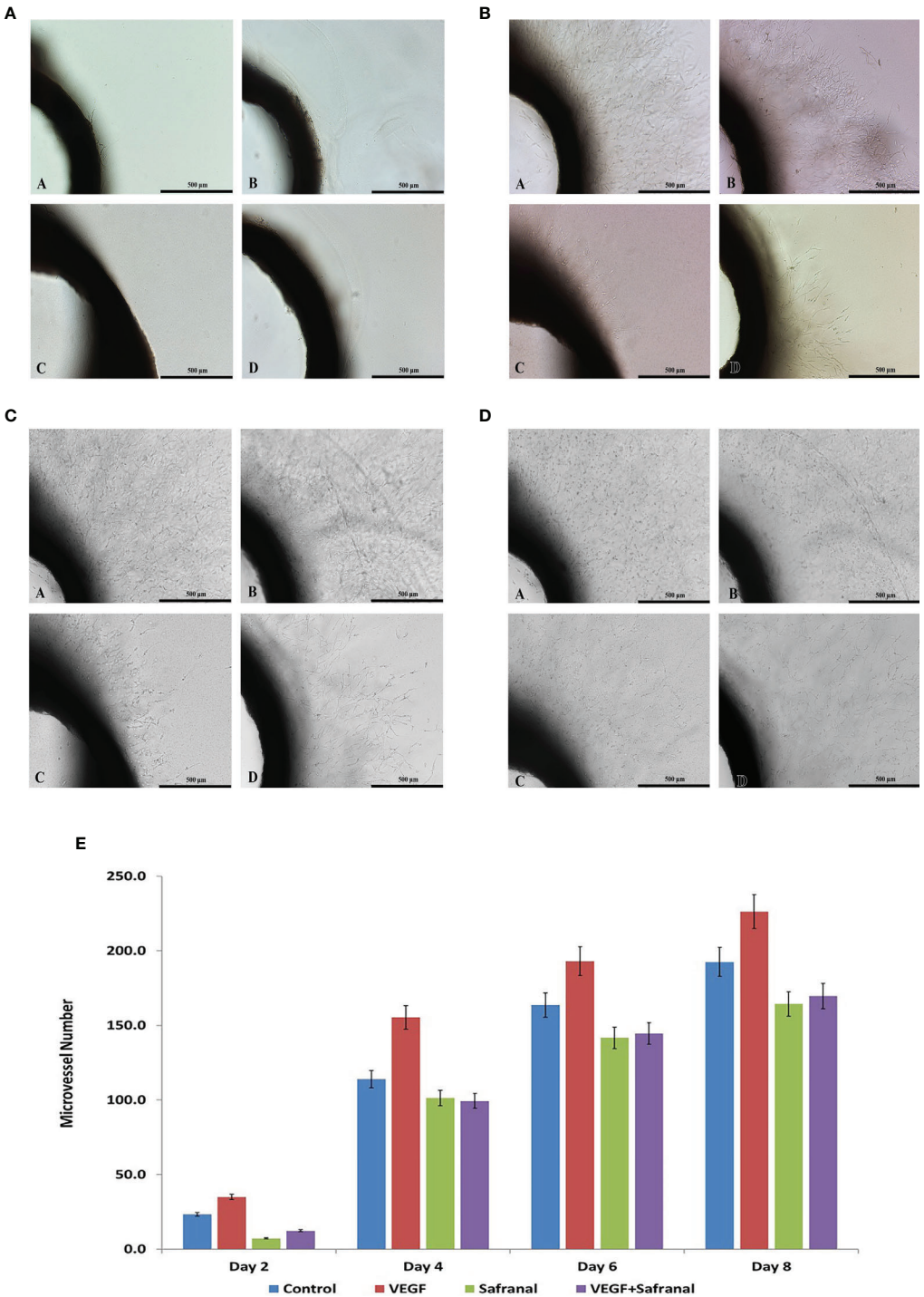

FIGURE 4 | Safranal inhibits angiogenesis *ex vivo*. **(A)** Effects of safranal on microvessels sprouting in aortic ring assay two days post treatment. Representative micrographs of sprouting microvessels from aortic ring grown in the absence **(a)** or presence **(c)** of safranal with VEGF added alone **(B)** or with tested drug **(d)**. **(B)** Effects of safranal on microvessels sprouting in aortic ring assay four days post treatment. Representative micrographs of sprouting microvessels from aortic ring grown in the absence **(a)** or presence **(c)** of safranal with VEGF added alone **(b)** or with tested drug **(d)**. **(C)** Effects of safranal on microvessels sprouting in aortic ring assay six days post treatment. Representative micrographs of sprouting microvessels from aortic ring grown in the absence **(a)** or presence **(c)** of safranal with VEGF added alone **(b)** or with tested drug **(d)**. **(D)** Effects of safranal on microvessels sprouting in aortic ring assay eight days post treatment. Representative micrographs of sprouting microvessels from aortic ring grown in the absence **(a)** or presence **(c)** of safranal with VEGF added alone **(b)** or with tested drug **(d)**.

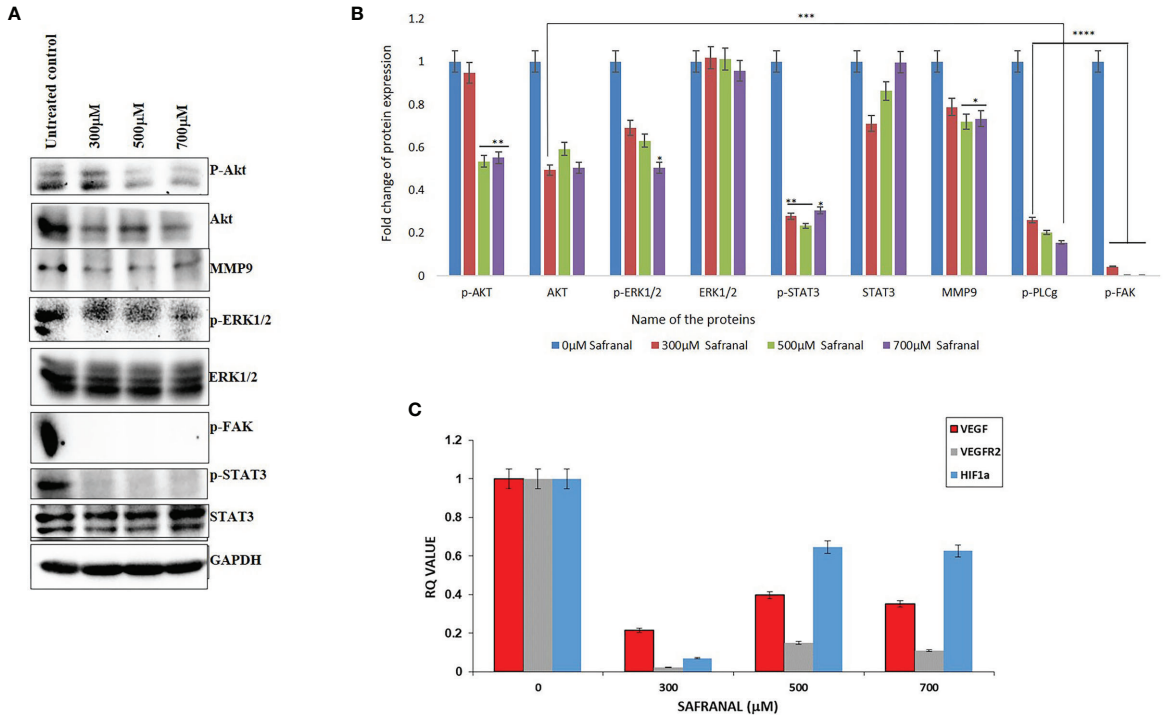

FIGURE 5 | Safranal inhibited VEGF-induced angiogenesis signaling pathway in HCC cells. HepG2 cells were starved with 0.1% FBS overnight and then incubated with various concentrations of Safranal for 24 hrs, with VEGF (30 ng/ml). **(A)** The cell lysates were subjected to western blotting and probed with indicated antibodies, GAPDH was used as loading control. **(B)** Each band intensity was quantified to analyse the protein expression using ImageJ, normalized relative to their respective loading control bands. Values are expressed as ratio of untreated control in log fold. Statistical analysis was carried out in all experiments by student's t-test using GraphPad Prism software and $p < 0.05$ was considered as statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(C)** Quantitative real-time PCR analysis shows that Safranal alters VEGF pathway family genes', VEGF, VEGFR1, MMP3, HIF-1α, expressions in the HEPG2 cells.

p-AKT (Ser473) and p-ERK1/2 were decreased by safranal without affecting the expression of non-phosphorylated ERK1/2. Expression of AKT remained the same with a slightly higher expression in the untreated control (**Figures 5A, B**). This could be due to stimulation *via* rhVEGF as it can elevate the expression of many target molecules downstream to the autocrine pathway in HCC cells (40). Matrix metalloproteinases (MMPs) does proteolytic modulation of ECM and cell surfaces to facilitate the release of signal molecules like VEGF, thereby participating in metastasis and vasculature (41). Safranal reduced the phosphorylation of FAK at Tyr-397 and MMP9 protein (**Figures 5A, B**) which further strengthens its interplay between VEGF signalling. Numerous studies have shown that FAK inactivation impacts the expression of MMPs, augmenting invasion and tumor angiogenesis (42). Signal transducer and activator of transcription 3 (STAT3), is an important member of STATs family which has a major role in inflammation and human cancers. Irregular STAT3 signalling directly stimulates the expression of MMP9, promoting metastasis (43). Safranal inhibited the activation of STAT3 by blocking the phosphorylation at Tyr705 (**Figures 5A, B**). Many studies have proven that the inactivation of STAT3 attenuates key regulators participating in tumor angiogenic events like the migration of vascular cells and the sprouting of vessels, thereby enriching the tumor (44, 45).

Hypoxia takes center stage in tumor environments, leading to the stabilization of hypoxia-inducible factor, HIF-1αγ an important transcription factor that activates many hypoxia-response genes such as VEGF (46). Interestingly, there was a correlated decrease in the mRNA levels of HIF-1α and VEGF (**Figure 5C**), which clearly demonstrates the antiangiogenic effect of safranal. Safranal mediated inactivation of AKT/ERK axis could have manifested the reduction in the HIF-1α mRNA (47). The mRNA level of VEGFR2 is significantly higher in HCC as compared to non-tumor cells (48). From the qPCR data, safranal significantly reduces the mRNA expression of VEGFR2 (**Figure 5C**). This must be due to decreased VEGF synthesis which must have auto regulated the expression of its receptor, VEGFR2. The higher expression of VEGF and its receptors in HCC have been an encouraging signal towards a possible targeted therapy (49).

Taken together, the data presented here suggests that safranal has a pleiotropic effect where it targets multiple key regulators of tumor angiogenesis making them major candidates for potential anti-angiogenic therapy (50). Safranal significantly affects the strong interplay of HCC cell, endothelial cell, and multiple signalling molecules involved in tumor angiogenesis. Being the natural food ingredient of a spice, safranal may be a promising candidate for developing targeted, non-toxic, chemotherapeutic agents for cancer treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by UAE University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

AAm designed the study and supervised all experiments. AAb and CM performed the experiments and did the statistical analysis. AAb and CM wrote the first draft of the manuscript. All authors contributed to the editing of the revised manuscript and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.789172/full#supplementary-material

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frontiers in Pharmacology

REVIEW
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Transforming Growth Factor-Beta (TGF-β) Signaling in Cancer-A Betrayal Within

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A ubiquitously expressed cytokine, transforming growth factor-beta (TGF-β) plays a significant role in various ongoing cellular mechanisms. The gain or loss-of-function of TGF-β and its downstream mediators could lead to a plethora of diseases includes tumorigenesis. Specifically, at the early onset of malignancy TGF-β act as tumour suppressor and plays a key role in clearing malignant cells by reducing the cellular proliferation and differentiation thus triggers the process of apoptosis. Subsequently, TGF-β at an advanced stage of malignancy promotes tumorigenesis by augmenting cellular transformation, epithelial-mesenchymal-transition invasion, and metastasis. Besides playing the dual roles, depending upon the stage of malignancy, TGF-β also regulates cell fate through immune and stroma components. This oscillatory role of TGF-β to fight against cancer or act as a traitor to collaborate and crosstalk with other tumorigenic signaling pathways and its betrayal within the cell depends upon the cellular context. Therefore, the current review highlights and understands the dual role of TGF-β under different cellular conditions and its crosstalk with other signaling pathways in modulating cell fate.

Keywords: TGF-β 1, signaling pathways, metastasis, tumor suppressor, tumorigenesis

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INTRODUCTION

What if your confidant turns into a foe? What if a trustworthy becomes a traitor? Does it hold true for transforming growth factor-beta (TGF-β) mediated signaling networks? This review highlights the story of TGF-β signaling and its betrayal within. The exciting story of TGF-β began nearly 4 decades ago, when in 1978, the ground-breaking efforts of De Larco, George Todaro (De Larco and Todaro, 1978) and later in 1981 the work carried out in the Harold Moses and Michael Sporn-Anita Roberts laboratory at the National Cancer Institute (NCI) resulted in the discovery and understanding of TGF-β (Todaro et al., 1981). The early experiments lead to the notion that TGF-β could be a key factor for tumorigenesis. This was based on the ability of TGF-β to "transform" the behaviour of normal fibroblasts forming progressively growing colonies hence the name "transforming" growth factor (Huang et al., 2014). The tumour suppressive role of TGF-β came as another twist when experiments involving epithelial and lymphoid cells showed growth-suppressive effects of TGF-β (Roberts and Wakefield, 2003). Further, evidence suggest that TGF-β promotes the activation of tumor suppressor genes such as *p15*, *p21* and attenuates the tumour promoting gene *c-MYC* expression thereby supports its antitumor effect (Katz et al., 2013). There was a division among the researchers, some believed that TGF-β could be tumour promoter, and some ended up saying that it has a role in tumour suppression. Dysregulation of TGF-β signaling hijacks the complexes of

biological functions that plays critical role in developmental processes and tumorigenesis, thus emerges as a promising signaling pathway to be targeted for the anticancer drug development at preclinical and clinical stages (Aashaq et al., 2021). TGF-β signaling pathway has a decisive and dual role in the human cancer progression. Besides promotes apoptosis, cell cycle arrest and autophagy in tumor cells, TGF-β also augments cell stemness, cell motility, angiogenesis, EMT and invasion of tumor cells, suggests that TGF-β plays both tumor supportive and suppressive role (Jena et al., 2021). Thus, TGF-β displays a tumor suppressor phenotype in normal cells and early stages of tumorigenesis, whereas in the later stages of cancer progression, it functions as proto-oncogene and promotes oncogenesis. Cellular signaling pathways are finely interconnected networks which regulate various cellular mechanisms such as cell proliferation and differentiation, embryonic development, angiogenesis, and apoptosis through a series of regulated molecular interactions (Kubiczkova et al., 2012). The complex molecular architecture of signaling pathways is controlled through a defined hub of various protein–protein interactions (Pawson and Warner, 2007). Aberrant alterations of key signaling molecules such as TGF-β could perturb the fine balance of signaling networks thereby leads to the acquisition of hallmark capabilities of cancer (Guo and Wang, 2009). Therefore, the current review highlights and summarizes the recent developments in TGF-β associated tumorigenesis, its antitumor effect as well as cross talks with associated signaling pathways. These findings could resurface new potential therapeutic targets of TGF-β associated signaling pathways in modulating cell fate and could predict new tumor biomarkers for future diagnostics.

TGF-β Signaling

TGF-β, a pleiotropic cytokine, plays a plausible role in a plethora of various physiological processes including growth, differentiation, cell death and migration (Neuzillet et al., 2015; Hata and Chen, 2016). The TGF-β family is further classified into two subfamilies: 1) TGF-β subfamily, which includes TGF-β, activin beta chains, and the protein Nodal, and 2) Bone Morphogenetic Protein (BMP) subfamily that includes BMPs, growth differentiation factors (GDFs), and mullerian inhibitory factor (MIF) (Akhurst and Hata, 2012; Caja and Vannucci, 2015). All these proteins which act as ligands are synthesized as dimeric pre-proprotiens. The pre-proproteins are processed for cleavage by proteases to generate mature functional growth factors which are then finally secreted as latent forms but remains interacted noncovalently with their respective polypeptides (Rossetti et al., 2020). Although, TGF-β activation requires release of active ligands, however, reports suggest that the precursor form of the protein nodal binds to the receptors directly to activate signaling without being processed (Schmierer and Hill, 2007). Mammalian TGF-β ligands exist in three isoforms; TGF-β1, TGF-β2, and TGF-β3. Each of these isoforms binds to their respective transmembrane serine/threonine kinases that bind to type I (TGF-βRI) and type II (TGF-βRII) receptors. Seven TGF-βRI (also known as activin-like receptor kinases {ALKs}, ALK1–7), five TGF-βRII (TGFBR2, BMPR2, ACVR2, ACVR2B, and AMHR2) and two TBRIIIs (betaglycan and endoglin) have been identified so far. Structurally, TGF-β receptors consist of a ligands binding extracellular N-terminal domain, an inner transmembrane region and a C-terminal cytoplasmic serine/threonine kinase domain (Santibañez et al., 2011; Kubiczkova et al., 2012; Hata and Chen, 2016). Binding of TGF-β to the receptors activates signaling via phosphorylation of Smads resulting in the formation of Smad complexes that are translocated to the nucleus where they bind to their respective DNA sequences to regulate the transcription of various target genes (Miyazawa et al., 2002) (**Figure 1**).

Smads are small intracellular effector proteins which are activated by TGF-β receptors to mediate intracellular TGF-β signaling (Kit Leng Lui et al., 2017). Smads are well conserved and classified into, the receptor-regulated Smads (R-Smads), which include Smad-1, -2, -3, -5 and -8; the common mediator Smad (Co-Smad), Smad-4; and the inhibitory Smads (I-Smads), Smad-6 and -7. R- and Co-Smads are characterized by two highly conserved domains at their N- and C-termini, known as Mad homology domains MH-1 and MH-2, respectively (Wrana and Attisano, 2000). The MH-1 and MH-2 domains are separated by a proline rich and serine/threonine rich linker domain that aids in phosphorylation (Lai, 2001). The linker region also contains phosphorylation sites for mitogen protein kinase and ubiquitin ligase SMURF1 for its recognition (Kamato et al., 2013). Besides, interact with DNA, MH1 domain can also bind with associated proteins which includes transcriptional factors, co-activators, and co-repressors as well as ubiquitination adaptors, and contains a nuclear localization sequence (NLS), whereas MH2 is responsible for oligomerization of Smads, transactivation of Smad nuclear complexes and transcription of key genes involved in various cellular signaling pathways (Xie et al., 2014; Macias et al., 2015; Ahmed et al., 2017). I-Smads have highly conserved C-terminal MH2 domain but lacks the MH1 domain in the N-termini. I-Smads, Smad-6 and Smad-7, function as negative-feedback regulators of TGF-β signaling. Smad-6 prevents the formation of R- and Co-Smad complexes, whereas Smad-7 recruits E3 ubiquitin ligases SMURF1 and SMURF2 for binding to activated TGF-β receptors leading to the ubiquitin-mediated proteasomal degradation (Xie et al., 2014; Tu et al., 2019).

Smad2 is proposed to be a tumor suppressor protein and encoded by the gene present at chromosome 18q21 (Samanta and Datta, 2012). Various malignancies where the mutation rate of Smad2 occurs at a low frequency are non small cell lung carcinoma (NSCLC) 2%, hepatocellular carcinoma (HCC) 3%, colorectal cancer 8% and cervical cancers 8% (Kim et al., 2000). Mutational analysis suggest that majority of mutations of MH1 and MH2 domains of Smad2 are missense mutations, however, in colon cancer Smad2 had two cases of homozygous deletion mutations (Macias et al., 2015). Besides act as a tumor suppressor, Smad2 plays crucial role in development. Smad2 knockout results in early embryonic lethality in mice (Liu et al., 2016). Smad2 missense or homozygous deletion

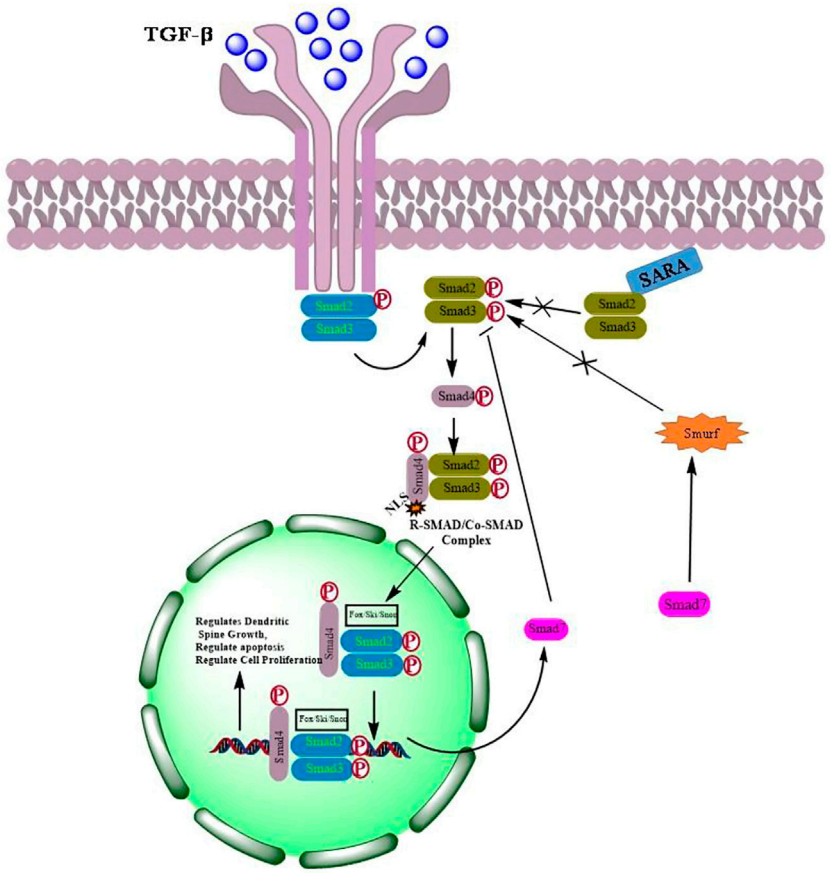

FIGURE 1 | Activation of TGF-β signaling by dimerization of respective receptors followed by phosphorylation and translocation of Smads into the nucleus to regulate transcription of genes involved in cell proliferation, apoptosis and differentiation.

mutations alters phosphorylation, nuclear translocation, and promotes Smad2 auto inhibition thereby leads its degradation. Smad2 is critical for the induction of p21 which regulates cell cycle by acting as a key CDK inhibitor (Moren et al., 2000).

Reports suggest that human tumors have increasing frequency of loss of expression of Smad3 (Vidakovic et al., 2015). Decreased TGF-β responsiveness was observed when Smad3 expression was lost in gastric carcinoma cells, however, TGF-β-mediated tumor suppressor activity was restored when ectopic expression of Smad3 was reintroduced in gastric carcinoma cells, suggests the Smad3 not only acts as tumor suppressor but might also be the target for epigenetic inactivation in gastric carcinoma (Li et al., 2015). Recent evidence suggests that loss of Smad3 expression downregulates TIMP1 expression in choriocarcinoma. This further promotes MMPs activity, thereby plays critical role in tumor invasion (Xu et al., 2003; Rah et al., 2012). Additionally, altered expression of Smad3 is reported to impair the TGF-β-mediated inflammatory response and immune suppression to contribute in tumorigenesis (Hao et al., 2019). Despite Smad3 plays crucial role as a tumor suppressor, recent reports suggest that no embryonic lethality was promoted when Smad3 gene was silenced or knockout. However, it does modulates immune function which later develops colon adenocarcinomas with ability to metastasise to distant secondary sites (Bellam and Pasche, 2010). Another important gene of Smad family located on chromosome 18q is Smad4 (Maru et al., 2004). Smad4 gene is remarkably absent in various cancers such as cervical, prostate, breast, pancreatic, and neuroblastoma due to greater frequency of loss of heterozygosity (LOH) of 18q (Zhao et al., 2018; Rah et al., 2021). The inactivation of Smad4 occurs by various mechanisms which includes frameshift, loss of entire chromosome segment, nonsense and small deletion mutations (Hata et al., 2018). Identified first as deleted in pancreatic carcinoma (DPC-4), Smad4 mutations mainly occurs in pancreatic cancer (Rah et al., 2021). Presence of germ line mutations in MADH4 of juvenile polyposis families further supports that the Smad4 act as a tumor suppressor (Harradine and Akhurst, 2006). Besides, playing critical role in regulating tumorigenesis in various gut associated cancers, Smad4 has been reported to have a crucial role in metastasis (Cheng et al., 2016). Using a cellular and mouse model of TGF-β-induced breast cancer progression, Dekers et al. demonstrated that Smad4 is required for TGF-β induced EMT and bone metastasis of breast cancer cells (Deckers et al., 2006). Further, Smad four knockdown in MDA-MB-231 resulted in the attenuation of EMT transition and bone metastasis thereby

highlighting the role of Smad4 in both tumor suppression and progression (Zhang et al., 2015). Collectively, the recent evidences suggest that Smad4 is a key player in regulating tumor progression and tumor suppression depends upon type and stage of malignancy. The inhibitory SMADs (I-SMADs), Smad 6 & 7 with conserved carboxy-terminal MH2 domains regulate TGF-β signaling through a negative feedback mechanism. The I-Smads inhibit TGF-β signaling through interaction with R-Smads and type I receptors. The inhibitory Smads prevent the complex formation of R-Smads and co-Smads. Smad6 particularly inhibits TGF-β signaling by BMP type I receptors ALK-3 and ALK-6 whereas Smad7 inhibits both TGF-β and BMP-induced Smad signaling. SMAD6 and SMAD7 have been shown to play a critical role in tumor progression. Aberrant expression of SMAD6 has been reported in many human cancers. The inhibition of Smad six is known to contribute to the reinstatement of TGF-β homeostasis and is one of the factors for poor survival in patients with NSCLC (Goto et al., 2007). SMAD6 has also been reported to determine the invasiveness of breast cancer cells in BMP-regulated zebrafish xenograft model. SMAD7, first identified in endothelial cells has a conservative Mad homology 2 (MH2) at its C-terminal with no SXSS domain and Mad homology 1 (MH1) domain at N-terminal which is different from the R-Smads and Co-Smads. The feedback inhibition of TGF-β signaling by Smad seven is due to the interaction of L3 loop of MH2 domain and L45 loop of the TGFβR1 kinase domain. In addition to L3 loop, a three finger-like structure in Smad seven provides additional support to bind to TGFβRI (Miyazawa and Miyazono, 2017). The binding of SMAD7 and TGFβRI blocks SMAD2/3 which further prevents the formation of R-Smad/Co-Smad complex, thereby inhibiting core signalling pathway (Pan et al., 2020). In a similar manner, BMP and activin membrane-bound inhibitor (BAMBI) forms BAMBI/SMAD7/TGFβRI complex which inhibits the activation of SMAD3 (Hernandez et al., 2018). Also, a number of proteins can interact with Smad seven to induce the degradation of TGFβRI. For example, the binding of E3 ubiquitin ligase SMAD ubiquitination regulatory factors (Smurfs) to the Smad 7 N-terminal region results in the degradation of TGFβRI (Koganti et al., 2018). In addition to the feedback regulation of TGF-β signaling, Smad7 also interacts with cellular pathways in an independent manner. SMAD7 is known to antagonize Wnt/β-catenin signalling. Smad seven forms complexes with β-catenin/Smurf2 which results in the degradation of β-catenin via proteasome (Vallée et al., 2017). In human prostate cancer cells, the SMAD7/β-catenin interaction plays a crucial role to provoke c-Myc transcription (Tripathi et al., 2019). Smad7 also promotes TNF-induced apoptosis by inhibiting the expression of several anti-apoptotic NF-κB target genes. In addition, Smad7 abrogates NF-κB activity by regulating the activation of TGF-β-activated kinase 1 (TAK1) (Gingery et al., 2008). Smad 7 augments STAT3 activation by directly interacting with the co-repressor gp130, an intracellular domain of leukemia inhibitory factor (LIF) resulting in the disruption of SOCS3-gp130 or SHP2-gp130 complex. Smad7 plays critical role in coordinating gp-130/STAT3 and TGF-β/Smad signalling pathways that promotes pathophysiological processes such as inflammation and tumorigenesis (Yu et al., 2017). Taken together, these findings revealed that I-Smads, Smad 6 and 7 regulate plethora of physiological and pathophysiological processes both TGF-β dependent and independent manner.

TGF-β as a Tumour Suppressor

TGF-β attains its tumour suppressive role by regulating cell proliferation, apoptosis and immune cell modulation. TGF-β signaling prominently abrogates malignant cell growth through both canonical SMAD-dependent and non-canonical pathway. Through canonical pathway, TGF-β inhibits cell cycle progression through G1-arrest by activating cyclin dependent kinase (CDK) inhibitors p21 and p15. TGF-β suppresses an important oncogene, c-Myc, which stimulates the proliferation and inhibits the transcriptional activation of p21 and p15 (Mukherjee et al., 2010; Katz et al., 2013). In addition, TGF-β inhibits DNA-binding protein inhibitor (ID1, 2, 3) and nuclear factors which plays a crucial role in cell differentiation and progression from G1 to S phase of cell cycle (Katz et al., 2013; Yoshida et al., 2018). TGF-β induces apoptosis in a variety of cell types by modulating the expression of B-cell lymphoma-2 (Bcl-2) family members, death receptor fibroblast associated antigen (FAS), growth arrest and DNA damage-inducible (GADD) 45-β, death-associated kinase (DAPK), and caspases to induce both the intrinsic and extrinsic apoptosis (Zhang et al., 2017). The role of TGF-β as a tumour suppressor has been demonstrated in several cancers (Meulmeester and Ten Dijke, 2011). The non-canonical TGF-β promotes tumor suppressor activity via p38 MAPK pathway to activate caspase-8-dependent programmed cell death. Besides induces tumor suppressive role by activation of programmed cell death, TGF-β promotes tumor suppressive role by regulating immune cell function in favour of tumor cell death (Schrantz et al., 2001). Taken together, TGF-β at the initial stage of tumorigenesis promotes tumor suppression activity, by arresting cell cycle, induces DNA damage and apoptosis is malignant cells.

TGF-β as a Tumor Promoter

In the later stages of cancer, TGF-β can paradoxically result in tumor progression and metastasis (Katz et al., 2013). Dysregulated expression of TGF-β signaling has been reported in many cancers such as hepatocellular carcinoma, colon, prostate, lung, and breast cancers (Sheen et al., 2013; Zhao and Chen, 2014; Villalba et al., 2017). TGF-β plays an important role in tumorigenesis and promotes tumour development by stimulating epithelial-to-mesenchymal transition (EMT), cell proliferation, invasion, metastasis, angiogenesis and evasion of immune surveillance (Melzer et al., 2017, 2019; Dufour et al., 2018). *In vitro* studies have demonstrated that increase in EMT is associated with the overexpression of Smad-3/4. TGF-β also promotes the secretion of matrix metalloproteases (MMP)-2 and -9, and inhibits the activity of tissue inhibitors of MMPs (TIMPs) (Moustakas and Heldin, 2016; Tan et al., 2017). Collectively, these reports suggest that constitutive activation or dysregulation of TGF-β signalling modulates the expression of various molecules which in turn can promote cell proliferation,

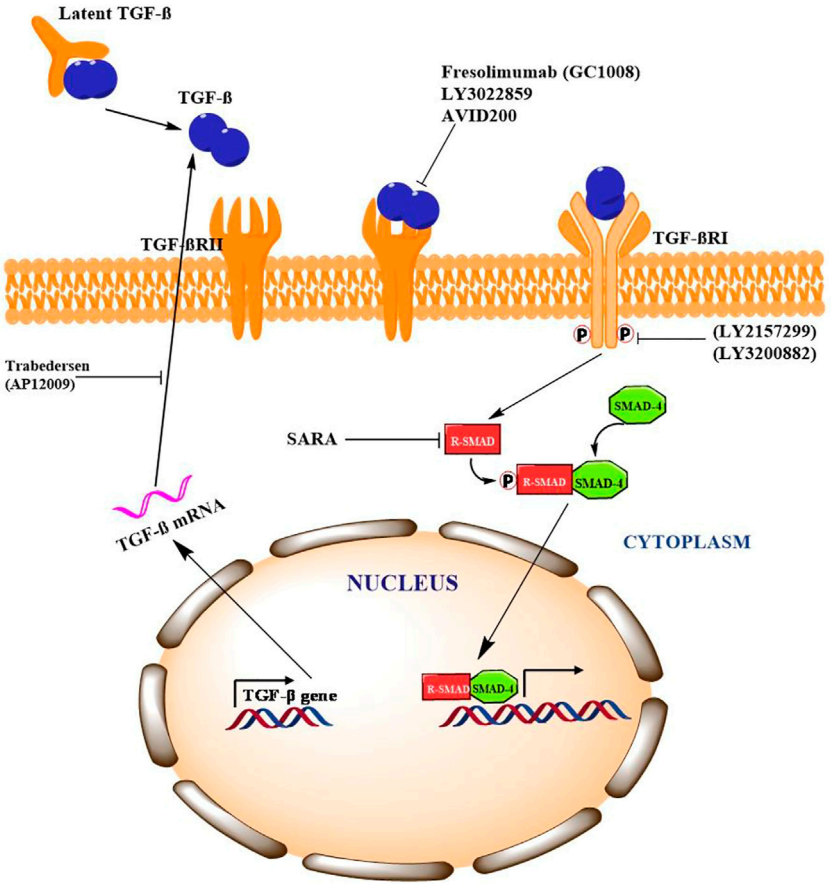

FIGURE 2 | Pharmacological intervention of TFG-β signaling and potential targets Signaling Cross Talk between TGF-β/Smad with other Signaling Pathways.

invasion, EMT and metastasis to distant sites during late stage malignancies.

TGF-β as a Therapeutic Target

The complex role of TGF-β in cancer necessitates the comprehensive understanding in order to strategize effective therapeutic approach. A number of pharmacological interventions that target different signaling components of TGF-β have shown promising results in number of preclinical and clinical trials. Different strategies including neutralizing antibodies, ligand trapping, small-molecule inhibitors and antisense oligonucleotides are being explored to target TGF-β signaling. In phase-I clinical trial for malignant melanoma patients, IgG4κ monoclonal antibody, fresolimumab (GC1008), has shown anti-cancer activity by neutralizing TGF-βI, II, and III (Morris et al., 2014). In addition, treating non-small cell lung cancer patients with fresolimumab is still in phase-II clinical trials. Studies in animal models have shown that IgG1 monoclonal antibody, an anti-TGF-βRII (LY3022859) blocks the binding of TGF-β to ectodomain of TGF-βRII which results in significant decrease in tumor growth and metastasis (Zhong et al., 2010). TGF-β ligand trapping by AVID200, a chimeric fusion protein, prevents binding of TGF-β to the receptor. *In vivo* study by Sanjabi et al., demonstrated that AVID200 enhanced the anti-cancer activity in immunocompetent host mice (Sanjabi et al., 2017). AVID200 is currently in phase-I clinical trials for advanced solid tumor patients (Yap et al., 2020). Galunisertib (LY2157299), a small-molecule inhibitor, binds to TGF-βRI thereby inhibiting its kinase activity. Preclinical study by Yingling et al., in *in-vitro* and *in-vivo* models demonstrated anti-tumour activity of galunisertib (Yingling et al., 2018). Phase-I clinical trials of galunisertib revealed promising anti-cancer activity in patients with pancreatic cancer, glioma, HCC and advanced solid tumours (Fujiwara et al., 2015; Ikeda et al., 2019; Wick et al., 2020). LY3200882, a potent ATP-competitive TGF-βRI inhibitor has shown antitumor activity in both preclinical mouse model of TNBC as well as patients with metastatic cancers (Pei et al., 2017). Another strategy is antisense oligonucleotides (AON) which are specifically designed to block the translation of genes. Trabedersen (AP12009), an AON, targeting TGF-βRII mRNA has shown promising effects in phase-I clinical trials for patients with pancreatic cancer, colorectal cancer and melanoma (Oettle et al., 2011). Nemunaitis et al., has shown that Belagenpumatucel-L or Lucanix, an AON vaccine targeting TGF-βRII improved the overall survival of NSCLC patients after chemotherapy

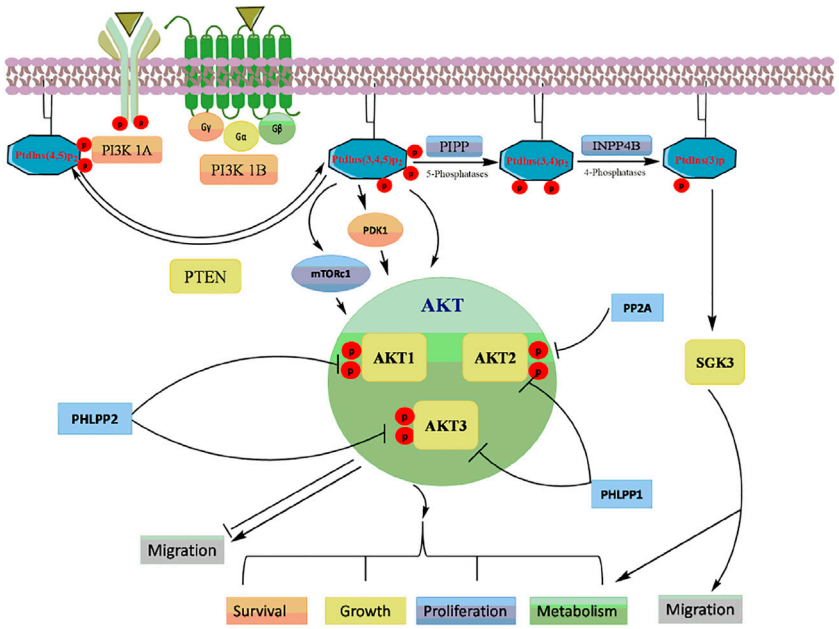

FIGURE 3 | A schematic representation of the PI3K/Akt signalling and its downstream targets.

(Nemunaitis et al., 2009). Collectively, these evidences indicate that TGF-β is a promising therapeutic target to inhibit tumorigenesis in plethora of cancers as described in **Figure 2**.

PI3K/Akt Signaling

The PI3K/Akt signaling pathway is a master regulator of various physiological and cellular processes including cell proliferation, growth, and survival (Rodgers et al., 2017). PI3Ks are classified into three classes based on the structure, distribution, substrate specificity and mechanism of action. PI3Ks are phospholipid kinases, existing as a heterodimer of a regulatory subunit p85 (p85α, p85β, p55α, p55γ and p50α) and a catalytic subunit p110 (p110α, p110β, p110γ, and p110δ) (Piddock et al., 2017; Giordano and Kiger, 2020). The multiple receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs) activate PI3K which inturn phosphorylate phosphatidylinositol 4,5-bisphosphate to form phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Mantamadiotis, 2017). PIP3 binds to the pleckstrin homology (PH) domains of various signaling proteins, including phosphoinositide-dependent kinases (PDK1) and its downstream target protein kinase B/Akt (Krygowska and Castellano, 2018; Gesmundo et al., 2019). The phosphorylation of the two critical amino acid residues, Thr308 and Ser473 is essential for full Akt activation (Yu and Cui, 2016). Akt has three isoforms: Akt1, Akt2 and Akt3, that are expressed from distinct genes located on separate chromosomes (Rahmani et al., 2020). Akt1 and Akt2 are ubiquitously expressed in human tissues, while Akt3 is restricted to brain and testes (Ji et al., 2015; Tian et al., 2019).

Akt activation causes the phosphorylation of many downstream targets in the cytoplasm and nucleus, explaining its relatively broad range of downstream effects and increases cell proliferation, invasion, and angiogenesis (Hinz and Jücker, 2019) (**Figure 3**). Activated Akt inturn phosphorylated wide range of target proteins including glycogen synthase kinase-3β (GSK-3β) (Hinds et al., 2016), forkhead box O transcription factor (FOXO) (Norambuena-Soto et al., 2017), Mouse double minute two homolog (MDM2) (Li et al., 2020), inhibitor of IkB kinase (IKK) (Ghoneum and Said, 2019), Bcl-2 interacting mediated cell death (BIM) (Kapoor et al., 2020), Bcl-2 associated agonist of cell death (BID) and Bcl-2 associated X protein (Bax) (Liu et al., 2020). The PI3K/Akt pathway is tightly regulated by lipid phosphatase enzyme phosphatase and tensin homolog (PTEN), which negatively regulates the kinase activity of PI3K (Haddadi et al., 2018).

Hyperactivation of the PI3K/Akt pathway is frequently seen in many cancers (Rasool et al., 2017; Rodgers et al., 2017). PI3K/Akt can activate NF-kB signaling by phosphorylation IKK or by stimulating nuclear translocation of NF-kB (p65) thereby inducing cell proliferation and apoptosis evasion (Tilborghs et al., 2017). Akt is known to inhibit proapoptotic proteins such as Bax, Bad and procaspase-9 (Wang et al., 2018). Akt also antagonizes p53-mediated apoptosis by phosphorylation MDM2 contributing to chromosome instability in cancer (Cao et al., 2020). Several studies have documented an increase in the expression of PI3K and Akt with suppression of PTEN in various human cancers (Han et al., 2018). Recent evidences suggest that PI3K/Akt pathway has been extensively linked with TGF-β signaling pathway majorly in stem cells and tumor cells of various tissues (Yeh et al., 2018). Although the cross-talk of these pathways is intricated, mutual regulation depends upon

cellular context and associated pathophysiological processes. Depending on cellular context this crosstalk can result in either inhibition or activation of various downstream molecules critical for biological processes. TGF-β can directly or indirectly activate PI3K-Akt pathway. The key components of TGF-β family Smad2/3 activation in coordination with hyperactivation of PI3K, modulates cell fate of human embryonic stem cells (hESCs) by upregulated the expression of Nanog which is a key pluripotent gene essential for self renewal (Gordeeva, 2019). Moreover, increased expression of PI3K inhibits ERK/MAPK signalling which results in GSK 3B activation leading to b-catenin inhibition. Since Smad2/3 in association with b-catenin is required for mesendoderm gene expression initiation, low PI3K activity allows β-catenin and Smad2/3 complex for direct mesoderm differentiation (Jason et al., 2015). In epithelial and lymphoid cells, smad dependent TGF-β signalling in known to inhibit cell proliferation and induces apoptosis in hepatocytes and resting B cells. PI3K/Akt signaling antagonizes TGF-β-mediated proapoptotic effect in B cells and hepatocytes by allowing interaction of Akt with Smad3 in cellular milieu. The binding of Akt to smad three results in sequestering smad3 which prevents Smad3 dependent apoptosis in hepatocytes (Papoutsoglou et al., 2019). Another study suggests that the cytostatic effect of TGF-β/Smad3 signaling is promoted via Akt-mediated phosphorylation of FOXO. The phosphorylated FOXO interacts with Smad3 to block its translocation into the nucleus thereby preventing transcription of genes involved in apoptosis (Yadav et al., 2018). This inhibition promoted by PI3K/Akt signaling switches the role of TGF-β from tumor suppression in early tumorigenesis to tumor promotion in the late stage tumorigenesis (Syed, 2016). Additionally, PI3K/Akt pathway in coordination with TGF-β signaling regulates EMT, cell invasion, and metastasis in various types of malignant cells (Luo, 2017). TGF-β phosphorylated Akt at Ser-473 and activates its kinase activity via integrin-linked kinase (ILK) (Tsirtsaki and Gkretsi, 2020). This activation promotes optimal transcriptional activity of Smad3 to upregulated expression collagen I in mesangial cells. A mechanistic study by Runyan et al. has demonstrated that PI3K/AKT signalling influences the expression of collagen I in mesangial cells stimulated by TGF-β (Runyan et al., 2004). Cancer cells in the tumor microenvironment require enhanced glycolysis to survive and proliferate. In glioblastoma cells, Smad dependent TGF-β signalling is known to target p38 MAPK and PI3K/Akt signaling pathway which in turn increases the expression of PFKFB3 and induces glycolysis (Rodríguez-García et al., 2017). Also, in normal murine mammary gland epithelial cells, TGF-β promotes the expression of connexin43 gene expression by activation p38 and PI3K/AKT signaling (Tacheau et al., 2008).

Together, these evidences suggest that PI3K/Akt signaling is linked with TGF-β signaling at multiple crosstalk points during tumor development. Depends upon the cellular context and influence of other signaling pathways, TGF-β could act as tumor suppressor by promotes apoptosis and/or tumorigenic regulates critical events such as EMT, invasion and metastasis of malignant cells (**Figure 7**).

NF-kB Signaling

NF-kB was first discovered as a transcription factor in the nucleus of B cells where it was reported to bind to the enhancer region of the k-light chain of immunoglobulin family. The NF-kB proteins are divided into two subfamilies, the 'NF-kB' proteins (p50/NF-kB1, and p52/NF-kB2) and the 'Rel' proteins (RelA/p65, c-Rel, RelB) (Tilborghs et al., 2017; Mitchell et al., 2019). These proteins are characterized by a highly conserved domain (Rel homology domain) of 300 amino acid residues essential for homo- or heterodimer formation to interact with DNA and IkB family of proteins (Kanapeckaitė et al., 2021). The C-terminal region of the RHD has a nuclear localisation signals that helps in the delivery of active form of NF-kB complexes into the nucleus, whereas the N-terminal region contains the DNA-binding domain (Serasanambati and Chilakapati, 2016). In addition, Rel proteins comprises of a transactivation domain (TAD) at C-terminal whereas NF-kB subfamily members contains multiple copies of ankyrin repeats which act to auto-inhibit these proteins (Collins et al., 2016). The activation of NF-kB can occur by two separate pathways, classical (canonical) or non-classical (non-canonical or alternate) pathway (Ichikawa et al., 2015; Park and Hong, 2016).

The Canonical Pathway: This pathway is activated primarily in response to many internal factors including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, epidermal growth factor (EGF), T- and B-cell mitogen, bacteria, and lipopolysaccharides, viral proteins, double-stranded RNA, and external agents involving physical and chemical stress (Chen et al., 2018a; Taniguchi and Karin, 2018). Initially NF-kB is inactive in naive cells that are not yet stimulated by external signals and the p50/p65 heterodimer is retained in the cytosol by inhibitor protein, IkB (Ghafoori et al., 2009). The IkB family consisting of IkB-α, IkB-β and IkB-ε subunits comprises of six ankyrin repeats that prevents the translocation of p50/p65 into the nucleus by shedding the activity of nuclear localisation signals of NF-kB (Morotti et al., 2017). The activity of IkB is tightly regulated by IKK, a large multisubunit kinase complex consisting of two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit IKKε (NEMO). In response to NF-kB inducing signals, both IKKα and IKKβ induce phosphorylation and degradation of IKB proteins (Galluzzi et al., 2012). The disintegration of IKB leads to the release and subsequent translocation of NFkB p65-p50 heterodimer into the nucleus, where it binds to the kB elements to mediate the transcription of responsive genes involved in cell growth, differentiation and survival, immune response, inflammation, apoptosis, invasion, metastasis, and angiogenesis (Yuan et al., 2016; Sun, 2017; Taniguchi and Karin, 2018).

The Non-canonical Pathway: Various members of TNF cytokine family such as lymphotoxin, B-cell activating factor belonging to the TNF family (BAFF), CD40 ligands or viruses such as Epstein-Barr virus (EBV) and T-cell leukaemia virus (Zhang et al., 2020b) activates the non-canonical pathway of or

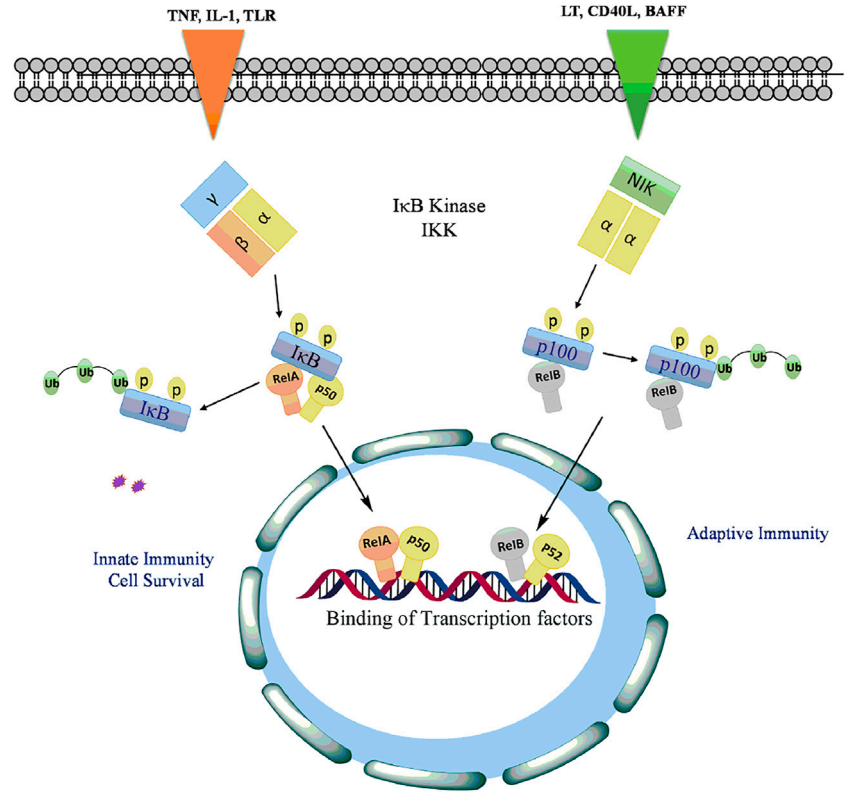

FIGURE 4 | Activation of NF-kB signaling by phosphorylation of IkB with TLRs and proinflammatory cytokines to release and allow translocation of RelA, p50, RelB into the nucleus to regulate transcription of genes involved in cell proliferation, antiapoptotsis, inflammation, cell survival, innate and adaptive immunity.

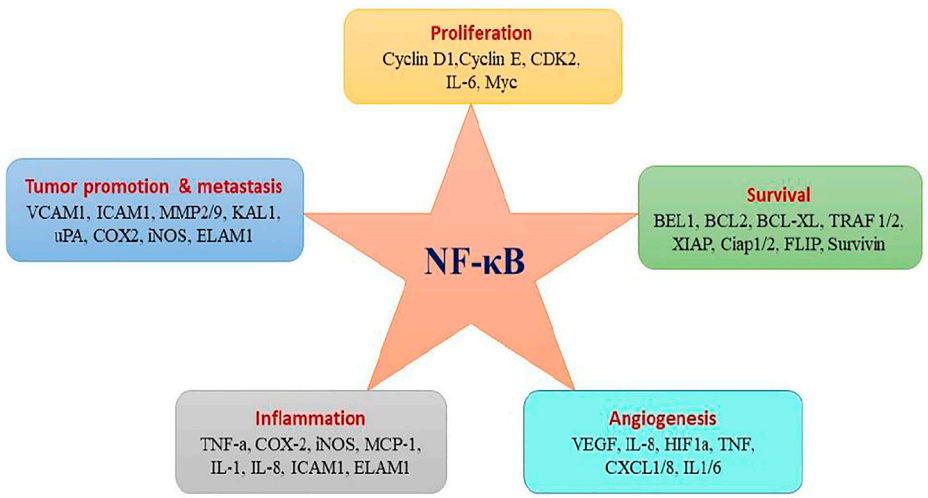

FIGURE 5 | NF-kB activation affects hallmarks of cancer trough the transcription of genes involved in cell proliferation, survival, and angiogenesis.

alternative NF-kB signaling pathway. This pathway involves phosphorylation and activation of IKKα by the NF-kB-inducing kinase (NIK) which in turn phosphorylated NF-kB2 (p52/p100) at Ser866 and Ser870 (Demchenko et al., 2014). The phosphorylation of p52/p100 by IKKα results in the proteasomal degradation of p100 leading to activation of RelB/p52 heterodimer (Roy et al., 2018). The active p52-RelB heterodimer translocated into the nucleus binds to respective

elements and regulates the expression of genes required for lymph organogenesis and B-cell activation (Park and Hong, 2016) as depicted in **Figure 4**.

Several *in vitro* and *in vivo* studies have revealed the constitutive activation of NF-kB and its association with many types of cancers including breast, lung, colon, pancreas, head and neck, oesophagus as well as melanomas, lymphomas (Xiao et al., 2018), and its role has been associated with various tumour-favouring cellular processes including cancer cell proliferation, preventing apoptosis, and increasing a tumor's angiogenic and metastatic potential (Park and Hong, 2016). **Figure 5** shows the various target genes of NF-kB. NF-kB is activated in cancer either from extrinsic signals in the tumor microenvironment or from intrinsic deregulation of the pathway within the tumor (Lee et al., 2017). Various factors such as autocrine secretion of inflammatory mediators (chemokines and cytokines), mutations and/or overexpression of ligands and receptors (EGF, hepatocytes growth factors and integrins), activation of kinases (IKK, NIK, GSK-3β, Akt/PKB, and mutation with defective function of IkB-α contribute to constitutive activation of NF-kB (Nagel et al., 2015; Lee et al., 2017).

Aberrant activation of TGF-β in association with NF-kB signaling has been documented in various cancers (Zappavigna et al., 2020). Activation of NF-kB by TGF-β has been reported to mediate the transcriptional activation of various TGF-β target genes (Torrealba et al., 2019). A study by Kon et al. (Khafaga et al., 2019), has shown that TGF-β triggers TNF-α or interleukin-1 to activate type VII collagen gene expression through NF-kB-binding site and SBE sites in various regulatory gene sequences (De La Cuesta et al., 2019). Aberrant activation of TGF-β/NF-kB signaling pathways has been documented to promote EMT and angiogenesis. TGF-β activates transcription of NF-kB target genes and promotes EMT in pancreatic cells as well as proliferation and differentiation of keratinocytes (Khatami, 2017). Activation of the NF-kB by TGF-β can be mediated by both canonical Smad pathway and non-canonical Smad pathway (Tripathi et al., 2019). In Canonical Smad pathway, Smad3 is shown to interact with the core proteins of NF-kB to activate various auxiliary proteins (Visconte et al., 2019). The physical interaction of Smad3 and p52/RelB is known to activate Jun B expression (Luo, 2017). Brandl et al., 2010 demonstrated that TGF-β-SMAD signaling is regulated by IKKα by interacting with SMAD3 thereby governing SMAD complex formation on DNA. Furthermore, the TGF-β-IKKα-SMAD signaling downregulates E-cadherin and activates transcription of genes encoding Slug and Snail in pancreatic cancer cells. In addition, IKKα also modulates canonical TGF-β-SMAD signaling in human MDA-MB231 breast cancer cells thereby highlighting the impact of IKKα on TGF-β-SMAD signalling. (Brandl et al., 2010). In non-Smad pathway, TGF-β can also activate NF-kB by TGF-β-activated kinase 1 (TAK1). TAK1 activates IKK, which in turn phosphorylate IκBα, leading to proteasomal degradation of IKBα and the release of NF-kB p65-p50 heterodimer resulting in NF-κB activation. (Hydarpoor et al., 2020). Studies have demonstrated that TGF-TAK1 also induces NF-kB activation in murine B cells, hepatocytes and head and neck squamous cell carcinoma (HNSCC) cells (Loren et al., 2021). Freudlsperger et al. has demonstrated the aberrant TGF-TAK1 expression and its association with nuclear NF-κB activation in HNSCC tumors (Freudlsperger et al., 2013). In response to TGF-β, TAK one also activates RhoA-Rho-associated kinase (ROCK) resulting in the phosphorylation and activation of IKKβ, leading to NF-kB activation (Kwon et al., 2018a). In addition, TGF-β also evokes cellular response through activation of PI3K-Akt pathway leading to the phosphorylation of IKKα/β, IkB and NF-kB which results in increased integrin expression and cell migration (Kwon et al., 2018b). Thus, the key players in NF-κB signaling pathway not only function as signaling components but also can act as the crossroad between NF-kB and TGF-β pathways (Torrealba et al., 2019). Although, many studies suggest the role of TGF-β in activating NF-κB, repression of NF-κB signaling by TGF-β has also been reported (Zhang et al., 2020a). Several studies have suggested a critical role of inhibitory Smad, Smad7 in the crosstalk between TGF-β and NF-κB signaling (Chen et al., 2018b). The upregulation of Smad7 and its interaction with NF-κB subunit p65 suppresses TGF-β-Smad signaling (Ciceu et al., 2021). On the other hand, an increase in the expression of Smad7 can also induce IκBα, thereby inhibiting NF-κB activation (Lu et al., 2017). This inhibition of NF-kB by TGF-β could be attributed to the negative feedback loop (Lang et al., 2020). A study by Arsura et al. demonstrated that in murine B cells and hepatocytes, the initial activation of NF-kB leads to the transcriptional activation of IkB that eventually causes inhibition of NF-kB signaling (Wang et al., 2017). This feedback loop could act as an important target in attenuating the cytostatic response of TGF-β during malignant progression. Collectively, TGF-β signaling modulates NF-kB signaling and promotes transcriptional activity of various genes which are involved in cell proliferation, invasion, metastasis, EMT and associated inflammatory signaling to promote tumorigenesis. In conclusion, although some studies suggest that TGF-β is also regulated by IKK to prevent tumorigenesis, however, significant number of studies demonstrated that the cross talk of TGF-β-mediated Smad/NF-kβ drives transcription of tumorigenic genes for tumor cell proliferation, growth, invasion, angiogenesis and metastasis to distinct secondary sites (**Figure 7**).

The JAK/STAT Signaling

The JAK/STAT pathway mediates cellular responses to a wide array of cytokines and growth factors (Maude et al., 2015; Pencik et al., 2016). JAKs were initially named as "just another kinase", but were later changed to "Janus kinase" which was attributed to being a unique class of tyrosine kinases that contain both a catalytic and kinase-like domain and possesses autoregulatory function (Genovese et al., 2017). Abundant evidence has supported the role of JAK/STAT in the regulation of various cellular processes including proliferation, differentiation, migration, apoptosis, and cell survival, depending on the signal, tissue, and cellular context (Pencik et al., 2016; Rios-Fuller et al., 2018). Mammalian JAK family contains four members: JAK1, JAK2, JAK3, and TYK2 each binding to different

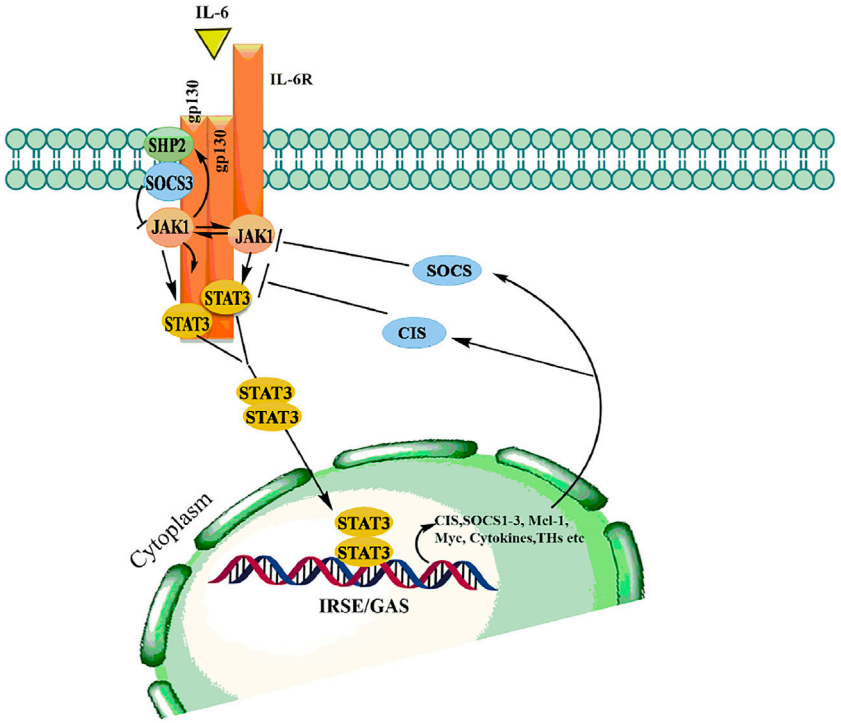

FIGURE 6 | Binding of ligand to a cytokine receptor results in dimerization and conformational changes leading to activation of JAK, which in turn phosphorylates downstream mediator STATs thereby allows dimerization followed by translocation into the nucleus to modulate transcription various genes involved in hematopoiesis, immunity, growth and differentiation.

receptors. STAT family is composed of seven members STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6, each having the tendency to bind to different cytokines (Siveen et al., 2018; Hammarén et al., 2019). The JAK proteins are relatively large kinases with more than 1,100 amino acids with a molecular mass between 120–130 KDa (Shahjahani et al., 2020). The JAK/STAT signaling is relatively simple and is activated by binding of extracellular ligands to the receptors that phosphorylated intracellular JAKs associated with them (Jan et al., 2021). Phosphorylated JAKs in turn create the docking site for downstream substrates, including both the receptor and the STATs (Bousoik and Montazeri Aliabadi, 2018). The activated STATs form homodimers in the cytoplasm followed by translocation to the nucleus where they bind to specific enhancer regions in target genes, thus regulating their transcription (**Figure 6**) (Hoi et al., 2016). Signal transducers and activators of transcription (STATs) belongs to a family of transcription factors, activated by Janus kinases (JAK) through phosphorylation of tyrosine residues in response to various cytokines and growth factors including macrophage colony-stimulating factor 1 (CSF-1), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR) (Singh et al., 2013) and interleukin-6 (IL-6) (Loh et al., 2019). The activated STAT3 forms a homodimers in the cytoplasm and transmits cytokine receptor generated signals by translocation into the nucleus (Mohassab et al., 2020). STAT3 binds to specific DNA response elements and regulates various processes that maintain the normal cellular homeostasis (Batista and Helguero, 2018).

The JAK/STAT signaling pathway plays a vital role in normal physiological processes. However, during the multistep process of carcinogenesis, JAK/STAT signaling pathway is persistently activated (Hu et al., 2020). Once in the nucleus, STAT3 homodimers binds to specific regulatory sequences and modulates the expression of many genes that have been shown to suppress apoptosis and induce cellular transformation (Abroun et al., 2015). Constitutive activation of JAK/STAT signaling has been implicated in various cancers including head and neck, gastric, breast, pancreatic, and prostate (Bose et al., 2020). Aberrant activation of JAK/STAT3 can mediate the recruitment of other molecules involved in tumorigenesis. STAT3 mediates its action by binding to the target genes involved in cell cycle regulation, cyclin D1 and inhibiting apoptosis by targeting anti-apoptotic Bcl-2, thereby contributing to cancer progression (Shao et al., 2021). The IL-6 associated JAK/STAT signaling pathway plays an important role in cancer development, and has proven to exhibit multifaceted properties to be considered as a therapeutic target for the treatment of cancer (Groner and von Manstein, 2017).

Several reports have described the regulation of JAK-STAT by TGF-β in either positive or negative manner. Interleukin12-induced activation of JAK2 in T lymphocytes is inhibited by

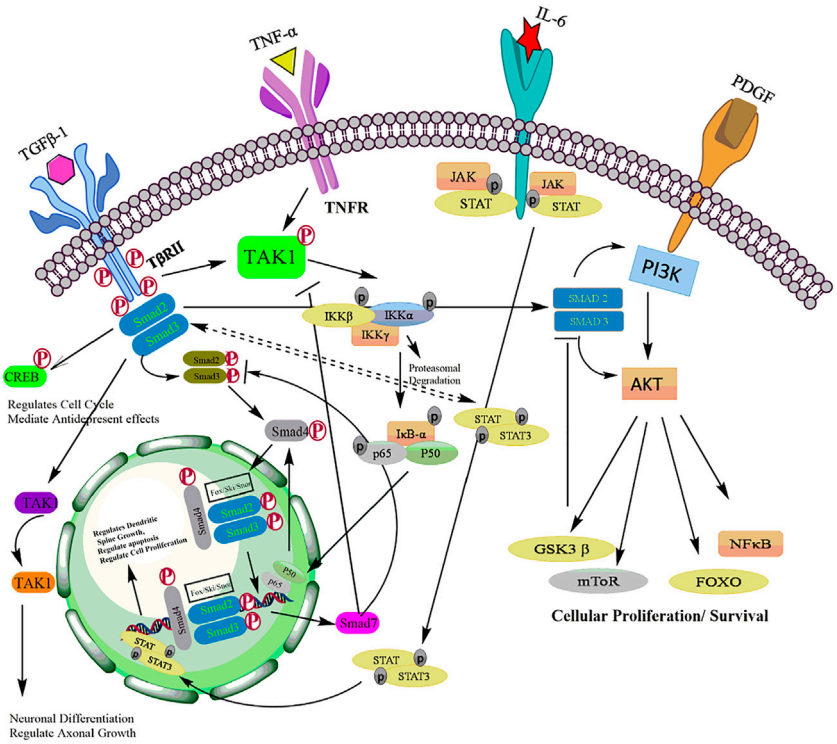

FIGURE 7 | Crosstalk of TGF-β with other major signaling pathways including PI3K/Akt, NF-kB, and JAK/STAT signaling pathways.

TGF-β resulting in inactivation of STAT3 and STAT4 (Salas et al., 2020). In contrary, TGF-β potentiates IL-6-induced STAT3 activation in hepatocytes and hematopoietic stem cells (HSC) (Rao et al., 2017). Moreover, it is also reported that TGF-β and Smad3 activation led to the elevated STAT3 phosphorylation in fibrosis and cirrhosis patient samples (Tang et al., 2017). The complex interaction between canonical Smads and STATS are highly involved in pluropotency and differentiation processes (Bertero et al., 2018). The Smad-mediated promoter activity requires Smad3/4 complex formation followed by nuclear translocation and activation of TGF-β responsive genes (Sakai et al., 2019). Conversely, STAT3 is known to interact with Smad3 to block Smad3/4 complex formation which attenuates the activity of TGF-β in inducing cell-cycle arrest and promoting EMT (Hao et al., 2019). JAK/STAT pathway indirectly regulates the activity of Smad3 by enhancing the expression of Smad7 (Syed, 2016). In human fibrosarcoma-derived cell line, INF-gamma induces expression of Smad7 mediated by phosphorylation and activation of the transcription factor STAT1 through JAK1 thereby preventing the interaction of Smad3 with TGF-β receptor (Majoros et al., 2017). Signal-transduction pathways induced by JAK/STAT and TGF-β signaling may be affected by transmodulating interactions between Smads and STATs (Chauhan et al., 2021). In conclusion, apart from regulating T-lymphocyte activation, TGF-β cross connects with JAK/STAT signaling to regulate plethora of pathophysiological process via Smads which includes activation of hematopoiesis, TGF-β fibrogenic responses in hepatic stellate cells, transcription of genes regulating EMT and regulating pluropotency and differentiations of cells (**Figure 7**).

CONCLUSION

Recent advances in the molecular biology led to deep understanding in the areas of signaling networks and their role in cancer. Signaling cross-talk between different pathways orchestrates various cellular functions in an accurate, effective, and balanced manner. However, aberrant activation of these cellular signals and their targets could lead to catastrophic events. Although, the dual role of TGF-β signaling has been extensively studied in various biological processes including cancer, it may still appear to be complex. TGF-β signaling cross-talk is context dependent, and can be direct or indirect or a part of feed-back mechanism. The key players of TGF-β signaling and their interaction with other cellular networks play a decisive role in embryonic development, stem-cell renewal, differentiation and specify cell fate within the physiological context. With the identification of new interconnections and their targets, the TGF-β pathway has emerged as networking hub of cell signaling. Several studies with different approaches have provided clues about the versatility of TGF-β and its interactions with other signaling pathways. Some studies have shown the contradictory results to the established role the TGF-β signaling. This discrepancy could be due to the disparities in

experimental conditions such as cell type, physiological/pathological status, developmental stage, localization of proteins, nature of modifying enzymes, co-factors etc. A future challenge for the researchers is to undergo in-depth mechanistic studies to identify the specific convergence point of these cellular pathways and to accurately predict biological outcomes. Recently, the role of TGF-β and associated signaling cascade has also been implicated in the regulation of microRNA, yet another unexplored area of TGF-β research. In addition, a number of studies have suggested the interconnection of TGF-β activity with energy metabolism (glucose uptake/consumption, AMPK and mTOR signaling) and NO (nitric oxide) signaling. The exciting progress in genome-wide mapping technologies and combinatorial approaches of therapies targeting the relevant signaling pathways along with the current techniques in genetics, molecular biology, and bioinformatics may reveal a detailed signaling network cascade and can also assist in elucidating the mechanism of the dual role of TGF-β, its functions and regulation under varying physiological contexts.

AUTHOR CONTRIBUTIONS

AB, BR, and GB contributed to manuscript writing, conceptualized and making figures; IM, SB, and RH read manuscript critically; DA contributed in reading, edited, and approved the submitted version.

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REVIEW
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JAK/STAT Signaling: Molecular Targets, Therapeutic Opportunities, and Limitations of Targeted Inhibitions in Solid Malignancies

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JAK/STAT signaling pathway is one of the important regulatory signaling cascades for the myriad of cellular processes initiated by various types of ligands such as growth factors, hormones, and cytokines. The physiological processes regulated by JAK/STAT signaling are immune regulation, cell proliferation, cell survival, apoptosis and hematopoiesis of myeloid and non-myeloid cells. Dysregulation of JAK/STAT signaling is reported in various immunological disorders, hematological and other solid malignancies through various oncogenic activation mutations in receptors, downstream mediators, and associated transcriptional factors such as STATs. STATs typically have a dual role when explored in the context of cancer. While several members of the STAT family are involved in malignancies, however, a few members which include STAT3 and STAT5 are linked to tumor initiation and progression. Other STAT members such as STAT1 and STAT2 are pivotal for antitumor defense and maintenance of an effective and long-term immune response through evolutionarily conserved programs. The effects of JAK/STAT signaling and the persistent activation of STATs in tumor cell survival; proliferation and invasion have made the JAK/STAT pathway an ideal target for drug development and cancer therapy. Therefore, understanding the intricate JAK/STAT signaling in the pathogenesis of solid malignancies needs extensive research. A better understanding of the functionally redundant roles of JAKs and STATs may provide a rationale for improving existing cancer therapies which have deleterious effects on normal cells and to identifying novel targets for therapeutic intervention in solid malignancies.

Keywords: solid tumors, signaling, molecular targets, therapeutic opportunities, inhibitors

INTRODUCTION

An intracellular signaling pathway is critical in regulating the cellular fate and modulates phenotypic modifications. JAK/STAT signaling is one such pathway that regulates embryonic development, stem cell maintenance, hematopoiesis and, inflammatory response (Bowman et al., 2018). JAK/STAT is a signal transduction pathway, which transmits the extracellular information or signals through a transmembrane protein, called Janus kinase (JAK). The JAK further directs the signal to an intracellular environment by phosphorylating the transcription factor known as STATs, which translocates into the nucleus to target the

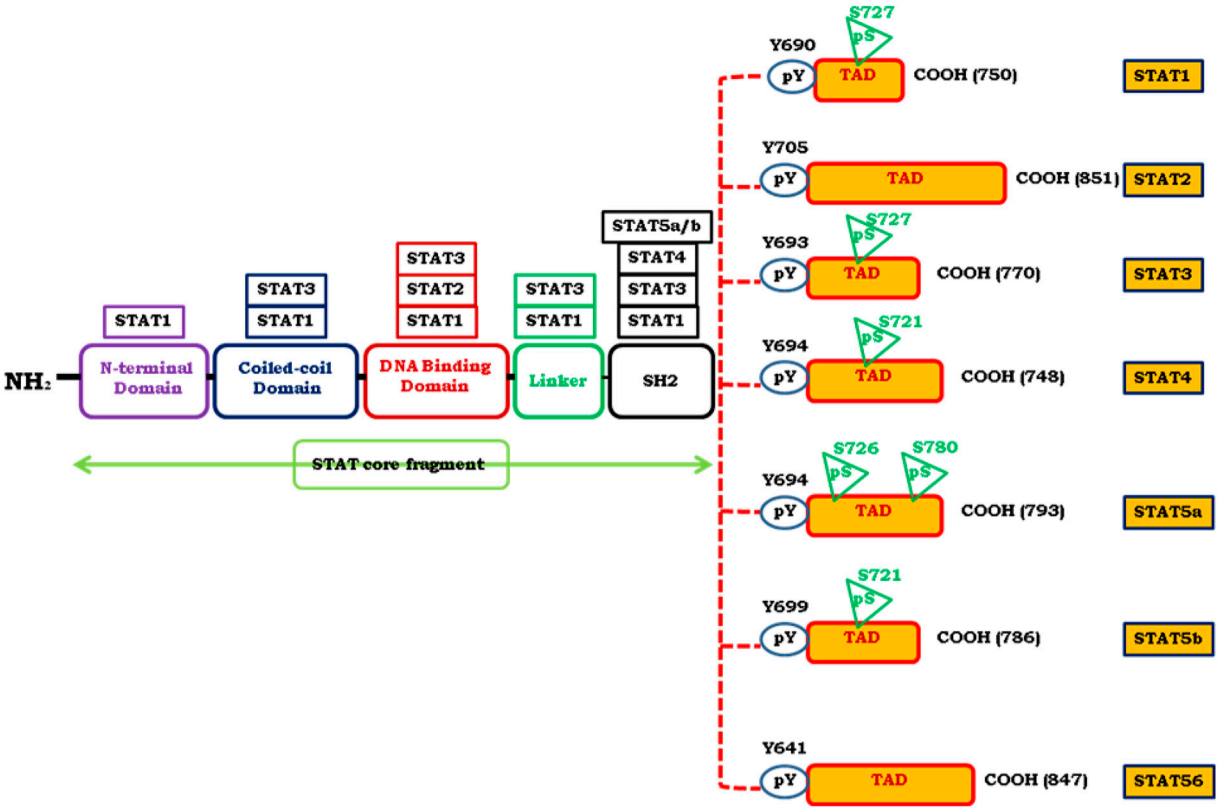

FIGURE 1 | Represents the structural and functional composition of various conserved regions of STAT proteins. STAT proteins composed of N-terminal domain, coiled-coil domain, DNA binding domain, a linker domain, Src-homology 2 (SH2) domain and a C-terminal transcriptional activation domain (TAD). TAD possesses tyrosine phosphorylation (pY) and serine phosphorylation (pS) sites which are essential for STAT activation. Location of pS and pY are depicted in figure. The length of amino acid chain defined by the carboxylic acid (COOH) group is indicated on the right side of each STAT member.

promoter region of a gene to regulate the mechanism of transcription (Fiebelkow et al., 2021). The evolutionarily conserved pathway in all eukaryotes, JAK/STAT is a principal signal transduction pathway in mammals for cytokines and growth factors (O'Shea et al., 2015). Structurally there are four members of the JAK family i.e., JAK1, JAK2, JAK3, Tyk2, and seven members of STAT, i.e., STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 in mammals (Abroun et al., 2015; Alunno et al., 2019) (**Figure 1**). As the ligands bind to the cognate receptor, the two JAK's come closer and allow *trans*-phosphorylation of both the receptors as well as STATs at their conserved tyrosine residue present near to the C-terminal region (Morris et al., 2018). The phosphorylation of tyrosine at the C-terminal region is responsible for the dimerization of STATs which further enhances the interaction of a conserved domain called as SH2 domain (Morris et al., 2018). STAT is a transcription factor that resides in the cytoplasm. The phosphorylated STATs then enter the nucleus from the cytoplasm through a mediator called importin α-5 and Ran nuclear import (Seif et al., 2017). These dimerized STATs then bind to the particular regulatory sequence for the activation or repression of the targeted genes (Seif et al., 2017) (**Figure 2**). Thus, JAK/STAT is a signal transduction pathway that converts the extracellular signals into the transcriptional message thereby regulating physiological processes. Aberrant activation of intracellular signaling pathways confers malignant phenotype to genetically and metabolically altered cells (Flavahan et al., 2017). Many of these alterations occur in signaling pathways that control cell growth and division, cell death, cell fate, cell motility, tumor microenvironment, angiogenesis, and inflammation (Jing et al., 2019). Besides regulating physiological processes, JAK/STAT signaling cascade is implicated in various pathophysiological disorders including malignancies by altering the JAK/STAT signaling. In solid malignancies, these alterations characteristically support progression from a relatively benign group of proliferating cells (hyperplasia) to a mass of cells with abnormal morphology (dysplasia), cytological appearance, cellular organization, genomic integrity, and metabolic state (Gale et al., 2016). As a result of the structural and functional complexity of solid tumors, cancer therapies exhibit variable responses in distinct patients and cancer types (Cabrera et al., 2015). Although, the number of signaling pathways are deregulated in malignancies, however; besides being invariably activated in hematological malignancies, JAK/STAT signaling is, altered in many solid tumors and showed deregulated activation (Joshi et al., 2015).

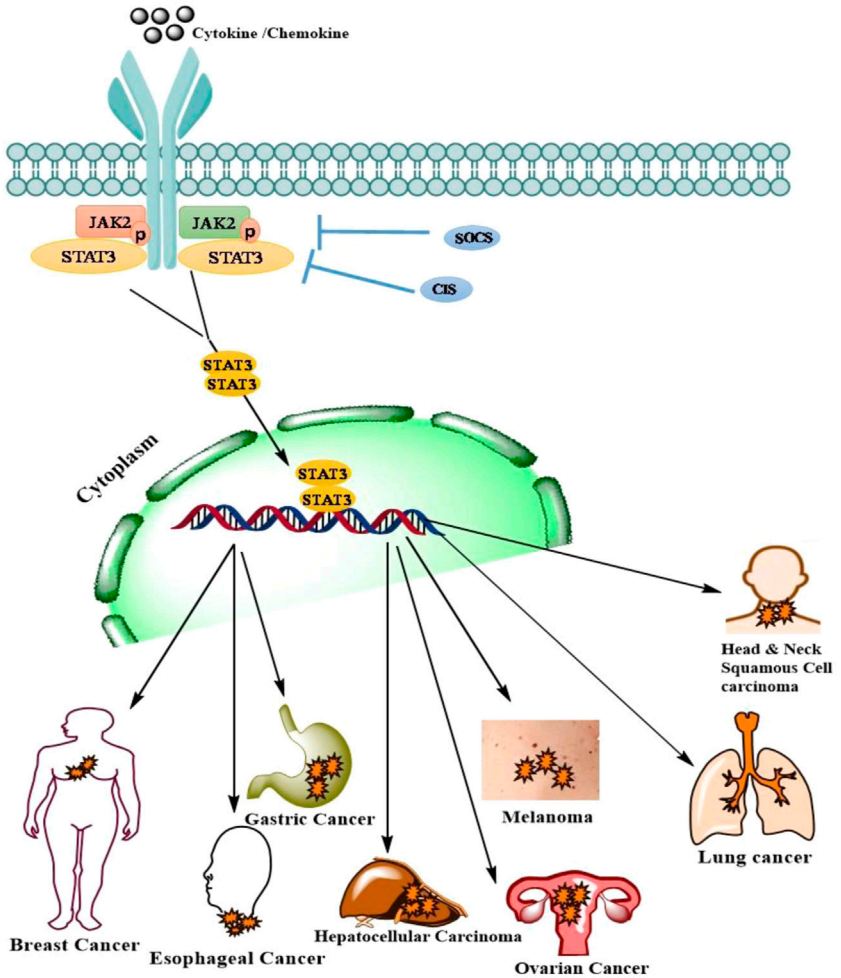

FIGURE 2 | Represents the signaling mechanism of JAK/STAT and its contribution in the pathogenesis of various solid malignancies. JAK/STAT signaling in Melanoma.

STATs are the effective downstream mediators of the JAK/STAT signaling cascade. STATs regulate the expression of a wide variety of genes both positively and negatively, identification of many of them is still imprecisely determined and warrants further investigation (Stark et al., 2018). The combination of STATs and the tissue in which they function at a given time often determines the subset of genes they control, which further contributes to the challenge of target gene identification. Constitutive phosphorylation of STAT1, STAT3, and STAT5 has been detected in many tumor cell models (Bellucci et al., 2015). The microarray-based expression analysis is used to comprehensively identify STAT target genes. Although STATs contributes to a malignant phenotype by regulating genes involved in cellular proliferation, survival, differentiation, angiogenesis, and invasion (Stark et al., 2018). However, how STATs execute these biological functions is critical for understanding the pathophysiology of solid tumors, signifying the need to understand the more precise role of JAK/STAT pathway in the pathobiology of solid malignancies. Despite this, some differences in STAT targets among cell types may be due to epigenetic variation among cells, which includes altered histone modifications or DNA methylation (Biswas and Rao, 2017; Garg et al., 2018). Thus, there is a need to identify the new target genes of STATs to fully understand how JAK/STATs can be used for the therapeutic intervention of solid tumors. Indeed, solid tumors contain aberrantly activated transcription factors—either through mutation of the transcription factor itself or through mutation of upstream signaling cascades leading to its activation (Qureshy et al., 2020; Gilmore, 2021). Therefore, understanding the genes regulated by STATs may provide insights into the pathophysiology of solid tumors in which they are inappropriately activated and may unravel novel targets for therapeutic intervention. Targeting intracellular signaling pathways has been a productive strategy for drug development, with several drugs acting on JAK-STAT signaling already in use and many more are being

developed. In this review, we provide a comprehensive review of the role of the JAK/STAT pathway in solid tumors, clinical evidence of targeted agents, and also discuss the therapeutic intervention of JAK/STAT in solid tumors. The knowledge that we gathered in this review can be used in the strategic design of future researches and in the development novel targets and therapeutic modalities.

JAK/STAT Signaling in Melanoma

Several intracellular and extracellular signals are transduced by the JAK/STAT pathway to regulate growth, proliferation, differentiation, development, and homeostasis (Dodington et al., 2018). Under normal physiological conditions, JAK/STAT pathway is activated to induce the secretion of a wide variety of growth factors, cytokines, interleukins, and hormones (Kraemer et al., 2020). Thus, the activation of JAK/STAT signaling may lead to increased proliferation, differentiation, migration, and cell death (Gu et al., 2016). However, aberrant activation of this pathway in skin cells leads to unregulated cell proliferation and cancer development (Khan A. Q. et al., 2019; de Araújo et al., 2019). Consequently, this signaling cascade plays an important role in the development of melanoma, a highly lethal and therapeutically resistant form of skin cancer (Lazăr et al., 2020). At the molecular level, this pathway is relatively uncomplicated. A wide range of ligands (cytokines, growth factors, hormones) and their cognate receptors participate in the activation of this pathway (Hubbard, 2018). In human skin cells and other mammalian cells, the JAK family typically consists of 4 membrane-associated Janus family kinases (JAKs), JAK1, JAK2, JAK3, and TYK2 which together constitute an important group of non-receptor tyrosine kinases. JAK-STAT pathway is activated upon ligand-mediated receptor multimerization which sets two adjacent JAKs into close proximity and facilitates *trans*-phosphorylation. Upon activation, JAKs phosphorylate signal transducer and activator of transcription proteins (STATs) and several other substrates, including both the receptors and the major non-receptor substrates (Xin et al., 2020). The STAT family comprises of 7 latent transcription factors (i.e., STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) that typically shuttle between cytoplasm and nucleus in response to extracellular stimuli or through deregulated activation of other aberrant signaling events (Guanizo et al., 2018). This shuttling mechanism of STATs is strongly influenced by phosphorylation. Once phosphorylated, phosphotyrosine residues allow STATs to dimerized through their interaction with conserved SH2 domains (Szelag et al., 2015). A negative regulation of the JAK/STAT pathway, which shuts this pathway off, is ensured by an array of kinases, phosphatases (PTPs), cytokine receptors, suppressors of cytokine signaling (SOCS) proteins, and protein inhibitors of activated STATs (PIAS) (Bang, 2019). In melanoma cells, STAT proteins are frequently constitutively phosphorylated or constitutively activated in response to intracellular or extracellular stimuli or through unregulated activation of other signaling molecules (Rovida and Stecca, 2015). Phosphorylated STATs enter into the nucleus in the form of dimers and bind to specific regulatory sequences, thereby activating or inhibiting the transcription of specific target genes (Wingelhofer et al., 2018). For example, STAT1-mediated gene expression is linked with growth retardation, apoptotic resistance, and reduced angiogenesis (Mohassab et al., 2020). Conversely, the gene expression induced by STAT3 has been found to be associated with cell proliferation, apoptotic resistance, metastasis, angiogenesis, and suppression of immune response (Chai et al., 2016). Thus, melanoma cells display distinct gene expression profiles depending on the level of activation of individual STAT proteins (Lopez-Bergami et al., 2008). A recently conducted study has shown that the ratio of phosphorylated STAT1 to STAT3 may be a biomarker that can predict the progression of melanoma (Verhoeven et al., 2020). Interestingly, STAT proteins have been found to be altered in both melanoma cells and in the immune system of melanoma patients (Tucci et al., 2019). Thus the JAK/STAT pathway imparts a mechanism that is used by melanoma cells to transduce extracellular stimuli into specific transcriptional responses.

Several strategies have been used to inhibit the STAT3 pathway as a therapeutic approach for treating malignant melanoma (Lee et al., 2019). Direct STAT3 inhibitors can be categorized into different classes of compounds: peptides, peptidomimetics, peptide aptamers, small molecules, platinum complexes, siRNAs, and plant polyphenols (Lee et al., 2019; Thilakasiri et al., 2021). Each of these STAT3 inhibitors has merits and demerits. For example, while peptide-based inhibitors exhibit a high degree of specificity, they possess limited cell permeability and *in vivo* stability (Aftabizadeh et al., 2021). More recently, cell-permeable, STAT3 SH2 domain mimetics with promising anti-tumor effects have been designed (Lai et al., 2015; Du and Lovly, 2018; Franke et al., 2018). Similarly, siRNA specific for the SH2 domain of STAT3 have been designed that have shown promising antitumor effects in prostate cancer cells and patient-derived murine xenograft tumor models (Son et al., 2017). Certain platinum complexes have shown remarkable antitumor activity by their ability to inhibit STAT3 in a variety of tumor models (Lazarević and Rilak, 2017). Likewise, natural products (e.g., resveratrol, flavopiridol, indirubin, magnolol, piceatannol, parthenolide, EGCG, cucurbitacin Q, and curcumin) have been demonstrated to downregulate the activity of STAT3 in cancer cells (Arshad et al., 2020). Besides, other studies also provide a rationale for inhibiting STAT3 as a therapeutic target in cancer cells. However, the specificity and selectivity of STAT3 inhibitors and inhibitors of other STAT proteins should be properly validated in various biological systems before final recommendations on the clinical use of these inhibitors are put forward (Huang et al., 2020). Arguably, certain small molecule inhibitors of STAT3 (e.g., Static, STA-21, S32-M2001, S3I-201) have shown remarkable antitumor activity by their ability to induce apoptosis [52]. A novel small molecule S3I-201.1066, a structural derivative of S3I-201, has recently been shown to disrupt phosphotyrosine interactions at the STAT3 SH2 domain with a high degree of efficacy (Beebe et al., 2018). Likewise, LLL-3, an early generation STAT3 inhibitor, has shown similar antitumor activity in different tumor models. FLLL32, a structural analog of curcumin, specifically inhibits STAT3 while retaining STAT1

TABLE 1 | STAT3 inhibitors in melanoma.

Small molecule inhibitor(s)	Mechanism of action
Stattic, STA-21, S32-M2001, S3I-201	Antitumor activity by their ability to induce apoptosis *via* STAT3 inhibition
LLL-3, an early generation STAT3 inhibitor	Antitumor activity in different tumor models
FLLL32, a structural analog of curcumin	Inhibits STAT3 while retaining STAT1 mediated signal transduction within melanoma and immune sensitive cells, FLLL32 triggers caspase-dependent apoptosis via its inhibition of STAT3
Resveratrol, flavopiridol, indirubin, magnolol, picetannol, parthenolide, EGCG, curcubitacin Q and curcumin	Downregulate the activity of STAT3 in cancer cells
Peptide aptamers	Interacts with a dimerized form of STAT3 thereby causes inhibition of antiapoptotic proteins and promotes apoptosis in melanoma cells
S31–1757 and S31-201 micelle formulation	Inhibits STAT3 dimerization in both cellular and animal models of melanoma with less undesirable effects on normal cells
Novel platinum (IV) compounds (CPA-1, CPA-7)	Disrupts STAT3 signaling, thereby abrogates tumor-promoting activity of STAT3
C48	Promotes alkylation of STAT3 at cys468 thereby attenuating the accumulation of activated STAT3 in the nucleus to inhibit the growth of melanoma cells

mediated signal transduction within melanoma and immune-sensitive cells (Paulraj et al., 2019). Treatment of different melanoma cells with FLLL32 triggers caspase-dependent apoptosis via inhibition of STAT3 (Paulraj et al., 2019) (**Table 1**).

JAK/STAT Signaling Gastric Cancer

Gastric cancer (GC) is a heterogeneous malignancy and is one of the leading cancer-related mortalities across the world (Jan et al., 2021; Rah et al., 2021). The major risk factors contributing to gastric tumorigenesis are environmental factors, geographical location, lifestyle factors, age, and chronic infection with *Helicobacter pylori* (Chia and Tan, 2016; Jan et al., 2021; Karimkhani et al., 2017). Besides environmental and other risk factors, recently conducted whole genome sequencing and genome-wide association studies have led to the identification of novel genomic alterations/mutations which profoundly contribute to the pathogenesis of GC (Pectasides et al., 2018). Recent shreds of evidence suggest that 474 hotspot mutations in 41 genes were identified in gastric adenocarcinoma patients, among them *PIK3CA* harbored mutation ranks at the top followed by mutations in *TP53, APC, STK11, CTNNB1,* and *CDKN2A* genes. These genomic mutations lead to aberrant activation of several signaling molecules, such as vascular endothelial growth factor receptor (VEGFR) family, epidermal growth factor receptor family (ErbB) members, and various components of JAK/STAT and PI3K/Akt/mTOR pathway thereby contributes in the molecular pathogenesis of GC (Khanna et al., 2015; Yeh et al., 2016; Zhu et al., 2019).

Although, several drugs have been developed for the treatment of GC, the overall prognosis for GC patients is still dismal (Ajani et al., 2017; Matsuoka and Yashiro, 2018). Currently available standard treatments for GC are surgery, chemotherapy, and radiation therapy (Sitarz et al., 2018). The most commonly used therapeutic regimen is ECX (epirubicin, cisplatin, capecitabine), ECF (epirubicin, cisplatin, 5-FU), EOX (epirubicin, oxaliplatin, capecitabine), and EOF (epirubicin, oxaliplatin, 5-FU) (Charalampakis et al., 2018). The second line regimen for GC therapeutics is irinotecan, docetaxel, and paclitaxel. Since GC is a heterogeneous disease, advances in personalized medicine should be improved with each patient being treated individually based on various genetic and epigenetic alterations regulating different signaling pathways with targeted drugs (Bonelli et al., 2019). Targeted therapy identifies and kills cancer cells specifically while leaving healthy cells unharmed. Monoclonal antibodies (such as HER2, VEGFR, or EGFR), multikinase inhibitors, and immune checkpoint inhibitor therapy (such as CTLA-4 or PD-1/PD-L1 are mainly involved in targeted therapy that has shown to be beneficial in the treating metastatic and advanced stages of GC patients (Seebacher et al., 2019). RNA interference and use of antisense oligonucleotides (ASOs) involving the silencing of target proteins has been explored as one of the STAT-inhibitory approaches which are targeting the JAK/STAT signaling pathways (Szelag et al., 2015). Recently, the use of short palindromic repeats (CRISPR) and its related proteins (Cas-9) have been explored as genome editing tools to mute the JAK/STAT pathway components (Bousoik and Montazeri Aliabadi, 2018).

Primarily known for immune regulation and plays a critical role in hematological malignancies harboring V617F driver mutation, JAK/STAT signaling cascade contributes strongly to the pathogenesis of GC. Besides, frequent amplification of the chromosomal region harboring JAK2 has been identified in GC. The most studied member of JAK/STAT is in GC is STAT3. Phosphorylation at tyrosine-705 residue of STAT3 leads to the aberrant activation of STAT3 resulting in increased growth, angiogenesis, invasion, and metastasis (Giraud et al., 2012; Loh et al., 2019). Hyperactivation of STAT3 was found in a number of GC cell lines and its elevated expression in histological sections of GC patients was strongly associated with angiogenic factors such as VEGF and microvessel density formation thereby contributing to GC progression (Qi et al., 2020). Furthermore, STAT3 expression was analyzed in gastric specimens with respect to a member of the regenerating gene family (REG Iα) to study its role in inflammation-associated with GC. The study further analyzed that REG Iα expression was strongly correlated with the phospho-STAT3 expression in GC specimens, suggesting that REG Iα plays a crucial role in inflammation associated gastric tumorigenesis by promoting antiapoptosis mechanism in gastric mucosal cells. Additionally, cytokine-associated (IL-6/IL-11) dependent elevated expression of STAT3 contributes to the development

TABLE 2 | JAK/STAT signaling inhibitors, target molecules, effects, and clinical indications in gastric carcinoma.

Inhibitor	Target	Effect	Indication (clinical trial phase)
Non-peptide small molecules			
Pravastatin	STAT1	downregulates IFN-γ levels	gastroesophageal cancer (phase IV)
Non-peptide small molecules			
pravastatin	STAT1	Phosphorylation inhibition of STAT1, decrease in IFNγ levels	gastroesophageal cancer (phase IV)
Natural products			
Vinorelbine	STAT3	Inhibition of STAT3 phosphorylation targets STAT3–tubulin interaction	gastric cancer (phase II)
Paclitaxel	STAT3	Prevents STAT3 phosphorylation, dissociation of STAT3 and, tubulin binding	Stomach cancer(phase I/II/III)
Tyrosine kinase inhibitors			
Sorafenib	JAK2, STAT3	Inhibits phosphorylation of STAT3	gastric cancer (phase I/II)
AZD148	JAK1, JAK2	Inhibition of STAT1, STAT3, STAT5, and STAT6 phosphorylation	gastric cancer (phase I)

of H. pylori-associated GC in gp130^{757FF} GC established mouse models. STAT3 is known to control the transcriptional epithelial-to-mesenchymal (EMT) regulators through G1 and G2/M cell-cycle progression, leading to the progression of metastasis in gastric malignancies (Azarnezhad and Mehdipour, 2017; Eissmann et al., 2019). STAT3 promotes angiogenesis in GC by stimulating the production of hypoxia-inducible factor (HIF)-1 and VEGF (He et al., 2019). Immunohistochemistry revealed that STAT3 expression was strongly associated with lymph node metastasis, TNM staging, and survival, suggesting that STAT3 could function as a predictive biomarker for poor prognosis in GC (Zhang et al., 2017). Furthermore, aberrant STAT3 signaling promotes tumorigenesis by deregulating the expression of genes including p21$^{WAF1/CIP2}$, MYC, BCL-2, cyclin D1, BCL-$_{XL}$, matrix metalloproteinase 1 (MMP1), MMP7, MMP9, and survivin which regulates cell proliferation and survival (Srivastava and DiGiovanni, 2016).

Besides, the JAK/STAT pathway which serves as a hub for a variety of signaling networks, exhibits a tremendous potential of crosstalk with other signaling pathways (Mullen and Gonzalez-Perez, 2016), thus acts as an appealing target cascade not only for hematological malignancies but also for other solid malignancy including gastric tumorigenesis. The currently used clinical trials involving targeted drugs mainly emphasize JAK family members as compared to those targeting STATs. Substantial effort has been made to develop STAT inhibitors, however, limited success has been achieved due to issues with bioavailability, *in vivo* efficiency, and selectivity (Shouse and Nikolaenko, 2019). Out of all STATs, STAT 3 has proven to be one of the promising targets for molecularly targeted treatment (27). The JAK/STAT signaling is activated in a substantial proportion of solid tumors and promotes to cancer cells aggressive characteristics, making it a promising target for novel therapies (Sabaawy et al., 2021). Several *in vitro* and *in vivo* studies have validated the potent part played by STAT3 in precancerous physiology of the stomach, implying that STAT3 could be used as a predictive marker for diagnosis of GC, thus inhibiting STAT3 activity with several inhibitory molecules might help to prevent cancer (Khanna et al., 2015) (**Table 2**). Taken together, JAK/STAT-associated signaling pathways play a multifaceted role in GC carcinogenesis and are ideal for the combinatorial targeted therapies which could be explored as a potential for the identification of novel biomarkers for GC treatment.

JAK/STAT Signaling in Esophageal Cancer

Esophageal cancer (EC) is among the top 10 ranked malignancies in terms of both incidence and mortality. Histopathologically two major types of EC are esophageal adenocarcinoma (EAC) and squamous cell carcinoma (ESCC). ESCC, on the other hand, accounts for almost 90% of all EC cases worldwide (Uhlenhopp et al., 2020). Regardless of recent breakthroughs in treatment tactics, the 5-year life expectancy runs from 30 to 50%, with over 70% of cases occurring in the male population (Sung et al., 2021). ESCC is highly aggressive and diagnosed at an advanced stage associated with distant lymph node metastasis. The majority of the patients with ESCC present with poor prognosis and high recurrence rates after surgery (Ohashi et al., 2015). Conventional treatment regimens including surgical intervention and radiotherapy have proven to be less effective in ESCC because of their high-grade invasive nature (Barzilai et al., 2019). Despite profound advancement in chemotherapeutic drugs, the overall survival time for the EC remains dismal and the management of disease outcome and eventually the quality of life is poor. Therefore targeted therapy is seemingly important for the treatment of EC. Signaling pathways have proven to be promising targets for targeted cancer therapies. Despite the signaling pathways being dysregulated at many levels, however, the critical junctures of signaling pathways that are frequently disrupted are receptors, transducer proteins, or transcriptional factors. Although a plethora of molecular signaling pathways plays a critical role in the EC pathogenesis, however, in the current review we mainly focus on JAK/STAT signaling in EC and its molecular targets for future therapeutics. Numerous studies have documented that the JAK-STAT pathway regulates molecular processes involved in EC progression including proliferation, survival, differentiation, and metastasis (Yang et al., 2020).

Aberrant activation of STAT3 contributes to esophageal cellular transformation, angiogenesis, epithelial to mesenchymal transition, and metastasis to distant sites. The

TABLE 3 | JAK/STAT signaling modulators, their targets, and mechanism in esophageal carcinoma/cells.

Compounds	Targets	Mechanism
Phlorizin	JAK2 STAT3	Inhibits malignant properties of esophageal carcinoma cells by dephosphorylation of JAK2 and STAT3 proteins
Stattic	STAT3	Inhibits dimerization of STAT3 by blocking tyrosine phosphorylation of SH2 domain of STAT3
Nimesulide	JAK STAT	Reduces the JAK/STAT expression signaling by blocking cyclooxygenase-2 enzyme expression
Ellagic acid	STAT3	Inhibits both cytokine driven STAT3 and endogenous STAT3 signaling and promotes apoptosis to ESCC cells
NVP-BSK805	JAK2	Inhibits kinase activity of JAK2 by dephosphorylation
Cryptotanshinone	JAK2 STAT3	Significant reduction of p-STAT3 (Tyr705) and *p*-JAK2 expression in ESCC cells

association of the JAK/STAT pathway and prognosis of patients with ESCC was first confirmed by immunohistochemistry in 100 ESCC tumors and adjacent normal esophageal epithelia (Sugase et al., 2017). Studies suggested a significant correlation between phosphor-JAK1 and phosphor-STAT3 expression and prognosis of the disease in which an increased expression of *p*-JAK1 and p-STAT3 in primary ESCC tumors predicted the poor prognosis of ESCC patients (Zhao et al., 2021). Moreover, the expression levels of both proteins were found to be statistically significant with lymph node metastasis and TNM staging (Cheng et al., 2019). Therefore, STAT3 is a promising target for designing specific inhibitors for future therapeutics against EC.

The JAK/STAT pathway plays a critical role in the process of inflammation and EC progression (Pencik et al., 2016). In *in vitro* settings, AG490, a JAK2 inhibitor, was reported to decrease the inflammation and development of ESCC by preventing the constitutive stimulation of STAT3 (Liu et al., 2018). Cell cycle experiments further confirmed the STAT3 mediated regulation of cell cycle and proliferation in ESCC progression (Lu et al., 2019). Furthermore, STAT3 phosphorylation and COX-2 an important mediator of inflammation were highly expressed and consistently co-related with the ESCC cell lines (Cho et al., 2017). AG490 decreased the STAT3 activation and COX-2 protein levels significantly. STAT3 was also found to be involved in IL-6 mediated inflammation in ESCC cell lines (Liu et al., 2018).

Recently, a diterpene Cryptotanshinone (CTS), purified root extract from medicinal herb *Salvia miltiorrhiza Bunge* (DanShen) was employed *in vitro* and *in vivo* as a potent compound for the treatment of ESCC (Ji et al., 2019) (**Table 3**). In a dose-dependent experiment carried out in CAES17 and EC109 esophageal cancer cells, CTS substantially decreased the phosphorylation of STAT3 (Tyr705) and JAK2 expression level cells in response to IL-6 stimulation (Ji et al., 2019). Phlorizin, a member of the dihydrochalcone family derived from sweet tea is commonly used as a traditional medicine in China. Owing to having promising pharmacological importance, phlorizin inhibits esophageal malignant cell properties by activating apoptosis, autophagy and attenuating JAK2/STAT3 signaling (Jia et al., 2021). Together, these studies suggest that although STAT3 is a well-known mediator for tumor progression in numerous malignancies, however, it could be used to design specific pharmacological inhibitors derived from different sources for future therapeutics against EC.

JAK/STAT Signaling in Hepatocellular Carcinoma

The most common type of primary heterogeneous malignancy affecting the liver is hepatocellular carcinoma (HCC) (Khemlina et al., 2017). HCC has gained the fifth rank in the list of most common cancers worldwide, and is responsible for the vast majority of cancer-related deaths (Bray et al., 2018). The most important factor that contributes to the development of HCC and HCC-related deaths is cirrhosis (Shiani et al., 2017). Nearly 80% of the population is developing HCC from chronic liver inflammation (Ghouri et al., 2017; Keenan et al., 2019). Besides, the high risk of hepatitis B virus (HBV) and exposure to aflatoxin-B1 (Kucukcakan and Hayrulai-Musliu, 2015; Irshad et al., 2017; Ivanov et al., 2017; Isaac and Burke, 2019) the other predominant molecular mechanisms responsible for the pathogenesis of HCC includes liver tissue damage which leads to cirrhosis and mutations in tumor suppressor and oncogenes (Alqahtani et al., 2019). These two mechanisms are associated with a dysregulation in the cell signaling pathway and lead to HCC (Gao JZ. et al., 2015). Although, various pathways such as wnt/β-catenin signaling pathway, PI3K/AKT/mTOR signaling pathway, receptor tyrosine kinase pathway, TGF-β signaling plays a critical role in the pathogenesis of HCC. However, JAK/STAT signaling equally contributes to the pathogenesis of HCC (Huang and Fu, 2015; Erkisa et al., 2021). In many human cancers including HCC, STAT3 is considered as a strong bona fide candidate promoting tumorigenesis (Khan AQ. et al., 2019). STAT3 activation as a transcriptional factor promotes a plethora of genes that contributes too many cancer hallmarks, thereby highlighting the tumorigenic role of STAT3 in HCC. STAT3 has a crucial role in G1 to S phase cell cycle progression by upregulating CCND1 expression. Additionally, STAT3 downregulates the expression of proapoptotic proteins TP53, Bax, Chop and upregulates the expression of antiapoptotic proteins Bcl-2, Bcl2-xl, Birc5, Mcl1, respectively in HCC cells. Many studies have reported the aberrant activation of JAK1 and STAT3 in the development of HCC which are the key members of the JAK/STAT signaling pathway and promote tumorigenesis (Hin Tang et al., 2020). It has also been shown that the JAK1 aberrantly phosphorylated STAT3 which results in cell proliferation, migration, invasion, and angiogenesis in HCC (Hin Tang et al., 2020). Aberrant activation of STAT3 in HCC is due to many growth factors and cytokines like TNF, IL-6, hepatocytes

TABLE 4 | JAK/STAT signaling modulators, their targets, and mechanism in esophageal carcinoma/cells.

Compounds/small molecule inhibitors	Targets	Clinical status	Mechanism
WP1066	JAK2	Phase I	Inhibits JAK2 phosphorylation thereby attenuating cell migration and invasion by inhibiting MMPs
Pacritinib	JAK2	Pre-clinical	Pre-clinical studies showed that fibrotic areas of the mouse liver were effectively reduced by reducing CK18 biomarker
CTS	JAK2 STAT3	Pre-clinical	In mouse models, CTS promotes apoptosis of tumor cells and helps to activate tumor-suppressive M1 cells
Ruxolitinib	JAK1/2	Pre-clinical	Inhibits cell proliferation and colony-forming ability of HCC cells
Stattic	STAT3	Pre-clinical	Stattic inhibits tumor cell function in HCC such as cell proliferation and invasiveness
OPB-111077	STAT3	Phase I	Inhibits STAT3 in patients who are at an advanced stage of HCC and are not responding to sorafenib therapy
OPB-31121	STAT3	Phase I/II	Inhibits STAT3 in patients who are at an advanced stage of HCC and are not responding to sorafenib therapy
Napanucasin (BBI608)	STAT3	Phase Ib/II	Promotes antitumor activity in advanced HCC patients with prior systemic Sorafenib therapy
AZD9150	STAT3	Phase Ib/II	Promotes antitumor activity in advanced HCC patients with prior systemic Sorafenib therapy

growth factor (HGF), and epidermal growth factor (EGF) family (Zhao et al., 2015; Ray et al., 2018). Abnormal release of cytokines stimulates JAKs, which eventually phosphorylated STAT3 at a critical tyrosine residue Tyr-705 or Ser-727 in HCC (Pinjari and Meerza, 2017). The activity of JAK and STAT3 is enhanced due to the overexpression of pro-inflammatory cytokines like IL-6, IL-10, IL-11, and TGF-α to regulate the tumor micro-environment, which eventually create the oncogenic conditions to inhibit apoptosis (Waters and Brooks, 2015; Ferrao et al., 2016; Yuan et al., 2017; Wu et al., 2018). Reports suggest that tumor aggression is associated with the activation of STAT3. Constitutive activation of STAT3 in HCC is also known to upregulate various miRNA's including miR-21. Missense mutation of JAK1 has also been identified in HBV-infected HCC patients (Tezcan et al., 2019). With the increase in JAK1 mutation, there is an increase in phosphorylation of JAK1 and STAT3 without cytokine stimulation (Sims, 2020).

A number of pharmacological inhibitors can be used for the inhibition of the JAK/STAT signaling pathway in controlling HCC (Mansour et al., 2014; Poulou et al., 2015; Gao et al., 2015b; Duffy et al., 2017; Luo, 2017; Zhu et al., 2017; He et al., 2018; Hiraoka et al., 2019; Hin Tang et al., 2020; Pinter et al., 2021). SSI-1 (STAT induced STAT inhibitor-1) is the inhibitor for STAT3 activation. Akira et al. and Nakajima et al. reported that the mRNA expression of SSI-1 inhibitor was induced by interleukins like IL-4, IL-6, and G-CSF (Rautela and Huntington, 2017). Ruxolitinib is one of the specific JAK1/JAK2 inhibitors approved by the FDA against myelofibrosis. Although, ruxolitinib for HCC is still in the preclinical stage, however, recent studies suggest that ruxolitinib attenuates cell proliferation and colony-forming ability in HCC cells (Shimoda et al., 2020). Filgotinib is a selective inhibitor for JAK1 (Westhovens et al., 2017) in combination with ruxolitinib inhibits cytokines like IL-11, G-CSF with a mild increase in hemoglobin (Schwartz et al., 2017). Stattic, a small molecule inhibitor, abrogates STAT3 dimerization and translocation which eventually inhibits STAT3 activation in a phosphorylation-independent manner in many cancers. While Stattic augments apoptosis thereby reducing dose-dependent cell survival and invasiveness in HCC cell lines prior treated with radiations. Another small molecule inhibitor OPB-111077 when used singly against HCC had minimal toxicity issues; however, when given in combination with sorafenib, OPB-111077 is effective against HCC with an acceptable safety profile. Additionally, OPB-31121, another STAT3 inhibitor in clinical phase-I against advanced solid tumors had acceptable antitumor activity against HCC, however, due to its side effects on the peripheral nervous system; its usage should not be long-term. Napabucasin in combination with paclitaxel is in clinical trials against GC; however, Napabucasin was reported to inhibit tumor growth of HCC cells and xenograft. AZD9150, a siRNA-based drug used against B-cell lymphoma patients to target STAT3. In HCC the AZD9150 was tested and was found to have good efficacy and well tolerable safety profile. There are various compound/small-molecule inhibitors having an impact on JAK/STAT signaling in HCC are listed in **Table 4**. Collectively, these results suggest that JAK/STAT signaling is promising signaling to target in HCC. Although the clinical outcomes of these inhibitors is still in the infancy stage for HCC, however, the beneficial effects documented against other malignancies could underline the possible clinical efficacy against HCC.

JAK/STAT Signaling in Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) is mostly derived from mucosal epithelial of the buccal cavity (Sung et al., 2021). The consumption of alcohol or exposure to tobacco-derived carcinogens increases the burden of HNSCC with prior infection to the oncogenic strain of the human papillomavirus (HPV). While the presence of a proportion of pre-malignant lesions could advance towards invasive malignancy, however, majority of HNSCC patients are clinically diagnosed at late stages of malignancy. Therapeutic modalities for HNSCC are surgical resection followed by chemoradiotherapy (CRT). Despite the frequent mutations in key tumors suppressor genes such as NSD1, CDKN2A, TP53, NOTCH1, PIK3CA, TGFBR2, and FAT1, the dysregulation of associated signaling pathways also contribute to the development of pathophysiology of HNSCC. Aberrant activation of key signaling pathways which contributes in HNSCC are PI3K/Akt/mTOR pathway and STAT3 (Chen et al., 2021). The constitutive activation of the JAK/STAT pathway in response to several upstream signaling pathways, especially the TGF/EGFR

TABLE 5 | JAK/STAT signaling modulators, their targets, and mechanism in head and neck squamous cell carcinoma (HNSCC)/cells.

Compounds/small molecule inhibitors	Targets	Mechanism
AG490	JAK2	Promotes *in vitro* efficacy against laryngeal carcinoma cells
Curcumin	JAK2	Promotes apoptosis, arrests HNSCC cells at G2/M phase of cell cycle, and augments tumor regression in animal models
FLLL12	JAK2 STAT3	Inhibits cellular growth of HNSCC and promotes apoptosis by upregulation of pro-apoptotic proteins, inhibits Tyr705 phosphorylation of STAT3
Stattic	STAT3	Stattic inhibits SH2 domain and decreases tumor growth in xenograft model of HNC
OPB-51602	STAT3	Binds SH2 domain of STAT3 thereby inhibits phosphorylation at Tyr705 and Ser727
AZD1480	STAT3	Inhibits cell proliferation and dephosphorylation of STAT3 in HNC cell lines
STAT3 decoy	STAT3	Inhibits STAT3 dimerization which is essential for translocation into the nucleus to act transcriptional factors for survival genes
Epigallocatechin Gallate (EGCG)	STAT3	Inhibits STAT3 action/function in HNSCC cells
Brusatol	JAK1/2, STAT3	Blocks upstream JAK1/2 and STAT3 activation in HNSCC cells
Dihydroartemisinin	JAK2 and STAT3	Exhibits antiproliferative and apoptotic activity by abrogating JAK2 and STAT3 activation in HNSCC cells
Honokoil	JAK and STAT3	Diminishes p-STAT3 tyr705, *p*-JAK tyr1007 as well as mRNA expression of respective genes in HNSCC cells

pathway, has been associated with aberrant STAT3 activation in HNSCC (Gkouveris et al., 2016). In several types of HNSCC cells, JAK/STAT signaling may also be activated by the alpha-7 nicotinic receptor, IL-6, IL-10, and IL22 receptor (Gkouveris et al., 2016). There is compelling evidence that strongly suggests that persistent activation of JAK/STAT in HNSCC is associated with an increase in STAT3 tyrosine phosphorylation (Gutiérrez-Hoya and Soto-Cruz, 2020). The tumorigenic role of JAK/STAT signaling in HNSCC has been emphasized in a number of studies. JAK/STAT signaling is constitutively activated and plays a crucial role in regulating various cellular processes including cell proliferation, differentiation, and apoptosis in HNSCC (Groner and von Manstein, 2017). The aberrant increase in STAT3 tyrosine phosphorylation is believed to be a potent inducer of HNSCC progression and initiation. The oncogenic potential of JAK/STAT signaling is mainly dependent on the Tyr705 phosphorylation status whereas STAT3 serine phosphorylation still remains elusive (Morgan and Macdonald, 2020). The aberrant activation of JAK/STAT signaling and its association with constitutively expressed p-STAT3 at tyrosine/serine and total STAT3, Erk1/2, and cyclin D1 has been observed in oral squamous cell carcinoma (OSCC) cell lines (Gkouveris et al., 2016). Besides regulating cell proliferation and cell cycle progression, JAK/STAT signaling is also implicated in apoptosis, angiogenesis, immune evasion, as well as exerting effects on cancer stem cells. (Groner and von Manstein, 2017). The constitutive activation of JAK/STAT signaling is documented in diverse malignancies and is associated with the transactivation of several genes in malignant cells both *in vitro* and *in vivo* settings (Mali, 2015). Increasing STAT3 phosphorylation in primary HNC tumors and normal mucosa from HNC patients indicates early STAT3 activation in carcinogenesis (Aggarwal et al., 2021). Furthermore, a study has been performed and reported the increased STAT3 expression in smokeless tobacco-mediated HNC with minimal STAT3 in normal tissues (Sinevici and O'sullivan, 2016). Moreover, immunohistochemistry staining for phosphorylated STAT3 has also revealed the increased expression of activated STAT3 in human squamous cell cancer of the tongue correlated with poor prognosis in patients with HNC (Lee et al., 2017).

The role of cancer stem cells in HNC has been extensively studied making them an appealing target for further investigation. In addition to providing insight into how JAK/STAT functions, understanding its potential crosstalk with other molecules may lead to new treatment strategies for patients with HNSCC (Byeon et al., 2019). A number of studies in human tumors and HNC cell lines have identified JAK/STAT signaling pathway, specifically, STAT3 as a potential therapeutic target (Liang et al., 2020). A study by Kowshik et al. demonstrated that dietary supplementation of astaxanthin inhibited tumor progression by attenuating JAK/STAT signaling and its downstream target molecules including cyclin D1, MMP-2, -9, and VEGF in the HPV-induced tumor models (Kowshik et al., 2014). STAT3 decoy inhibitors inhibit STAT3 dimerization which is essential for translation into the nucleus to act as a transcriptional factor for a plethora of tumor survival genes. These inhibitors are taken in the form of injections and are used currently against the patients of HNSCC with a safe toxicity profiles. Another study by Ahn et al. showed Guggulsterone (GS) and other inhibitors (**Table 5**) decreased p-STAT3 expression in multiple myeloma and HNSCC cell lines (Siveen et al., 2014). Honokoil, a phytochemical derived from Magnolia Officinalis diminishes p-STAT3 tyr705, *p*-JAK tyr1007 as well as mRNA expression of respective genes in HNSCC cells. Leeman-Neill et al. also demonstrated growth inhibitory effects of guggulsterone in HNSCC preclinical models mediated by modulation of STAT3 signaling (Leeman-Neill et al., 2009). A natural triterpene, brusatol has been reported to act as a blocker for STAT3 and inhibits upstream kinases such as JAK1/2 thereby preventing the HNSCC progression. An artemisinin analog, dihydroartemisinin exhibits cytotoxicity potential and promotes apoptotic activity by diminishing activated JAK2/STAT3 expression in HNSCC cells. A significant association of smokeless tobacco consumption habits and accumulation

of nuclear p-STAT3 was observed in clinical oral squamous cell carcinoma tissues (Zhou et al., 2016). Wang et al. suggested that OSCC cell proliferation and cell cycle regulation might be associated with the overexpression of JAK/STAT and cyclin D1 (Wang et al., 2019). Taken together, these findings collectively suggest that JAK/STAT signaling plays a critical role in HNSCC and is a promising signaling pathway to be targeted for drug development in HNSCC.

JAK/STAT Signaling in Breast Cancer

Breast cancer (BC) is the most common heterogeneous malignancy and the second leading cause of cancer-associated mortalities in females. Thanks to worldwide screening for early diagnosis, BC has a better survival time compared with other gynecological malignancies. Apart from the genetic predisposition of BRCA1/2, PTEN and TP53 mutations in high-risk groups, the other genetic expression profile of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor-2 (HER-2) contributes to the pathogenesis and stratification of BC (Chen et al., 2020). Besides, mutations and epigenetic modifications in various critical genes, BC pathophysiology is also contributed by various signaling pathways. The major pathways that are dysregulated in BC are MAPK, PI3K/Akt/mTOR, and JAK/STAT signaling pathway. A significant number of growth factors and cytokines activate JAK/STAT signaling pathway (O'Shea et al., 2015). Various studies have shown that acquiring a functional mutation, and polymorphisms in various components of JAK/STAT pathway are responsible for the overactivation of the JAK/STAT pathway (Lokau and Garbers, 2019). Several mechanisms include autocrine/paracrine production of cytokines, activating mutations of different isomers of JAKs, its receptors, and various distant oncogenes constitutively stimulate this pathway which eventually further activates downstream STATs (Banerjee and Resat, 2016). The role of JAKs and STATs is not only restricted to inflammation, survival, and proliferation of cells but also implicated in a plethora of organ-associated tumorigenesis. Many reports in the recent past have shown that JAK/STAT has a significant role in the development of BC by acting either as oncogenes or tumor suppressors (Xing et al., 2021). In cancer, somatic mutations are rare in members of the JAK family. Regardless of this, it has already been described that JAK1, JAK2, and JAK3 have harbored somatic mutations in BC specimens (Kalimutho et al., 2015). Recent research exhibits the promising role of JAK2 and TYK2 in BC development. Studies have shown that the V617F point mutation causes constitutive activation of JAK2 in epithelial mammary cells, resulting in hyperactivation of STAT5 which eventually enhanced the proliferation of epithelial memory cells (Rani and Murphy, 2016; Velloso et al., 2017).

Other STAT proteins like STAT1, STAT3, STAT5, and STAT6 have also been associated with the progression, prediction, and prognosis of BC (Wang et al., 2018). In terms of physiological dependency perspective, STAT1 has been shown to act as a tumor suppressor or oncogene. Nearly 45% of androgen receptor (AR)-positive and 22% of AR-negative cases of BC showed evidence of reduced STAT1 levels in malignant cells compared to benign breast tissues (Haddon, 2018). On the contrary high expression levels of STAT1 are associated with therapeutic resistance and metastasis. This indicates that STAT1 acts as a tumor suppressor in menopausal ER^+ BC, but STAT1 actively promotes tumor growth in ER^- tumors or ER^+ tumors in premenopausal BC (Dong et al., 2020).

Solid tumors including BC usually have high activity of STAT3. Its aberrant turnover may advance invasion and metastasis, apart from regulating inflammatory reaction in breast tumorigenesis (Edsbäcker, 2019). It has been shown by various studies that STAT3 is perhaps the most often activated in primary BCs and is associated with tissue invasion and poor prognosis (Banerjee and Resat, 2016). The tumor microenvironment appears to get affected by the constitutive activation of STAT3 through the secretion of several cytokines, e.g., Interleukin-10, Interleukin-6, Interleukin1β, by cancer cells which activate noncancerous cells, tumor associated macrophages and T-helper (Th)-17 cells to release more cytokines, thereby contributing a positive feedback circle (Jeannin et al., 2018; Duan et al., 2020). This further elevates tumor cell growth and differentiation. IL-10 secretion directed by STAT3 in cancer cells also results in the arrest of antitumor immunity (McCaw et al., 2017).

In BC STAT5 like STAT3 is constitutively activated, but is deemed to be feebly oncogenic in mouse models of BC. STAT5 may possibly help in carcinogenesis, but it is not an acceptable proto-oncogene in BC according to current data available (Braicu et al., 2019; Recio et al., 2019). STAT6 regulates the balance of Th1 and Th2 cells, which promotes tumor growth by allowing tumor cells to evade immune responses. Depending on the cytokine secretion in the environment of T lymphocytes, T cells can differentiate either into Th1 or Th2 cells (Tough et al., 2020). Th1 cells helps in recognizing tumor antigens and eliciting an immune response, but Th2 cells are oncogenic, promote invasion and metastasis of tumors. STAT6 is required for T lymphocyte development in Th2 cells, which is mediated by the interleukin 4 (IL4) (Kim et al., 2018).

A large data shows the significance of the JAK/STAT pathway in various diseases of the immune system and in various cancers thereby drawing attention to therapeutic targets on key members of this pathway (Pectasides et al., 2018). In myeloproliferative diseases, JAK and STAT protein mutations have been comprehensively characterized and have been linked to hyperactivity of the JAK/STAT pathway which eventually leads to unrestricted cell proliferation (Vainchenker et al., 2018; Baldini et al., 2021). In myeloproliferative diseases, JAK and STAT protein mutations have been comprehensively characterized and have been linked to hyperactivity of the JAK/STAT pathway which eventually leads to unrestricted cell proliferation (Vainchenker et al., 2018; Baldini et al., 2021). Similar alterations have not been well studied in BC. However constitutive phosphorylation of STAT1, STAT3, and STAT5 is often found due to higher levels of cytokines and their respective receptors (Qin et al., 2019). Various studies have shown that the IL-6/JAK2/STAT3 pathway is upregulated in basal-like BC cells, and it has been stated by Marotta LL et al. that NBP-BSK805 and other inhibitors (**Table 6**) abrogates the growth of patient-derived

TABLE 6 | JAK/STAT signaling modulators, their targets, and mechanism in breast cancer/cells.

Compounds/small molecule inhibitors	Targets	Mechanism
Pentagalloylglucose (PGG)	JAK1	Promotes dephosphorylation of JAK1 in MDA-MB-231 cancer cells and reduces tumor development in MDA-MB-231-induced xenograft tumor models
Methylsulfonylmethane	JAK2	Decreases phosphorylation of JAK2 in T47D and MCF-7 cells of breast cancer
Curcumin-BTP hybrids	STAT3	Represses STAT3 phosphorylation and nuclear translocation
BMA097	STAT3	Downregulation of STAT3 activated genes by dephosphorylation of the SH2 domain of SH2
Furanoditerpenes (Crispenes F and G)	STAT3	Inhibits STAT3 dimerization in MDA-MB-231 cancer cells which is essential for STAT3 activity
Alantolactone	STAT3	Reduces the pSTAT3 expression in MDA-MB-231 cancer cells
WMJ-8-B	STAT3	Increases SHP-1 driven reduction in MDA-MB-231 cancer cells by decreasing phosphorylation of STAT3
Galiellalactone	STAT3	Inhibits Tyr705 STAT3 phosphorylation

breast tumor xenografts (Banerjee and Resat, 2016). Pyridone 6 another JAK inhibitor that was introduced in the early 2000s by Merck, has potential *in vitro* activity against JAK family members (Buchert et al., 2016). Ruxolitinib (Novartis) is another approved inhibitor for the treatment of myelofibrosis, which targets both JAK1 and JAK2 and is currently being used in a number of clinical studies (phase I, II, and III) in solid tumors including BC [173]. Apart from JAK inhibitors, (Winthrop, 2017) many molecules have been developed that primarily inhibit STAT3 and STAT5 e.g., a STAT3 inhibitor CJ1383 which shows good results in two BC cell lines having elevated expression of phosphorylated STAT3 (Roskoski, 2015; Bharadwaj et al., 2016; Beebe et al., 2018; Kim and Strober, 2018). IS3295 and FLL32 inhibitors have also shown promising results by inhibiting STAT5 in quite a few BC cell lines (Bharadwaj et al., 2016). Bcl-XL mediated expression and apoptosis of various tumor cells are regulated by STAT3 and STAT5. STAT3 and STAT5 are targeted by another anti-apoptotic Mcl-1 protein, a member of the Bcl-2 family (Maeda et al., 2018). Moreover, it was also demonstrated by Real *et al* that the overexpression of Bcl-2 mediated by STAT3 inhibited chemotherapy-induced apoptosis in BC cells (Liao et al., 2017), while another study by Masuda *et al* demonstrated that STAT3 inhibition improved the sensitivity of head and neck cancer cells to 5-Fluorouracil. Furthermore, survivin expression has also been shown to be induced by STAT3 (Aziz et al., 2021). In view of its anti-apoptotic activity and activation frequency in cancers, STAT3 could be used as an appealing therapeutic target in a number of cancers.

JAK/STAT Signaling in Ovarian Cancer

Ovarian cancer (OC) is the most common cause of cancer-related deaths from gynecologic malignancies accounting for 4.3% of female deaths worldwide (Sung et al., 2021). The high mortality rate is due to the late-stage diagnosis, lack of effective early detection methods, and strong drive towards metastasis. Depending on the origin of tissue, OC has been classified into epithelial ovarian cancer (EOC), germ cell tumor, and stromal endocrine cell tumor. EOC, the most lethal of all gynecological malignancies, accounts for 90% with various other subtypes including serous, endometrioid, clear cell, and mucinous carcinoma (Cortez et al., 2018). Non-epithelial OC includes germ cell tumors and sex cord-stromal carcinoma accounting for 10% of OC (Maoz et al., 2020). Accumulating evidence indicates extensive crosstalk between various oncogenic signaling pathways such as PI3K/Akt, RAS/RAF/MEK/ERK, and JAK/STAT in a wide range of malignant neoplasms including OC (Yap et al., 2009). Interestingly, a number of studies have highlighted the significance of the JAK/STAT signaling pathway in the pathogenesis of OC. Aberrantly activated JAK/STAT signaling has been observed in various OC cell lines (OCC) as well as clinical tissue samples (Masoumi-Dehghi et al., 2020). The JAK/STAT signaling pathway plays a critical role in promoting cell proliferation, invasion, survival, stemness, angiogenesis, and chemo-resistance in OC (Liang et al., 2020) **Figure 3**. The critical role of JAK/STAT in enhancing tumor progression and survival is well established. Numerous studies have indicated that the aberrant activation of STATs strongly influences the expression of Bcl-2 (Groner and von Manstein, 2017). In addition, an increase in the expression of cyclin D1 and c-Myc has also been correlated with the constitutive activation of STAT3 thereby contributing to malignant transformations in human OC (Poli and Camporeale, 2015). Several studies have demonstrated the critical role of STAT3 in the migration and invasion of OC. Increased expression of pY-STAT3 in association with loss of protein inhibitor of activated STAT3 (PIAS3) resulted in the progression of high-grade serous carcinoma (Wu et al., 2020). The PKM2–STAT3/NF-κB axis activated by AKT2 enhanced the migratory and invasive capabilities of EOC cells. VEGF and HIF-1α, the two critical molecules in angiogenesis play a pivotal role in angiogenesis (Wu et al., 2020). Activated STATs (STAT3 and STAT5) have been found to regulate the expression of VEGF by directly binding to its promoter, thereby strengthening VEGF expression and tumor angiogenesis (Wu et al., 2020). In comparison with benign and normal tissues, immunohistochemistry showed increased expression of pY-STAT3, pY-STAT5, and VEGF in patient-derived ovarian epithelial carcinoma tissues (Wu et al., 2020). Also, immunohistochemical analysis revealed an increase in the nuclear expression of pY-STAT3 and HIF1α in ovarian clear cell carcinoma (Wu et al., 2020). IL-6 is known to induce transcription of VEGF and activates expression of downstream HIF-1α *via* STAT3. The upregulation of IL-6, STAT3, HIF-1α observed in ovarian clear cell cancer samples

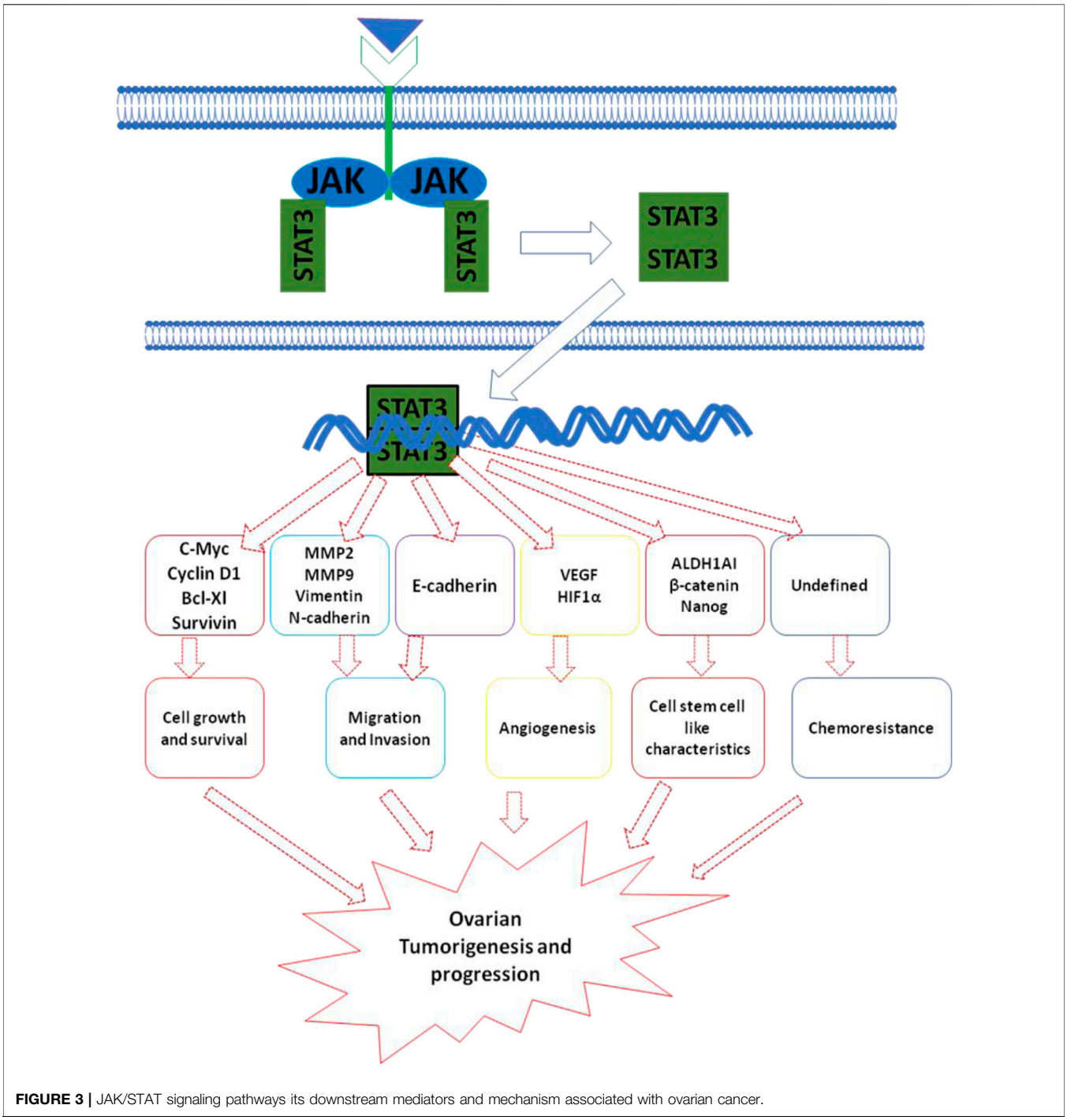

FIGURE 3 | JAK/STAT signaling pathways its downstream mediators and mechanism associated with ovarian cancer.

indicates an IL-6/STAT3/HIF1α/VEGF autocrine activation loop in EOC thereby facilitating tumor angiogenesis (Tawara et al., 2019). Recent advances in understanding the cellular signaling pathways have led to the development of several strategies and targets for various cancers including ovarian cancer (Wen et al., 2020). Despite chemotherapy being a crucial part of OC treatment, a significant number of patients develop chemoresistance. In addition, to killing cancer cells, chemotherapeutic intervention can also have cytotoxic effects on adjacent normal tissues and cells as well (Norouzi-Barough et al., 2018). To date, various alternate approaches have been carried out to inhibit JAK/STAT signaling including small molecules derived from natural sources, synthetic molecules, and antisense oligonucleotides (Yaswen et al., 2015). In preclinical or clinical trials, a number of natural and synthetic agents targeting JAK/STAT in OC are being explored (Bose et al., 2017, 2020). Resveratrol derived from red grapes and berries

TABLE 7 | JAK/STAT signaling modulators, their targets, and mechanism in ovarian cancer/cells.

Compounds/small molecule inhibitors	Targets	Mechanism
AG490	JAK2	Reduces JAK2 phosphorylation in ovarian cancer cells
MLS-2384	JAK/Src	Promotes apoptosis and decreases cellular growth of ovarian cells by inhibiting JAK2 phosphorylation
Ruxolitinib	JAK2	Inhibits JAK2 phosphorylation
Resveratrol	STAT3	Suppresses tumor growth, cell proliferation and promotes apoptosis of ovarian cancer cells by targeting STAT3 phosphorylation
Diferuloylmethane	STAT3	Reduces STAT3 phosphorylation in ovarian cancer cells
Corosolic acid	STAT3	Decreases STAT3 expression by inhibiting M2 polarization of macrophages in the ovarian tumor microenvironment
Diarylidenylpiperidone (DAP)	STAT3	Inhibits activation of STAT3 by reducing phosphorylation

is known to possess anti-cancer activity in various cancers, including OC. Another study demonstrated that resveratrol significantly inhibited cell proliferation and induced apoptosis in OC patients (Rauf et al., 2018). Also, resveratrol inhibits growth, increases apoptosis and autophagy activity in OC cells by suppressing the STAT3 signaling pathway (Rauf et al., 2018). Curcumin (diferuloylmethane) is known to suppress STAT3 activation, resulting in decreased cell viability in OC cells (Perrone et al., 2015). Curcumin not only inhibits characteristics of OC through STATs but also blocks another signaling, including PI3K/Akt and NF-kB pathway (Perrone et al., 2015). Similar to curcumin and resveratrol, Corosolic acid, a potent STAT3 inhibitor, abrogates STAT3 activity resulting in the suppression of cell growth in OCC. In OC cells, Corosolic acid is also known to enhance the cytotoxicity of various chemotherapeutic drugs *via* inhibition of STAT3 activation (Willenbacher et al., 2019). The rapid metabolism or delivery systems and low bioavailability of natural compounds are some of the major concerns. Hence, the designing of corresponding analogs of natural compounds or synthesizing other novel small molecules that can abrogate JAK/STAT signaling has received great attention (ElNaggar et al., 2016). Curcumin analog diarylidenylpiperidone (DAP)-based synthetic compound, HO-3867 is known to block STAT3 activation by directly binding to the STAT3 DNA-binding domain. Treatment of human ovarian cancer cells with HO-3867 strongly decreased their migration (Liang et al., 2020). In a similar manner to HO-3867, DAP compound derivatives, HO-4200 and H-4318, also inhibit STAT3 by interacting with its DNA-binding domain (Liang et al., 2020). A study by El Naggar et al. demonstrated that HO-4200 and H-4318 significantly decreased the expression of STAT3 target proteins cyclin D1/D2, Bcl-xl, Bcl-2, survivin, and c-myc in cisplatin-resistant OC cell line suggesting cell survival inhibition and induction of apoptosis (ElNaggar et al., 2016). Also, HO-4200/H-4318 inhibited expression of VEGF, decreased migration, and invasion activity of OC cell line (ElNaggar et al., 2016). Besides DAP compounds, another STAT3 inhibitor, LC28 targets the DNA-binding domain of STAT3 (Liang et al., 2020). Huang et al. demonstrated that LC28 significantly blocked STAT3 interaction with DNA to inhibit the growth of cisplatin-resistant OC cells (Huang et al., 2018). Several other inhibitors of JAK/STAT signaling such as WP1066154, AZD1480153, MLS-2384155, and SD-102945 are also known for their anti-tumor property in OC models (Liang et al., 2020). Various upstream kinases or diverse cytokines, including IL-6, EGFR, and Src are known to activate JAK/STAT signaling pathway and are attractive strategies to abrogate JAK/STAT signaling (Wee and Wang, 2017; Gutiérrez-Hoya and Soto-Cruz, 2020). The decrease in the expression of the STAT3 by EGFR inhibitors such as Cetuximab, lapatinib, Erlotinib, and Gefitinib have shown minimal clinical activity in patients treated for OC (Seshacharyulu et al., 2012). Src, a cell membrane-associated non-receptor tyrosine kinase is known to activate STAT3 pathway and plays a decisive role in cell proliferation differentiation and migration of tumor cells. Src inhibitor, Dasatinib has shown minimal efficacy in patients with recurrent epithelial ovarian cancer (Zhang and Yu, 2012). However, combinatorial study has revealed the synergistic effect of dasatinib and paclitaxel on the inhibition of growth of ovarian granulosa cell tumor cells. IL-6, one of the critical cytokines that recruits gp130, forms IL-6/IL-6R/GP130 complex to activate STAT3 (Roze et al., 2021). The IL-6/gp130/STAT3 signaling axis is frequently aberrant in many tumors. Guo et al. demonstrated that a Monoclonal anti-IL-6 antibody, siltuximab significantly decreased the expression of STAT3 by inhibiting IL-6-induced STAT3 activation in OC cells (Liang et al., 2020). Another study by Coward et al. showed a significant decrease in nuclear expression of pY-STAT3 expression in intraperitoneal EOC xenograft (Wu et al., 2020) (**Table 7**). Collectively, these findings suggest that targeting JAK/STAT and an associated pathway is the ideal strategy for the future development of anticancer therapeutic lead molecules against OC.

CONCLUSION

JAK/STAT is one of the versatile signaling pathways which has been extensively studied for its crucial role in tumor progression. The potential crosstalk of JAK/STAT with multiple alternative pathways has made it a promising target for the development of new lead molecules. Aberrant activation of JAK/STAT signaling

has been frequently observed in a wide range of malignant neoplasms. A number of studies have provided compelling evidence that inhibition of the JAK/STAT pathway provides significant therapeutic benefits. Several JAK/STAT inhibitors (natural as well as synthetic) have been found to modulate the expression of molecules involved in the JAK/STAT signaling network. Preclinical studies in cancer models have shown the effect of various inhibitors of JAK/STAT signaling which resulted in inhibition of cellular proliferation and tumor progression.

Thus, JAK/STAT signaling appears to be an important target with the potential for a high therapeutic index. Although it takes more than two decades to get approval from FDA to target JAK/STAT signaling. In the coming future, we expect more specific compounds with the least deleterious effects on normal cells which target particularly the kinase activity of JAK/STAT mediators to attenuate its amplification in malignancies. However, the big question is whether next-generation compounds/inhibitors will be able to attenuate the functional part of mutated JAKs and leave wild-type JAKs unaffected. This approach of inhibition could decrease the deleterious effects of compounds/inhibitors on normal cells and will improve the drug efficacy. Besides various preclinical and experimental settings, effective STAT inhibitors in clinical settings are still a dream to come true. Although targeting transcriptional factors is not common, however, this approach will lead to more specific inhibition and in the coming future could be a promising therapeutic approach against JAK/STAT signaling in solid malignancies. However, it is important to validate the mechanism of action of small molecule inhibitors (SMIs) of the JAK-STAT pathway before conclusions can be drawn about their clinical use. The hope is to administer SMIs of JAK-STAT pathway with minimum risk to human subjects and that these inhibitors will produce a significant therapeutic effect against the solid malignancies.

AUTHOR CONTRIBUTIONS

BR, RR, IM, MF, ABB, TY, and GB contributed to manuscript writing, conceptualized and contributed in manuscript writing, conceptualized and made tables. SP, RH, AB, SB, and MHZ read manuscript critically; DA contributed to reading, edited and approved the submitted version. All authors agreed to manuscript submission.

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Epigallocatechin-3-Gallate Delivery in Lipid-Based Nanoparticles: Potentiality and Perspectives for Future Applications in Cancer Chemoprevention and Therapy

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Chemoprevention is a strategy aimed to not only reduce the risk but also delay the development or recurrence of cancer. An ideal chemopreventive agent is not dangerous and ought not to result in side effects or damage to human health. In this context, epigallocatechin-3-gallate (EGCG) is considered a suitable chemopreventive agent, but its clinical use is limited by many factors, namely, the difference in source, administration, individual metabolism, absorption, and distribution. Genetic and dietary differences greatly cause this variability, which has limited the rational use of EGCG in chemoprevention and, particularly, the definition of a safe and efficient concentration. In the present mini review, the main limitations to a complete understanding of the use of EGCG as a chemopreventive agent will be briefly illustrated. This review also indicates the introduction and trialing of lipid-based nanoparticles (NPs) as a proper strategy to deliver EGCG at a well-defined concentration for better investigation of the chemopreventive activity. Finally, some examples of cancers that might benefit from EGCG treatment in different stages of the disease are proposed.

Keywords: cancer, catechins, medicinal chemistry, nanomedicine, nutraceutics, EGCG

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INTRODUCTION

EGCG, the most abundant catechin in green tea, is considered a suitable chemopreventive agent based on epidemiologic (Yuan et al., 2011; Yi et al., 2019; Zhao et al., 2021) and animal model studies (Khan and Mukhtar, 2007; Yang et al., 2011; Yiannakopoulou, 2014). *In vitro* studies on human cancer cells have also provided a large set of data that indicate the cytotoxicity of EGCG on cancer cells (Singh et al., 2011; Gan et al., 2018; Shirakami and Shimizu, 2018; Aggarwal et al., 2020). On the other hand, many contradictory results have also been published, and doubts on its potential use in humans exist (Filippini et al., 2020; Kim et al., 2020). The complexity of unraveling the activity of EGCG against cancer cells is due to the very high number of variables, which are difficult to interpret correctly (Mereles and Hunstein, 2011). A first issue concerns the poor physicochemical stability and low bioavailability of EGCG (Lambert and Yang, 2003; Chow and Hakim, 2011; Sang et al., 2011). Oral consumption of EGCG bioavailability after oral intake can be reduced or increased by assuming various food and beverages (Kale et al., 2010; Peters et al., 2010; Naumovsky et al., 2015). Thus,

EGCG, which is only partly degraded in the stomach at low pH, reaches the intestine, where the pH is neutral-alkaline, and is further degraded (Neilson et al., 2007). The quantity of EGCG that crosses the enterocytes is low: EGCG enters the enterocytes mainly by passive diffusion since no specific receptors carrying EGCG exist on the surface of enterocytes. Active outflow by multidrug resistance-associated protein 2 (MRP2) may occur, further lowering EGCG absorption (Vaidyanathan and Walle, 2001; Hong et al., 2003; Scholl et al., 2018). Once into the enterocytes, the EGCG is actively metabolized by phase II enzymes, conjugated with glucuronic acid and sulfate, or by methylation or methylated by catechol-Omethyltransferase (COMT). Glucuronidation and sulfation mainly occur in the intestine, whereas glucuronidation, sulfation, and methylation occur later in the liver (Singh et al., 2011). Some conjugates are further methylated. Genetic heterogeneity due to a polymorphism involving COMT results in a low-activity variant, which possesses a 40–75% less catalytic capacity than the enzyme coded with wild-type alleles and can introduce a high variability in EGCG activity (Wu et al., 2003; Miller et al., 2012; Lai et al., 2019). A large part of orally taken EGCG is effluxed from the enterocytes into the intestinal lumen or from the liver to the bile and excreted in the feces, and hence lost. Gut microbiota plays a critical role in the metabolism of EGCG. Microbiota can deconjugate and degrade EGCG (Zhang et al., 2013; Liu et al., 2020). In contrast, it has been found that metabolites of green tea, including EGCG, produced by gut microbes, have significant health benefits: small molecules derived from breakdown can show antioxidant and anti-inflammatory capacity, correct dysbiosis, and decrease harmful metabolites (Zhang et al., 2019; Xu et al., 2020). Then, ultimately, microbiota may concur to improve the health effects of EGCG. In addition to cancer cell cytotoxicity, indirect advantages of EGCG on cancer onset and development are also related to the metabolic (antidiabetic and contrasting obesity effects) (Hursel et al., 2009; Hara-Terawaki et al., 2017; Quezada-Fernández et al., 2019), antioxidative, and anti-inflammatory actions of EGCG (Chen et al., 2018), all these conditions being clearly associated with cancer development (Thielecke and Boschmann, 2009; Yang et al., 2016; Oz, 2017; Potenza et al., 2020).

Then, why should EGCG be proposed as a chemopreventive agent? The relative potency of all these variables to stability and bioavailability makes defining the health effects of green tea catechins and EGCG unpredictable. Several observational and interventional studies have been reviewed extensively over the last years (Clement, 2009; Fujiki et al., 2018; Almatroodi et al., 2020). The findings are conflicting, but a consistent number of studies on several human malignant neoplasia support the potential use of EGCG as a chemopreventive agent despite this variability: any improvement in EGCG stability and bioavailability is, therefore, supposed to improve the efficacy. When we turn to animal models, there is a general agreement that a clear chemopreventive effect independent of the experimental model used (chemical carcinogenesis, xenograft tumors, knock-out animals spontaneously developing cancer, etc.) occurs. A large majority of studies clearly demonstrated that EGCG, usually used as a beverage, delayed cancer onset and the metastatic process and reduced the size and number of neoplastic foci (Ju et al., 2007; Fujiki et al., 2018; Gan et al., 2018). In the context of animal models, the number of variables under evaluation and concurring to the final target decreases: animals are genetically related, frequently inbred, exposed to the same environmental conditions that are controlled from the beginning to the end of the experimental treatments, and consume the same food, which is very simple and not varied. In these conditions, EGCG shows chemopreventive efficacy (Ju et al., 2007; Yang et al., 2011).

Another conflicting point concerns "*in vitro*" experiments on cell lines. In this case, the EGCG concentrations used to obtain significant results, including cytotoxicity and downregulation of molecular pathways involved in cancer onset and development, are far from those measured *in vivo*, in blood and tissues, after EGCG oral administration (Gan et al., 2018; Almatroodi et al., 2020). This discrepancy seems to be related to the fact that EGCG dissolved in culture medium is barely taken up by cells and is unstable at pH greater than 5 (Hong et al., 2002). Furthermore, oxidation processes may occur, and they are considered misleading with respect to the interpretation of the final cytotoxic effects (Lambert and Elias, 2010; Wei et al., 2016). On the other hand, currently a large number of studies are underway on human cancer cell lines: extensive meta-analysis in the last years reported coherent results concerning the molecular targets (Singh et al., 2011; Gan et al., 2018; Shirakami and Shimizu, 2018; Aggarwal et al., 2020; Farooqi et al., 2020) and stressed another crucial point: EGCG is safe for normal cells since very high concentrations can only induce cell death (Weisburg et al., 2004; Papi et al., 2013; Tyagi et al., 2015; Luo et al., 2018; Ni et al., 2018; Xie et al., 2018). So, EGCG is preferentially taken up by cancer cells rather than by normal cells, fulfilling one of the basic characteristics of a molecule suitable for chemoprevention. Can the potentiality of EGCG as a chemopreventive agent be improved in view of future applications?

APPLICATION OF NANOTECHNOLOGY IN EPIGALLOCATECHIN-3-GALLATE DELIVERY SYSTEMS TO CANCER CELLS

The use of nanotechnology, namely, the development of drug delivery systems, especially biocompatible nanoparticles (NPs), constitutes a promising approach to increase the bioavailability and stability of this natural compound (Granja et al., 2017; Cai et al., 2018; Granja et al., 2019). Different types of NPs, including lipid-based NPs, polymeric NPs, and gold NPs, among others, can be used, and for more comprehensive information, the reader can consult the following studies: Min and Kwon (2014), Granja et al. (2017), and Li et al. (2020). NPs are extremely versatile and can be easily modified at their surface to selectively target cancer cells and, therefore, selectively release ECGC, especially inside tumor cells (Li et al., 2020). The main ligands that are used include antibodies that recognize tumor cells and folic acid and analogs because of the higher expression of folic acid receptors in cancer cell lines than in healthy cells (Granja et al., 2017). Among the different types of NPs, lipid-based NPs emerge as the most

TABLE 1 | Lipid-based NPs, including liposomes and lipid NPs, as EGCG delivery systems.

Nanodelivery system	Particle size (nm)	EE (%)	Type of cancer	Primary outcome	References
Liposomes	157	57	Skin	Accumulation of EGCG in the tissues in a mouse model of basal cell carcinoma	Fang et al. (2005)
Liposomes	100	100	Skin	Liposomes allowed a higher EGCG accumulation within tumor cells	Fang et al. (2006)
Liposomes	56	90	Breast	Antiproliferative and proapoptotic effect in MCF7 breast cancer cells	Cohen de Pace et al. (2013)
Liposomes	127	59	Breast	Synergistic outcome of EGCG combination with an anticancer drug PTX in an MDA-MB-231 breast cancer cell line	Ramadass et al. (2015)
Solid lipid NPs	157	67	Breast and prostate	The NPs increase the cytotoxicity of MDA-MB-231 and DU-135 cell lines by 8.1- and 3.8-fold, respectively, in comparison with unloaded EGCG	Radhakrishnan et al. (2016)
Nanostructured lipid NPs	300	90	Not specified	Folic acid to make it easier for NLC-based nanoparticles to transport EGCG across the intestinal barrier to reach the cancer cells	Granja et al. (2019)
Solid lipid NPs	163	67	Breast	C57/BL6 mice showed greater survivability and reduction in tumor volume in mice treated with functionalized lipid NPs as compared to those treated with a nonfunctionalized formulation or with unloaded EGCG	Radhakrishna et al. (2019)

EGCG, epigallocatechin gallate; NPs, nanoparticles; NLCs, nanostructured lipid carriers; PTX, paclitaxel; SLNs, solid lipid nanoparticles; EE, encapsulation efficiency.

promising drug delivery systems because their composition is based on lipids, which also exist in the human body, making the NPs biocompatible and biodegradable (Nature Reviews Materials, 2021). One important advantage of their use is also their production simplicity, which is based on nanoemulsion oil/water production, and their high physicochemical stability, which is demanding for their scale-up (Frias et al., 2016).

Lipid-Based Nanoparticles

Lipid-based NPs constitute a broad and diverse group that includes liposomes and lipid NPs (Garcia-Pinel et al., 2019). Liposomes were discovered in 1965; are vesicular structures, constituted by phospholipid bilayers, enclosing an aqueous medium; and have been attracting interest as nanocarriers for many years (Bangham et al., 1965a; Bangham et al., 1965b). Thus, liposomes are already used in the clinical industry in several marketed formulations, including Doxil®, Ambisome®, and DepoDur™, among others (Bulbake et al., 2017). Solid lipid nanocarriers (SLNs) were developed in the 1990s, and next-generation nanostructured lipid carriers (NLCs) found almost 10 years later to improve the stability and capacity loading of SNLs (Naseri et al., 2015). The lipid NPs approved for clinical use were recently used in COVID-19 mRNA vaccines (Nature Reviews Materials, 2021). This mini review highlights the main contributions of EGCG lipid-based NPs developed in recent years and applied in cancer therapy and their enormous advantages and potential in clinical use. The main characteristics of lipid-based NP EGCG delivery systems in cancer therapy are summarized in **Table 1**. In 2005, Fang et al. developed liposomes loaded with EGCG of 157 nm diameter and with an encapsulation efficiency (EE) of 57%. The authors demonstrated that the formulation allowed the accumulation of EGCG in the tissues of Balb/c-nu, a mouse model of basal cell carcinoma. One year later, the authors developed liposomes of size 100 nm and with an EE of 100%. The authors demonstrated that in comparison with unloaded EGCG, the liposomal formulations demonstrated a 20-fold higher EGCG deposition (Fang et al., 2005; Fang et al., 2006). In 2013, Cohen de Pace et al. developed nanoliposomes loaded with EGCG of diameter 56 nm and with an EE of 90% and demonstrated that this formulation significantly enhanced EGCG stability, improved its sustained release, and increased the intracellular EGCG content in MCF7 cells, inducing apoptosis of MCF7 cells and inhibiting MCF7 cell proliferation compared to unloaded EGCG. In addition, the authors demonstrated that the developed formulation retained its antiproliferative and proapoptotic effectiveness at 10 μM or lower, at which unloaded EGCG does not have any beneficial effects (Cohen de Pace et al., 2013). In 2015, Ramadass et al. developed liposomes with EGCG of diameter 127 nm and with an EE of 59%. They demonstrated the synergistic outcome of a combination of EGCG with an anticancer drug paclitaxel (PTX) in an MDA-MB-231 breast cancer cell line and concluded on the suitability of PTX/EGCG co-loaded liposomes for the treatment of invasive breast cancer (Ramadass et al., 2015). In 2016, Radhakrishnan et al. developed EGCG solid lipid NPs of diameter 157 nm and with an EE of 67%. This formulation was tested in MDA-MB-231 and DU-135 cell lines, and an increase in cytotoxicity of 8.1- and 3.8-fold, respectively, was observed. In the same year, Frias et al. developed SLNs and NLCs loaded with EGCG of diameter 300–400 nm that demonstrated a higher EE of 80% and 90%, respectively, and a high stability during long-term storage (Radhakrishnan et al., 2016). Later, in 2019, Granja et al. developed NLCs of 300 nm diameter and with an EE of 90% and functionalized the NPs with folic acid to increase their transport across the intestinal barrier to reach the cancer cells (Granja et al., 2019). Recently, Radhakrishnan et al. developed solid lipid NPs loaded with EGCG and functionalized the NPs with a gastrin-releasing peptide receptor (GRPR)-specific peptide as GRPRs are overexpressed in breast cancer. The "*in vivo*" studies performed on C57/BL6 mice showed greater survivability and reduction in tumor volume in mice treated with functionalized solid lipid NPs than in mice treated with a nonfunctionalized formulation or with unloaded EGCG (Radhakrishnan et al., 2019). **Table 1** summarizes examples of successful nanoformulations with EGCG. An update on this topic can be found in Yang et al. (2020), Kazi et al. (2020), and Rashidinejad et al. (2021).

POTENTIAL APPLICATIONS AND KEY ISSUES FOR FURTHER RESEARCH

Neoplastic disease is extremely heterogeneous, and even in the same tissue and organ, the multiplicity of cancer types having different degrees of malignancy and evolution is very high. Chemoprevention is classified as primary, secondary, or tertiary. *Primary chemoprevention* aims at preventing the development of premalignant lesions (often assessed by appropriate markers) and subsequent cancer in high-risk cohorts. *Secondary chemoprevention* prevents the evolution of premalignant markers/lesions into cancer. Finally, *tertiary chemoprevention* prevents the recurrence of cancer (Landis-Piwowar and Iyer, 2014).

Premalignant Lesions in Sporadic Cancer

Sporadic cancer represents the most common human cancer. In some cases, premalignant lesions precede the development of frankly malignant neoplasms with invasive growth. Here, we discuss some aspects of a few neoplasms largely found in the human population, for example, prostatic intraepithelial neoplasia (PIN) and high-grade PIN in prostatic cancer, atypical hyperplasia and lobular/ductal carcinoma *in situ* (LCIS, DCIS) of the breast, and colon premalignant lesions, which can be identified by screening or are detected occasionally, and some open questions that might be addressed by a chemopreventive intervention based on EGCG. In some cases, surgery is the first option, but evolution of a premalignant lesion is difficult to predict and the risk of overtreatment must be evaluated too (Curtius et al., 2017). For a critically reviewed list of premalignant diseases potentially subject to chemopreventive interventions, the reader can consult Maresso et al. (2015). Chemoprevention might be an option to be investigated in all those cases where surgery is not considered necessary or a second-line intervention after surgery to target potentially evolving situations. A successful example of a green tea catechin (GTC) extract, orally administered in patients with high-grade PIN, was reported by Naponelli and coworkers (Naponelli et al., 2017). After 1 year of treatment, only one tumor was diagnosed among 30 GTC-treated men, while nine cancers were found among 30 placebo-treated men. Despite the limitations of stability and bioavailability of GTCs, many successful trials on the use of green tea or EGCG in prostate cancer prevention have been reported and critically analyzed (Guo et al., 2017; Jacob et al., 2017; Perletti et al., 2019).

In breast atypical hyperplasia and LCIS or DCIS, tamoxifen and raloxifene have been demonstrated to reduce breast cancer risk, but they must be used in postmenopausal women, reducing the number of patients who might obtain significant risk reduction benefits without incurring serious harm (Moen and Keating, 2008; Sauter, 2018). Aromatase inhibitors, which increase osteoporosis risk, are also preferentially used in postmenopausal women, but estrogen-insensitive breast neoplasms do not respond to this kind of treatment (Trivedi et al., 2017; Thorat and Balasubramanian, 2020). Therefore, premenopausal women and women with estrogen-negative neoplasms cannot benefit from this plan of chemoprevention. Patients with premalignant lesions, especially those in premenopause, might be eligible for a chemoprevention trial based on EGCG or green tea extract. In early breast carcinoma, EGCG administration 4 weeks before the surgery resulted in a higher concentration of EGCG in the tumor than in the adjacent normal tissue, which correlated with a lower Ki-67 index with respect to untreated patients (Lazzeroni et al., 2017). Mammographic density was found to be reduced by GTE supplementation in women at high risk of breast cancer, similar to tamoxifen treatment (Samavat et al., 2017). On the basis of numerous studies indicating a protective effect of green tea catechins in breast cancer development (Yu et al., 2019), EGCG nanoformulations might have the potential to reduce the risk in patients who develop premalignant breast lesions, independent of the presence or absence of estrogen receptors and age.

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the world (Arnold et al., 2017). Although recommendations for aspirin-based chemoprevention strategies have recently been established, hazards of the long-term use of aspirin make identification of individuals for whom the protective benefits outweigh the harms important (Katona and Weiss, 2020). Some premalignant diseases develop in the context of inflammatory bowel diseases (Nadeem et al., 2020) and require surgery to alleviate symptoms, independent of possible future cancer, such as colectomy for patients suffering from severe colitis symptoms. EGCG might be suggested as a chemoprevention strategy, particularly in patients suffering from severe colitis, due to the anti-inflammatory property of EGCG (Sokolosky and Wargovich, 2012; Wang et al., 2020). The development of a suitable nanoformulation for proper delivery to the colic mucosa might be an achievable target for these patients and an opportunity to delay and reduce cancer risk.

Disease-free intervals between chemo- and radiotherapy treatments occur in many cancer patients. In these phases, patients often do not receive any support (apart from some nutrition suggestions) to achieve remission or to prevent relapse. Compounds such as EGCG that have anti-inflammatory, antioxidant, and chemopreventive properties might also offer an opportunity of treatment for all patients experiencing undesired consequences of therapies. A special mention concerns childhood neoplasms: treatments in young cancer patients may open the way to secondary neoplasms that have time to insurge. Especially for very young cancer survivors, the option of safe intervention is mandatory (Gebauer et al., 2019).

CONCLUSION

This mini review summarized the main contributions of lipid-based NPs for EGCG delivery in cancer prevention and therapy and demonstrated that lipid-based NPs offer a great opportunity to increase the potential of EGCG as a chemopreventive and therapeutic agent. Adequate clinical trials to establish the safety and efficacy of nanoformulations are urgently needed to validate EGCG as a crucial component in experiencing a new innovative chemoprevention and treatment strategy for cancer.

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FF wrote and revised the manuscript. MP wrote and revised the manuscript and prepared **Table 1**.

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BRIEF RESEARCH REPORT
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Phenethylisothiocyanate Potentiates Platinum Therapy by Reversing Cisplatin Resistance in Cervical Cancer

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Acquired cisplatin resistance in cervical cancer therapy is principally caused by reduction in intracellular drug accumulation, which is exerted by hyperactivation of the oncogenic PI3K/Akt signaling axis and overexpression of cisplatin-exporter MRP2 along with prosurvival effectors NF-κB and IAPs in cervical cancer cells. These activated prosurvival signaling cascades drive drug efflux and evasion of apoptosis for rendering drug-resistant phenotypes. Our study challenges the PI3K/Akt axis in a cisplatin-resistant cervical cancer scenario with phenethylisothiocyanate (PEITC) for chemosensitization of SiHa^{R}, a cisplatin-resistant subline of SiHa and 3-methylcholanthrene–induced cervical cancer mice models. SiHa^{R} exhibited higher MRP2, p-Akt^{Thr308}, NF-κB, XIAP, and survivin expressions which cumulatively compromised cisplatin retention capacity and accumulated PEITC better than SiHa. SiHa^{R} appeared to favor PEITC uptake as its accumulation rates were found to be positively correlated with MRP2 expressions. PEITC treatment in SiHa^{R} for 3 h prior to cisplatin exposure revived intracellular platinum levels, reduced free GSH levels, generated greater ROS, and altered mitochondrial membrane potential compared to SiHa. Western blot and immunofluorescence results indicated that PEITC successfully downregulated MRP2 in addition to suppressing p-Akt^{Thr308}, XIAP, survivin, and NF-κB expressions. In mice models, administration of 5 mg/kg body-weight PEITC priming dosage prior to treatment with 3 mg/kg body-weight of cisplatin remediated cervical histology and induced tumor regression in contrast to the group receiving the same dosage of cisplatin only. This suggested PEITC as a potential chemosensitizing agent in light of acquired cisplatin resistance in cervical cancer and established its candidature for Phase I clinical trial.

Keywords: PEITC, chemosensitization, cisplatin resistance, MRP2, PI3K/AKT

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INTRODUCTION

Cervical cancer, the fourth-leading cause of morbidity among women worldwide (Globocan, 2018), is reported to cause maximum deaths among Indian women (NICPR, 2018). Relapse and recurrence catered by failure in treatment owing to acquirement of resistance to chemo/radiotherapy is a common occurrence (Dasari and Tchounwou, 2014; D'Alterio et al., 2020). Conventionally, chemotherapy with platinum-based drugs such as cisplatin, alongside other chemotherapeutics or radiation (Concurrent

Chemoradiotherapy; CCRT), is used for treating invasive cervical cancer (Stage IIB onward), where cisplatin is used as a radiosensitizer (Todo and Watari, 2016). Therefore, loss of cisplatin sensitivity with eventuation of resistance can be highly detrimental (Zhu et al., 2016).

Cisplatin (cis-diamminedichloroplatinum; CDDP) renders its action by attacking DNA to generate complex irreparable DNA adducts following 'intracellular hydrolytic activation' (Lorusso et al., 2014). Pleomorphically, cancer cells evade drug effects through some epigenetic and genetic changes that get triggered upon drug treatment (Kim et al., 2018). Similar changes also desensitize cervical cancer cells to CDDP for augmenting resistance (Seol et al., 2014). Overcoming CDDP resistance for improved treatment is the utmost requisite for better therapy outcome.

The Phosphatidylinositol 3 Kinase/Protein Kinase B or Akt (PI3K/Akt) signaling axis plays a pivotal role in conferring cells with CDDP-resistant properties by metabolically transforming them for reducing drug accumulation (Muggia et al., 2015). AKT/protein kinase B, being serine threonine kinases, gets activated by PI3K upon induction by various extracellular triggers such as chemotherapeutic drugs (Shi et al., 2019). Activated phospho-Akt1 inhibits IκB to evoke NF-κB for promoting evasion of apoptosis by triggering Inhibitor of Apoptosis proteins (IAPs) (Fu et al., 2014; Barra et al., 2019). This prosurvival axis domineers the event of acquired CDDP resistance by translationally activating CDDP exporters, namely, P-glycoprotein-1(Pgp1) and Multidrug Resistance Protein (MRP2), which conveniently pump out CDDP and critically reduce its intracellular levels, thereby restraining apoptosis (Zhang et al., 2020; Liu et al., 2021). Targeting of MRP2 and its upstream regulators, that is, PI3K/Akt axis, therefore, may provide a therapy-rationale for cervical cancer.

Natural phytochemicals from cruciferous vegetables such as phenethylisothiocyanate (PEITC) potentiate apoptosis by negatively regulating prosurvival molecules (Wang et al., 2011; Gupta et al., 2014; Dai et al., 2016). Reports suggest that PEITC can induce apoptosis in cancer cells to enable resistance reversal (Sarkar et al., 2012; Biswas et al., 2021). PEITC chemosensitizes many cancer cells by scavenging free radicals (Soundararajan and Kim, 2018). In biliary tract cancer cells, PEITC was reported to induce apoptosis *via* glutathionylation-dependent degradation of Mcl-1 (Li et al., 2016). Additionally, in gastric cancer cells, PEITC was reported to inhibit Multidrug Resistance gene (MDR1), MRP1, Akt, and NF-κB (Tang et al., 2014). Thus, mechanistic insight into PEITC-mediated targeting of PI3K/Akt signaling may be considered for improvising the therapy and prognosis of cervical cancer. The present study aims to explore the mechanism of PEITC-mediated regulation of this prosurvival signaling axis for overcoming acquired CDDP resistance in cervical cancer scenarios.

MATERIALS AND METHODS

Cell Culture

SiHa cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (gentamycin 40 μg, penicillin 100 units, and streptomycin 10 μg/ml) at 37°C in a humidified CO_2(~ 5%) incubator.

A CDDP-resistant subline ($SiHa^R$) was developed from the parental SiHa cell line by 'pulse treatment' (Sharma et al., 2010). Thereafter, the doubling time was calculated. Dissimilarities in cellular morphology were studied under a phase-contrast microscope (Olympus). Respective protocols have been elaborated in **Supplementary Data S1**.

Animal Experimentation

A cervical cancer mice model, developed by chronic 3methylcholantherene (3MC; chemical carcinogen) treatment of virgin female Swiss Albino mice (*Mus musculus*; 5–6 weeks old; weight: 23–25 gms), was used in this study (Mahapatra et al., 2020). All animals were obtained from the Central Animal Facility of CNCI and housed in polyvinyl cages within well-ventilated rooms (temperature: ~ 22°C; relative humidity: 50–60%; 12 h day/night cycle). The details of animal acclimation have been provided in **Supplementary Data S1**.

Based on body weight, 50 mice were randomized into five broad groups (Group IV), each consisting of ten animals separated into two batches [No. of mice (n) = 5/cage x 2]. Group I was kept as an "untreated" control group where mice did not receive any treatment. Invasive cancer-bearing mice were randomized into Group II (no intervention), Group III (3 mg/kg body weight CDDP), Group IV (2.5 mg/kg body weight PEITC), and Group V (PEITC followed by CDDP). These doses were selected after proper dosimetry. During the concurrent chemocycles of two weeks, body-weight fluctuations and tumor-growth alterations were recorded periodically. Food and water were provided *ad libitum*.

MTT Assay

The MTT assay was performed in SiHa and $SiHa^R$ cells after exposure to a wide range of PEITC (SIGMA Aldrich) and CDDP (CIPLA) concentrations as per the detailed description of the process provided in **Supplementary Data S1**.

Cyclocondensation Assay

Enumeration of the 'optimum period' and quantitation of intracellular PEITC in SiHa and $SiHa^R$ following 1, 2, 3, 4, 5, and 6 h of its administration was undertaken by the cyclocondensation assay (Zhang and Talalay, 1998). SiHa and $SiHa^R$ cells were treated with 2 μM of PEITC (maximum tolerated dose or MTD). PEITC concentrations were determined by spectrophotometry against a standard curve (**Supplementary Data S3**). The experimental discourse has been described in **Supplementary Data S1**.

Flameless Atomic Absorption Spectroscopy

The frozen cell pellets (-20°C) of SiHa and $SiHa^R$ were brought to room temperature, lysed in radio-immunoprecipitation assay lysis buffer (RIPA), and acid-digested in concentrated nitric acid at 60°C (Federici et al., 2014) for 2 h. All samples were quantitated for platinum levels at an absorbance of 265.9 nm in an inert argon gas chamber supplied with a platinum lamp being operated at 10 mA current. The measurements were recorded against varied

concentrations (0.1 nM-25 μM) of platinum using an atomic absorption spectrometer (VARIAN). Each experiment was repeated five times.

Assessment of Cell Viability by Trypan Blue Dye Exclusion Assay

Calculation of cell viability in SiHa and SiHa^R was performed following the trypan blue dye exclusion method. Accordingly, the cells were pelleted down by centrifugation at 1,500 rpm for 5 min after trypsinization. Equal volumes of cell suspension and 0.4% trypan blue stain were thereafter incubated for 1 min, followed by differential counting of the live and dead cells under a Phase Contrast Microscope (Olympus) using a hemocytometer. The results were graphically (% viable cells *vs.* treatment points) represented. The experiments were repeated thrice.

Histopathological Study

The dissected mice cervix tissues were washed in cold normal saline (0.87%), fixed in 10% neutral buffered formalin (NBF; MERCK), and processed for histology sectioning (Mahapatra et al., 2020).

Flow Cytometry and Fluorescence Microscopy

Intracellular ROS was quantitatively estimated by flow cytometry (BD FACS Calibur; BD Biosciences) followed by qualitative analysis under a fluorescent microscope. Equal densities of SiHa and SiHa^R were seeded in 55-mm plates (2.5×10^6 cells) and over coverslips in 6-well plates (2.5×10^5 cells) for flow cytometry and microscopic analysis, respectively. The plates were trypsinized and incubated with 10 μM 2′,7′-dichlorofluorescein dihydroacetate (DCFH-DA; Santa Cruz) for 45 min, followed by flow cytometric analysis in FL1-H. Scatter plots and histograms were generated in replicates using Cell Quest software. Respective coverslips from the corresponding 6-well plates were scanned for qualitative analysis of generated ROS using an FITC filter under a fluorescent microscope (Leica).

Rhodamine 123 Assay

SiHa and SiHa^R cells, seeded in densities of 2.5×10^5 cells/well in 6-well plates, were stained with 5 μg/ml of rhodamine (Rh-123) and incubated at 37°C for 30 min following PEITC and CDDP treatment. The results were generated spectrofluorimetrically (VARIAN; excitation-488 nm, emission-525 nm) and represented graphically (fold-change) for each replicate point.

Estimation of Free GSH Level

Free glutathione reductase [E.C1.8.1.7] levels were spectrophotometrically assessed as per the protocol of the glutathione assay kit (Cayman Chemical) for triplicate experimental sets.

Western Blotting

The expression status of prosurvival effectors (Akt/p-Akt, NF-κB, XIAP, and survivin) and cisplatin exporter pump (MRP2) was comparatively studied by Western blotting after standardized laboratory protocol (Mahapatra et al., 2020). The particulars of the antibodies and inhibitors used have been provided in **Supplementary Data S1**.

Semi-Quantitative Reverse Transcription PCR Analysis

Isolation of total cellular RNA was performed using TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μg of total RNA using a RetroScript kit (Ambion/Applied Biosystem) which was amplified by PCR using respective forward and reverse primer sequences (**Supplementary Data S1**). The PCR product was analyzed by electrophoresis in ethidium bromide (EtBr) containing 2% agarose gel and visualized under a gel documentation system.

Immunofluorescence

The cells were seeded (2.5×10^5 cells/well) onto coverslips placed within 6-well plates for performing immunocytochemistry. PEITC and CDDP-treated cells were immunostained with respective antibodies and fluorophore-tagged secondary antibodies as per laboratory protocol (Biswas et al., 2021).

Cytopathological Study

The smears of cervical exfoliated cells suspended in PBS were fixed with 100% ethyl alcohol and stained as per the protocol given by Mahapatra et al. (2020), followed by microscopic (ZEISS) analysis.

Systemic ROS Quantitation

Reactive oxygen species (ROS) generated due to chronic treatment with 3 MC in animal models were spectrofluorimetrically quantitated according to the protocol given by Biswas et al. (2010), which is detailed in **Supplementary Data S1**.

In Silico Studies

AutoDock Vina (Trott and Olson, 2010) was utilized in all the docking experiments with the optimized protein models as the docking target against the ligand PEITC. The detailed protocol is enclosed in **Supplementary Data S1**.

Statistical Analysis

The mean values of the PEITC, CDDP, and PEITC + CDDP points were compared by factorial analysis of variance (ANOVA). The relationship between the studied parameters was analyzed by calculating Pearson's correlation coefficient using the CORREL function of Microsoft Excel. Data were expressed as mean ± standard deviation (S.D.) A p-value < 0.0001 was considered statistically significant.

RESULTS

Elevated Expression of MRP2 in SiHa^R Facilitates Better Intracellular Uptake and Accumulation of PEITC

The finally isolated subline SiHa^R, developed by "pulse treatment" of SiHa, displayed terminal resistance to the parental IC_{30}

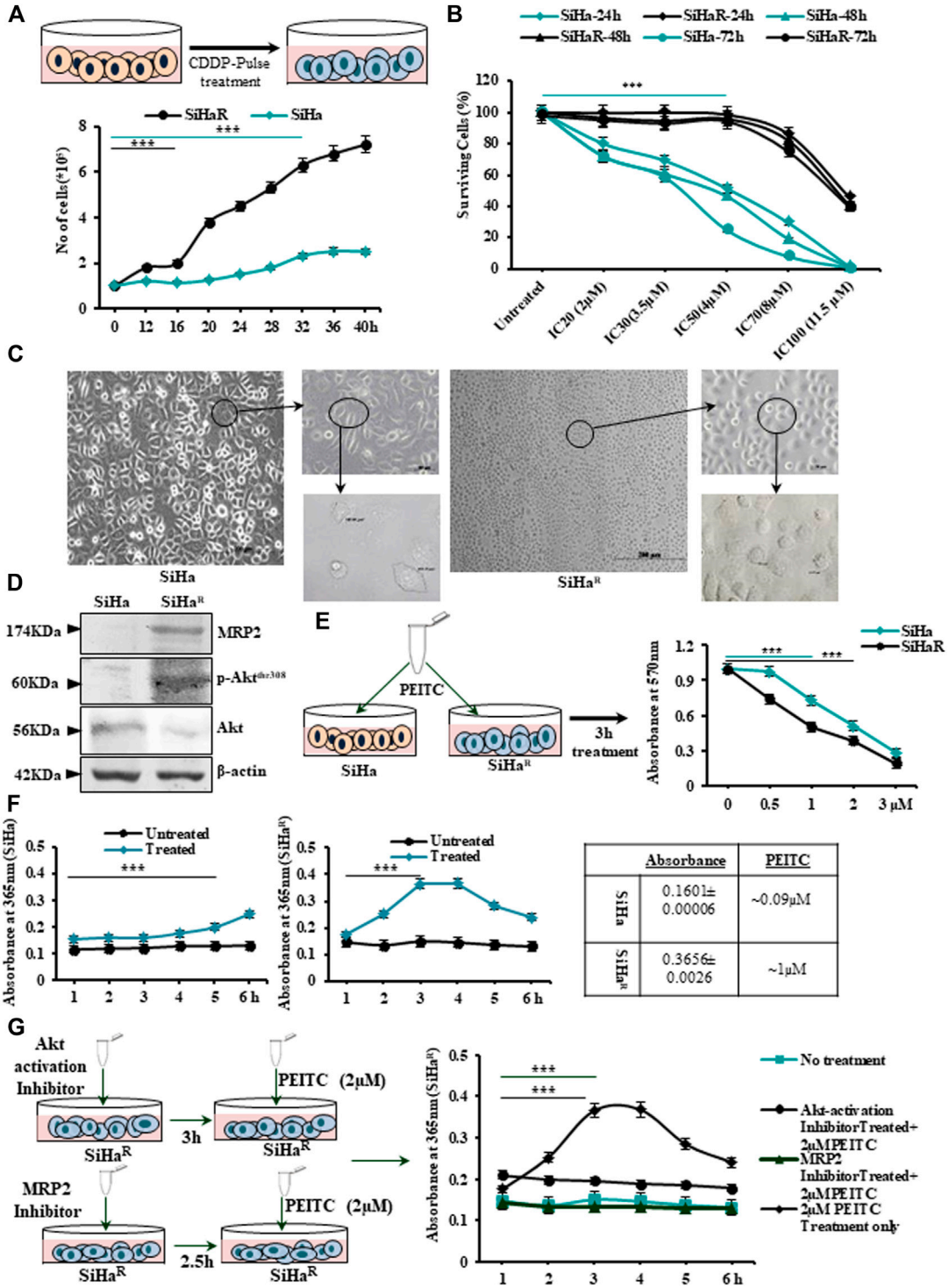


FIGURE 1 | (A) Schematic outline of the "pulse treatment" methodology followed for the development of cisplatin (CDDP)-resistant subline SiHa^R from the parental CDDP-sensitive SiHa cell line (upper panel). Representation of the cell-growth patterns depicting differences in the doubling time (SiHa-32h and SiHa^R-16 h) in a graphical format (lower panel). **(B)** Graphical anecdote of the MTT survivability assay results for SiHa and SiHa^R cells following treatment with CDDP for 24, 48, and 72 h with IC_{20} (2 μM), IC_{30} (3.5 μM), IC_{50} (4 μM), IC_{70} (8 μM), and IC_{100} (11.5 μM) CDDP doses of SiHa at 24 h. **(C)** Phase-contrast micrographic snippets of SiHa and SiHa^R representing remarkable differences in their morphology. Magnification for the main image is ×100 (Scale bar: 100 μm), while that of the subsequent insets are 200x (Scale bar: 50 μm) and ×400 (Scale bar: 20 μm). **(D)**Western blot results depicting differential expression patterns of Akt, p-Akt^{Thr308}, and MRP2 proteins in SiHa and SiHa^R. Respective band intensities as calculated by ImageJ software have been provided in **Supplementary Data S2**. **(E)** Pictorial demonstration of the experiment and the subsequent graphical representation of MTT assay results for identifying the respective IC_{50} doses of

(*Continued*)

FIGURE 1 | phenethylisothiocyanate (PEITC) in SiHa and SiHa^R cells. **(F)** Graphical description of the findings of cyclocondensation assay performed to quantitate intracellular PEITC uptake levels in SiHa and SiHa^R. **(G)** Pictorial emulation (left panel) with corresponding graphical representations (right panel) of cyclocondensation assay performed particularly with SiHa^R after treatment with Akt and MRP2 inhibitors, respectively. All experiments **(A–G)** were repeated thrice. The values have been represented as the mean of three independent determinants (Mean ± SD), where ***p represented $p < 0.0001$ compared to untreated cells.

(3.5 μM) dose of CDDP (**Figure 1A** upper panel). SiHa^R exhibited proliferative enhancement, as evident from the graph (**Figure 1A**, lower panel). The resistant subline SiHa^R doubled within 16 h, unlike SiHa, which doubled in 32 h (**Figure 1A**, lower panel). Consequently, the surviving potential of SiHa^R was validated by the MTT assay, wherein SiHa^R cells showed appreciable survival in the parental IC_{20} (2 μM), IC_{30} (3.5 μM), and IC_{50} (4 μM) CDDP concentrations for 24, 48, or even 72 h, respectively. Death of SiHa^R cells in comparison to SiHa at IC_{70} (8 μM) and IC_{100} (11.5 μM) CDDP doses was notably less (**Figure 1B**). Relatively, the calculated IC_{70} and IC_{100} doses of CDDP in SiHa turned out to be the IC_{30} and IC_{60} in case of SiHa^R, while the IC_{50} of CDDP for SiHa^R was calculated to be around 11 μM. Therefore, SiHa^R was estimated to be 2.75-fold resistant to CDDP.

Furthermore, a comparative morphological characterization of SiHa and SiHa^R by microscopic examination (**Figure 1C**) vividly delineated morphological differences among the sublines. SiHa^R had an increased nucleus-to-cytoplasmic ratio (666.81 μm^2) compared with that of SiHa (367.97 μm^2). As per Western blot results (**Figure 1D**), SiHa^R expressed remarkably higher levels of MRP2 (2.1 fold) and p-Akt^{Thr308} (1.91 fold), unlike the parental SiHa. A significantly higher ratio of p-Akt/Akt (1.7 fold) in SiHa^R cells **(Supplementary Data S2A,B)** indicated the contributory role of the upregulated PI3K/Akt pathway in the acquirement of the CDDP-resistant phenotype.

Before exploring the mechanistic role of PEITC in overcoming acquired CDDP resistance, it was important to calculate the growth inhibitory dosage of PEITC in SiHa and SiHa^R cells. The MTT assay was therefore undertaken following treatment of cells with PEITC doses ranging between 0.5 and 3 μM for 3–12 h (data not shown). Accordingly, the time-point of 3 h was selected (**Figure 1E**) in which the IC_{50} dose of PEITC was calculated to be 1 and 2 μM for SiHa^R and SiHa, respectively. This indicated that 50% killing in SiHa^R got mediated by exactly half of the PEITC IC_{50} dose of SiHa cells. This was supported by spectrophotometric results of the cyclocondensation assay, where SiHa^R was found to accumulate higher intracellular PEITC (0.3656 ± 0.0026; 1 μM) than SiHa (0.1601 ± 0.00006; 0.09 μM) for 3 and 4 h. (**Figure 1F**). Treatment extension for 5 h yielded reduced absorbance of 0.2836 ± 0.00021 (0.7 μM) in SiHa^R cells (**Figure 1F**). However, a subtle rise (0.2 ± 0.0005; 0.5 μM) in PEITC level among SiHa cells was noted after 5 h (**Figure 1F**). Apparently, SiHa^R accumulated PEITC better than SiHa.

When these experiments were repeated with SiHa^R following treatment with MRP2 and Akt inhibitors, interesting observations were attained (**Figure 1G**). Inhibition of Akt activation compromised PEITC accumulation by SiHa^R, unlike its usual nature. Surprisingly, upon MRP2 inhibition, the uptake reduced significantly (**Figure 1G**). These findings affirmed that increased PEITC uptake in SiHa^R was a result of higher MRP2 expression.

PEITC Expedited CDDP Retention and Enabled CDDP-Mediated Intracellular ROS Generation to Curb the Growth of Resistant Cervical Cancer Cells

In alignment with the spectrophotometric findings of **Figure 1F,** mass spectroscopic analysis of PEITC-treated SiHa and SiHa^R for the same time intervals was performed in order to identify the retention time of PEITC (**Figure 2A**). Respective mass-peak intensities of the cyclocondensed intracellular PEITC intermediate (1, 3-benzodithiol 2 thione) were found to be significant for SiHa^R in 3 h, which sustained till 4 h, followed by a decline. PEITC levels in SiHa could only be detected from 3 h and onward. Conclusively, the results permitted the selection of the 3 h time-point as the 'optimum treatment time' in successive experiments.

Therefore, SiHa and SiHa^R were treated with 3.5 μM of CDDP following a 3 h pretreatment with their respective IC_{30} PEITC doses (SiHa: 1μM; SiHa^R: 0.5 μM) for exploring the association between their PEITC accumulation and CDDP-retention capacities (**Figure 2B**). Intracellular platinum levels, as quantified by flameless atomic absorption spectroscopy (**Figure 2C**), revealed an improved and increasing trend in the drug retention capacities of PEITC-enriched SiHa^R cells with respect to PEITC-deficient SiHa for 24 h. In addition, PEITC pretreatment could also efficiently restrain SiHa^R growth in even higher CDDP doses, wherein it would normally grow in the absence of the phytochemical (**Figure 2D**). As evident from the graphical anecdote (**Figure 2D**), 50% of PEITC pretreated SiHa^R got killed by only 2 μM of CDDP, while the same for SiHa cells was achievable with a higher CDDP concentration of about 3.7 μM. These results highlighted the chemosensitizing potentials of PEITC.

Mechanistic insights of the chemoenhancing potentials of PEITC were further explored by checking the cell-killing ability of the retained CDDP by generating ROS *via* disruption of mitochondrial membrane potential followed by depletion of free-GSH levels. Depictions of flow cytometry results (**Figure 2E**, left panel) portrayed a clear peak shift for DCF generation in CDDP-treated SiHa^R cells in the presence of PEITC pretreatment as compared to SiHa. The frequency of DCF-bearing SiHa^R cells was greater than that of SiHa for combinatorial treatment modalities (**Figure 2E**, right panels). In addition, the findings were strengthened by the corroborating fluorescent microscopic results representing the ROS content of SiHa and SiHa^R cells (**Figure 2F**). In fact, the relative mitochondrial-membrane potential was found to be

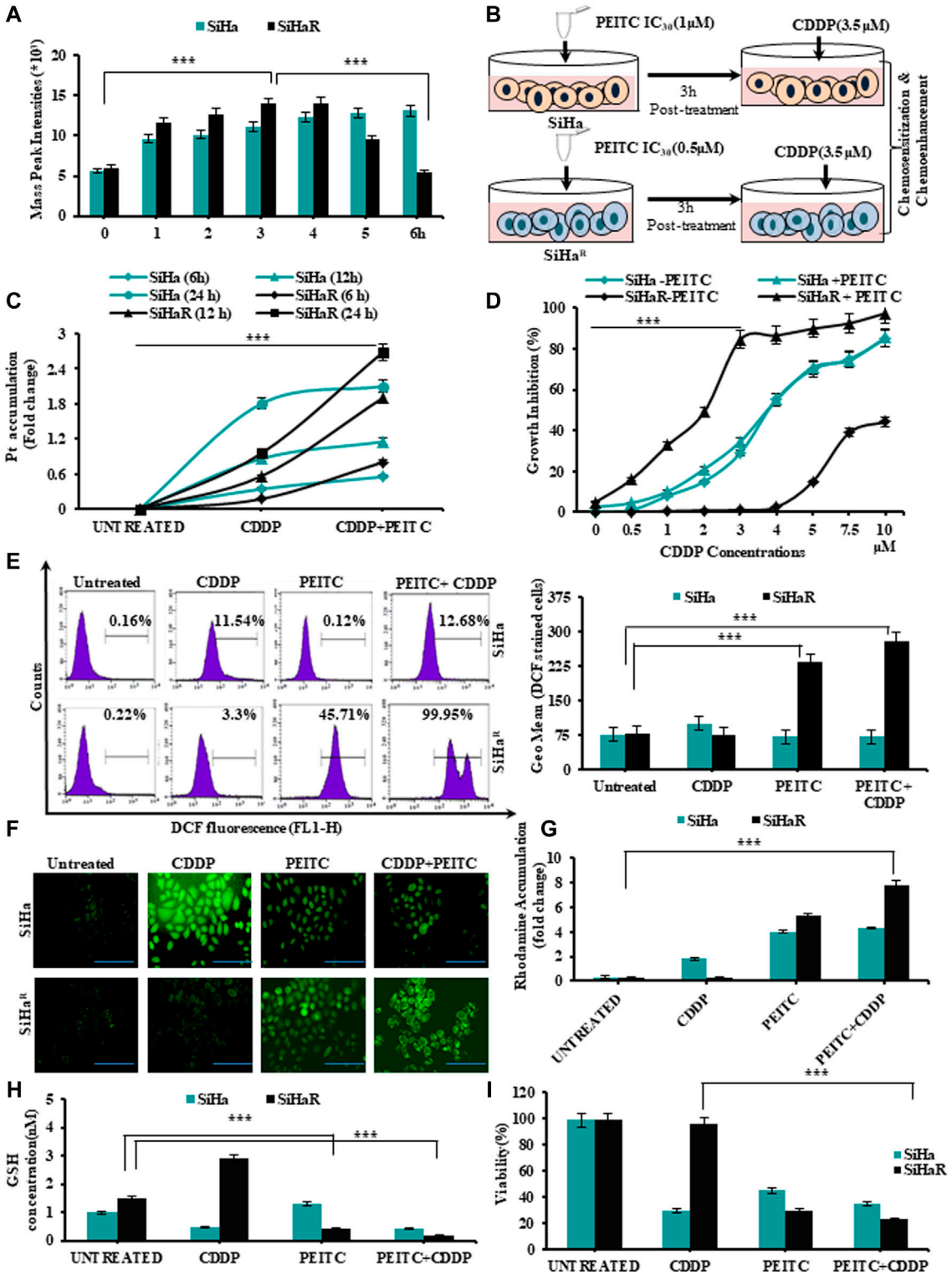


FIGURE 2 | (A) Mass spectroscopic analysis to identify the optimum time point of PEITC uptake and retention in CDDP-sensitive and -resistant cells. **(B)** Schematic outline of the entire treatment design followed for validating the effects of PEITC as a cisplatin chemosensitizer and chemoenhancer. **(C)**Time-dependent intracellular accumulation of CDDP in SiHa and SiHa^R cells upon exposure to 3.5 μM of the drug following PEITC (IC_{30} dose) pre-treatment for 3 h. **(D)** Comparative growth inhibition of SiHa and SiHa^R in a varied range of CDDP doses for 24 h in absence and presence of PEITC pretreatment for ascertaining optimum combinatorial dose. **(E)** Histogram plots displaying FL1 peak shifts due to ROS generation represented by DCFHDA stained cells (SiHa^R and SiHa) subjected to treatment with PEITC and CDDP

(*Continued*)

FIGURE 2 | either in a pretreatment mode or solely (left panel). The geometric mean (Geo.Mean) values of these DCFHDA stained cells were plotted graphically (right panel). **(F)** Relative fluorescent microscopic images showing ROS content of $SiHa^R$ and SiHa cells treated either with PEITC or CDDP or both (Magnification: ×400; Scale bar: 20 μm). **(G)** Graphical representations of the spectrofluorimetric findings corresponding to mitochondrial membrane potential status of $SiHa^R$ and SiHa with reference to rhodamine 123 dye accumulation. **(H)** Kit-based spectrophotometric quantification of free GSH levels in $SiHa^R$ and SiHa. Absorbance values were recorded for five kinetic cycles at an interval of 1 min at 340 nm. **(I)** Relative trends of cellular viability (%) as determined by the trypan blue dye exclusion method. All the experiments **(A–I)** were performed in triplicate, and the values were expressed as mean ± SD; ***$p < 0.0001$ with respect to untreated cells.

highly disrupted in the rhodamine 123 staining assay (**Figure 2G**) because $SiHa^R$ cells accumulated rhodamine 16.7 times more than SiHa in a combination treatment setup. Interestingly, the free-GSH levels of $SiHa^R$ were reduced by 0.39 folds upon CDDP treatment only in the case of prior PEITC priming, unlike SiHa, which showed no noteworthy alterations (**Figure 2H**). Finally, the trypan blue dye exclusion methodology revealed a significant ROS-mediated reduction in $SiHa^R$ viability to 66% ($p < 0.0001$) from 98.5% upon PEITC treatment ahead of CDDP treatment (**Figure 2I**). All these results also entitled PEITC as a CDDP chemoenhancer in $SiHa^R$ cells.

PEITC Increased the Efficacy of CDDP by Negatively Regulating Prosurvival Markers and Drug Exporter MRP2

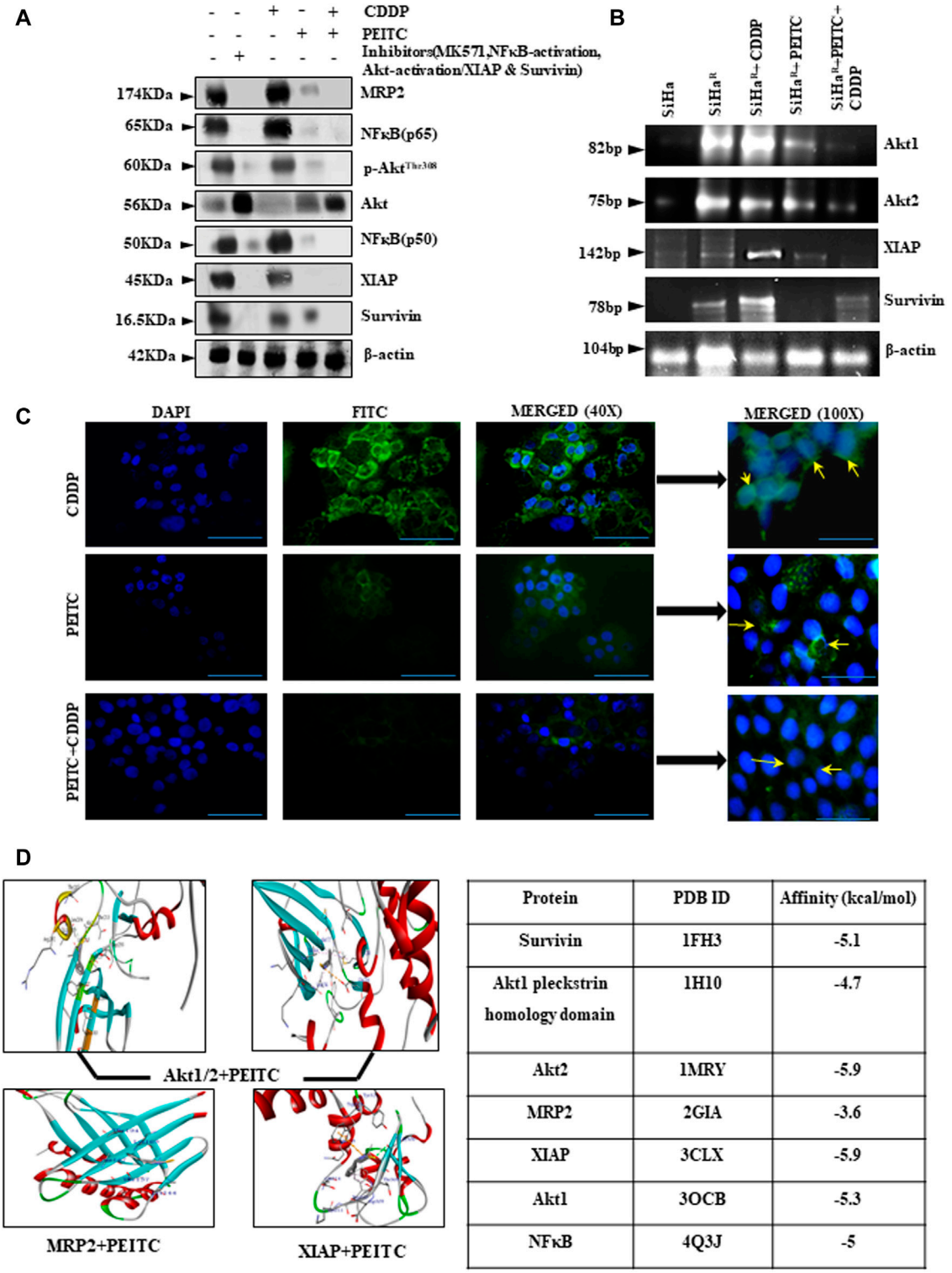
In an effort to investigate the regulatory effect of PEITC over deregulated proteins (Akt, NF-κB, MRP2, XIAP, and survivin) for chemoenhancing CDDP, the $SiHa^R$ cells were treated with the respective pharmacological inhibitors alongside differential treatment with either CDDP or PEITC or both. The proteins isolated thereafter were comparatively studied for deciphering the inhibitory role of PEITC. Western blot results (**Figure 3A**) depicted remarkable decrease in the expression patterns of $pAkt^{(Thr308)}$, total-Akt, XIAP, survivin, NF-κB (p65), NF-κB (p50), and MRP2 in combinatorial treatment modalities of PEITC and CDDP in comparison to single-agent treatments and untreated $SiHa^R$. Respective band intensities (**Supplementary Data S4**) also revealed that the results were comparable with pharmacological inhibition of the respective molecular markers (**Figure 3A**). This inhibition was not delimited at protein levels of these cells as the RT-PCR blots (**Figure 3B**) suggested a depleted expression of the relative mRNAs. Moreover, PEITC was observed to directly affect the subcellular localization of MRP2 in $SiHa^R$. As portrayed in immunofluorescence micrographs (**Figure 3C**), PEITC restricted MRP2 localization in the nuclear periphery of $SiHa^R$ cells which was originally found in their membrane in the presence of sole CDDP treatment. On the contrary, neither the cell membranes nor the nuclear periphery harbor any MRP2 in $SiHa^R$ when subjected to CDDP after PEITC pretreatment (**Figure 3C**).

PEITC was screened for the top-ranked poses based upon the docking score and non-bonded contact potential with the target protein conformations. Docking results (**Figure 3D**) delineated the highest affinity between PEITC and Akt2 (-5.9 kcal/mol) and XIAP (-5.9 kcal/mol). Specifically, the Thr213 residue of Akt2 formed a hydrogen bond, while its Leu204 formed pi–pi electrostatic interaction with PEITC. XIAP exhibited one hydrogen bond (Gly306), three pi–pi electrostatic, and two electrostatic interactions with Gln319 and Trp323 residues. An overall strong affinity was also observed between NF-κB and PEITC (-5 kcal/mol). Detailed portrayal of considerable interactions between PEITC and other molecules also supported the notion (**Supplementary Data S5**).

PEITC Successfully Restricted Tumor Growth in an *In Vivo* Model by Augmenting CDDP Responses

The chemoenhancing effect of PEITC in an *in vivo* invasive cervical cancer model was explored by intraperitoneal administration of CDDP and PEITC either in combination or singularly (**Figure 4A**). Pictorial and graphical representations showed that combination treatment efficiently regressed tumor size (**Figure 4B,** upper panel) and subsequently decreased its weight (**Figure 4B,** lower panel) among the treatment groups. Nevertheless, sole treatment with 3 mg/kg body weight of CDDP failed to reduce the tumor size as the tumor weight remained unaltered, reflecting the acquirement of potential drug-insensitive nature. The results corroborated with the harmonizing patterns in their relative body weight fluctuations (**Figure 4C**).

These findings prompted an investigation of the protein-expression status of MRP2. For this purpose, the tumor cortex, stroma, and cortico-medullary regions were differentially studied for the expression profiles of MRP2, both with and without PEITC interventions during CDDP chemocycles of two weeks. Interestingly, MRP2 levels were characteristically high in all the tumor regions (**Figure 4D**). However, 2.5 mg/kg body weight of PEITC decreased these increasing expression patterns of MRP2, which went unchecked in the presence of sole CDDP intervention (**Figure 4D**). This observation verified the *in vitro* findings which claimed that effective PEITC function was an outcome of MRP2 upregulation. Elevations in systemic ROS levels were noted in these combinatorial treatment groups, indicating direct control of PEITC administration over CDDP-mediated free-radical generation (**Figure 4E**). Histopathology (**Figure 4F**) and cytopathology (**Figure 4G**) of these groups supported the findings as administration of PEITC in individual and in combination with CDDP was found to restrict the invasive basal cells from intruding the stromal region of the cervical epithelium, besides checking their extensive keratinization (**Figure 4G**).



Protein	PDB ID	Affinity (kcal/mol)
Survivin	1FH3	-5.1
Akt1 pleckstrin homology domain	1H10	-4.7
Akt2	1MRY	-5.9
MRP2	2GIA	-3.6
XIAP	3CLX	-5.9
Akt1	3OCB	-5.3
NFκB	4Q3J	-5

FIGURE 3 | (A) Relative protein expressions of Akt/p-Akt^{Thr308}, NF-κB (p50/p65), MRP2, XIAP, and survivin by Western blotting. β-actin was used as a loading control. Respective band intensities of these proteins have been represented graphically in **Supplementary Data S4**. **(B)** Relative effects of PEITC (pre-treatment) along with CDDP upon Akt and IAP mRNAs as represented in the RT-PCR blots. Lane # 1: SiHa, Lane # 2: SiHa^R, Lane# 3: SiHa^R + CDDP (3.5 μM), Lane # 4: SiHa^R + PEITC (0.5 μM), Lane # 5: SiHa^R + PEITC (0.5 μM) + CDDP (3.5 μM) **(C)** Spatiotemporal distribution of MRP2 upon similar treatment conditions in SiHa^R and SiHa cells as displayed by indirect immunofluorescence data. Main images are magnified up to ×400, while the insets displayed alongside are magnified up to 1,000x. Scale bar of each field measures to about 50 μm (main images) and 20 μm (side-insets). About 50 fields were scanned under the microscope for data acquisition. **(D)** AutoDock Vina results highlighting PEITC interaction with Akt, MRP2, and survivin in the best poses. Scores of all the possible interactions have been incorporated in tabular format. Images displaying PEITC interactions with other prosurvival molecules have been provided in **Supplementary Data S5**. All experiments were performed in triplicate.

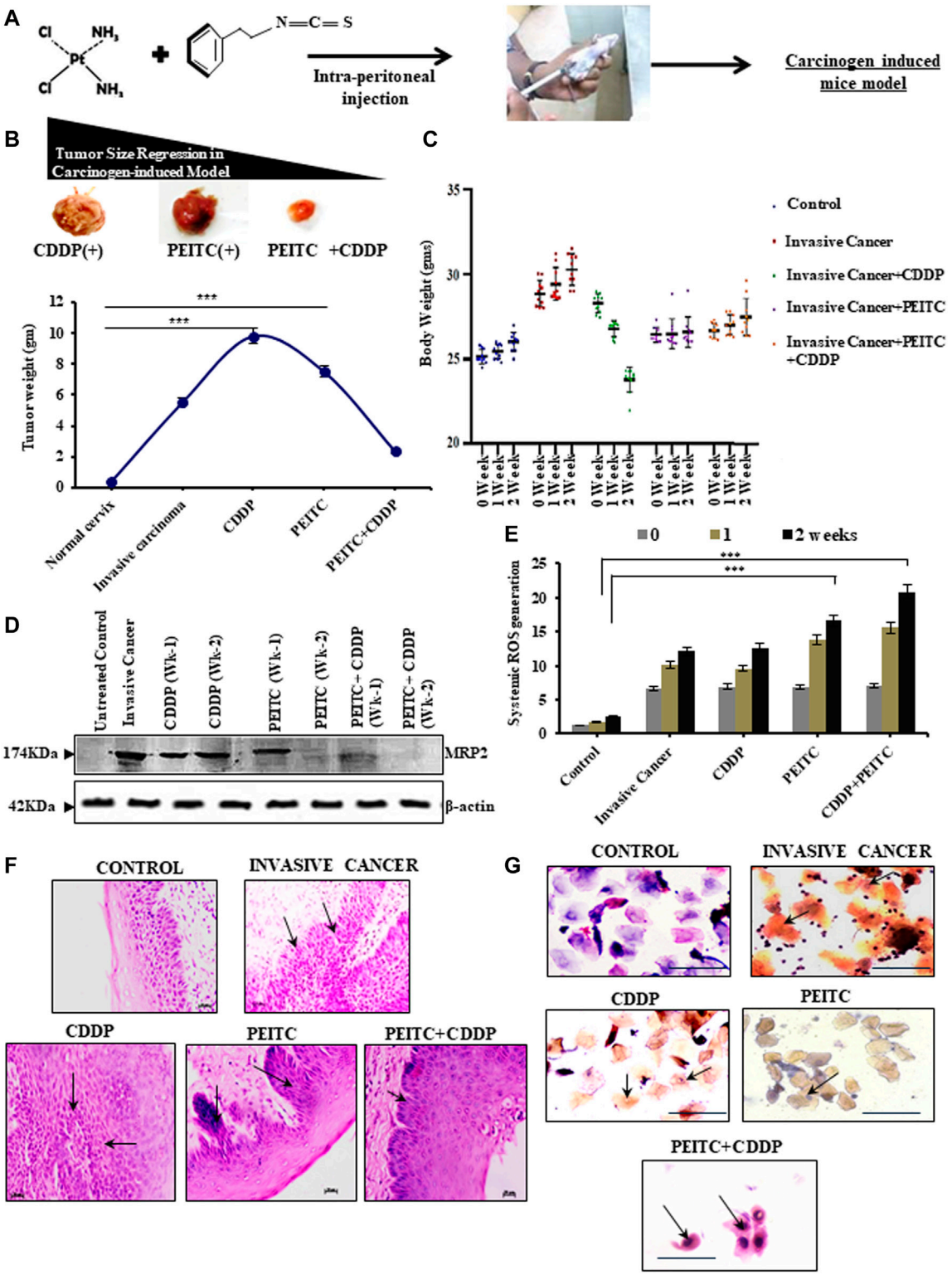


FIGURE 4 | (A) Clear outline of the treatment protocol observed for animal experimentations in a carcinogen-induced cervical cancer model. **(B)** Pictorial (upper panel) and corroborative graphical descriptions (lower panel) of tumor size regression among invasive cancer-bearing mice after treatment with CDDP or PEITC or CDDP + PEITC. **(C)** Trends in body-weight alterations in mice upon interventions with PEITC (5 mg/kg body weight) and CDDP (3 mg/kg body weight) either in combination or solely with respect to untreated healthy control and non-intervened invasive cancer-bearing mice. **(D)** Western blot results depicting the modulatory effect of PEITC upon the expression profiles of MRP2 proteins in the carcinogen-induced cervical tumor microenvironment in the isolated or combination mode. **(E)** Graphical

(*Continued*)

FIGURE 4 | representation of the systemic ROS levels upon PEITC and CDDP. **(F)** Histopathological (left panel; Magnification: ×400; Scale bar: 20 μm) and **(G)** cytopathological changes (right panel; Magnification: ×400; Scale bar: 20 μm) represented by hematoxylin–eosin and PAP staining, respectively, displaying the effect of the treatment regimen. Marked areas (arrow indicated) in histology demonstrate alterations in epithelial growth within the stroma, while cellular keratinization and nuclear–cytoplasmic ratio variations within cervical exfoliated cells in cytology have been delineated (arrow indicated). Numerical results were displayed as mean ± SD; ***$p < 0.0001$ with respect to untreated. All experiments **(B–G)** were performed in triplicate.

DISCUSSION

Resolving the problem of acquired CDDP resistance by finding specific chemosensitizers is the utmost need of the hour. In this regard, natural isothiocyanates can be considered better options due to the exploitation of drug exporters for cellular entry. Paramount reports suggested that efflux pumps (Pgp, MRP1/2 and BCRP) promote the intracellular import of isothiocyanates (Telang et al., 2009; Mi et al., 2011; Morris and Dave, 2014). PEITC, in this study, was found to chemosensitize MRP2 overexpressing CDDP-resistant $SiHa^R$ cells, which also promoted its intracellular uptake and retention. Hence, PEITC in this study was identified as a better cell growth inhibitor for $SiHa^R$ than SiHa. Previous experimental evidences have firmly established PEITC as an anticancer, chemopreventive (Chen et al., 2012; Ioannides and Konsue, 2015; Suvarna et al., 2017), and chemosensitizing agent (Yang et al., 2014; Souvick et al., 2020). Correlatively, $SiHa^R$ cells, which grew prolifically in 3.5 μM of CDDP owing to acquired CDDP resistance, had surprisingly ceased to grow in the same and higher drug doses following PEITC pretreatment for 3 h. This permitted negation of relative toxicity of CDDP, which often impedes therapy. This pre-treatment improved drug-retention capacities of $SiHa^R$ and thereafter delivered adequate growth inhibition. However, PEITC-primed SiHa cells exhibited no enhancement in their platinum levels, and their viability remained unaffected. This corroborated with the preferential PEITC accumulation in $SiHa^R$ over SiHa cells. Chemosensitization is just not enough for the reversal of acquired chemoresistance among aggressive cancers as their deregulated molecular signaling conveniently deteriorates the chemotherapeutic pharmacodynamicity. ROS overproduction in $SiHa^R$ cells upon PEITC treatment (sole/combinatorial) supported the abovementioned viewpoints and aligned with pre-existing reports (Hong et al., 2015). The present study recorded high free-GSH levels in CDDP-treated $SiHa^R$ cells which went down manifold when 3 h of PEITC pre-treatment was ensued. This abided with reports suggesting the prevalence of inverse correlation between increased cellular GSH levels and CDDP-accumulation (Okazaki et al., 2019; Liu et al., 2021) as the drug also attacks GSH for allowing cellular cytotoxic death by free-radical outburst (Achkar et al., 2018). Among SiHa cells, tracer amounts of PEITC could not mediate pro-oxidant functions neither in the presence nor in the absence of CDDP. This necessitated the importance of "exposure-time" and "exposure-dose" of PEITC in relaying antioxidant or pro-oxidant functions in a cell-specific manner. Mitochondrial membrane potential disruption furthermore confirmed that PEITC acted as a CDDP chemoenhancer in $SiHa^R$ cells.

Considering the pioneering role of the PI3K/Akt signaling cascade in orchestrating the scenario of acquired CDDP resistance in cervical cancer, it was intended to concentrate upon the ways in which PEITC modulated this cascade for altering MRP2 distribution in $SiHa^R$ particularly. It was observed that the phosphorylation status of Akt had remarkably reduced in $SiHa^R$ with PEITC followed by CDDP treatment. Expression profiles (protein/mRNA) of downstream effectors of the signaling cascades aligned with those of p-Akt. The expression profiles of NF-κB (p50/p65) decreased, resulting in cumulative inhibition of XIAP, survivin, and MRP2. PI3K/Akt is reported to ubiquitously modulate the multidrug-resistant phenotype in cancer (Zhang et al., 2020; Liu et al., 2021). Therefore, shutting down the activity of the upstream effectors would concoct the acquired resistant phenotype of cancer cells. Accordingly, the spatiotemporal distribution of MRP2 proteins was apparently disturbed. PEITC pretreatment for 3 h among $SiHa^R$ cells considerably reduced MRP2 accumulation in the membrane even upon CDDP treatment. This unveiled the root cause of increasing CDDP levels within the resistant cells upon PEITC pretreatment. A milieu of reference studies also reported that apparently, PEITC reduces the expression of drug exporters to promote the reversal of acquired chemoresistance (Morris and Dave, 2014; Suvarna et al., 2017). *In silico* observations pinpointing at specific interactions of PEITC with Akt, XIAP, and MRP2 proteins further established the role of PEITC as a chemosensitizer.

Upon validation in a 3 MC induced *in vivo* cervical cancer model, reiterations of similar results were attained. The mice group when treated with CDDP alone did not restrict the tumor growth, as evident from histopathological study and tumor images. However, prior PEITC administration alongside CDDP injection controlled tumor growth and improved the relative histology and cytology by permissibly surging systemic-ROS levels. These aggregated evidences were enough to confirm PEITC as a CDDP sensitizer in cisplatin-resistant cervical cancer. Although, detailed insight into PEITC-mediated cisplatin sensitization in *in vivo* set-up is mandatory. The present study has laid down a foundation for the candidature of PEITC as a cisplatin sensitizer and enhancer in Phase I clinical trial.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Ethics Committee, certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

AUTHOR CONTRIBUTIONS

EM predominantly acquired all the data, analyzed them to design the concept for this article, and composed the manuscript with extensive assistance from DS and SD, who helped analyze the data of some experiments. RK and BD performed molecular docking studies. SM conceptualized and designed the project work, analyzed results, and critically reviewed the manuscript. MR provided scientific inputs and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.803114/full#supplementary-material

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REVIEW
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Role of microRNA/lncRNA Intertwined With the Wnt/β-Catenin Axis in Regulating the Pathogenesis of Triple-Negative Breast Cancer

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Objective (s): In this mini-review, we aimed to discuss the Wnt/β-catenin signaling pathway modulation in triple-negative breast cancer, particularly the contribution of lncRNAs and miRNAs in its regulation and their possible entwining role in breast cancer pathogenesis, proliferation, migration, or malignancy.

Background: Malignant tumor formation is very high for breast cancer in women and is a leading cause of death all over the globe. Among breast cancer subtypes, triple-negative breast cancer is rife in premenopausal women, most invasive, and prone to metastasis. Complex pathways are involved in this cancer's pathogenesis, advancement, and malignancy, including the Wnt/β-catenin signaling pathway. This pathway is conserved among vertebrates and is necessary for sustaining cell homeostasis. It is regulated by several elements such as transcription factors, enhancers, non-coding RNAs (lncRNAs and miRNAs), etc.

Methods: We evaluated lncRNAs and miRNAs differentially expressed in triple-negative breast cancer (TNBC) from the cDNA microarray data set literature survey. Using *in silico* analyses combined with a review of the current literature, we anticipated identifying lncRNAs and miRNAs that might modulate the Wnt/β-catenin signaling pathway.

Result: The miRNAs and lncRNAs specific to triple-negative breast cancer have been identified based on literature and database searches. Tumorigenesis, metastasis, and EMT were all given special attention. Apart from cross-talk being essential for TNBC tumorigenesis and treatment outcomes, our results indicated eight upregulated and seven downregulated miRNAs and 19 upregulated and three downregulated lncRNAs that can be used as predictive or diagnostic markers. This consolidated information could be useful in the clinic and provide a combined literature resource for TNBC researchers working on the Wnt/β-catenin miRNA/lncRNA axis.

Conclusion: In conclusion, because the Wnt pathway and miRNAs/lncRNAs can modulate TNBC, their intertwinement results in a cascade of complex reactions that affect TNBC and related processes. Their function in TNBC pathogenesis has been highlighted in molecular processes underlying the disease progression.

Keywords: breast cancer, lncRNA, microRNA, wnt/β-catenin, pathogenesis

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INTRODUCTION

Breast cancer represents the most common type of cancer worldwide, with high morbidity and mortality rates, (Agustsson et al., 2020). Breast cancer shows high heterogeneity, which impacts the clinical course of the disease. Differential expression profiles among patients lead to tumor tissue heterogeneity, resulting in variations in malignant behavior, prognosis, and responsiveness to standard therapies (Padmanaban et al., 2019). This cancer is prevalent in women and is the first leading cause of cancerous death in women. Breast cancer has been progressively increasing in most nations (Ferlay et al., 2015). According to WHO data (https://www.who.int/news-room/fact-sheets/detail/breast-cancer), 2.3 million women were diagnosed with breast cancer in 2020, with about 685,000 fatalities. This year, in the United States alone, around 281,550 cases of invasive and 49,290 cases of non-invasive breast cancer cases were diagnosed in women, according to the American Cancer Society. Most deaths occur due to metastasis instead of the primary tumor in breast cancer (Geng et al., 2014). Metastasis is linked with stem cells, which have characteristics such as self-renewal, differentiation ability, drug resistance, etc. (Phi et al., 2018). These properties favor aggressive behavior in breast cancer stem cells, leading to recurrent and aggressive tumors on and away from the primary site. Upregulation of Wnt/β-catenin signaling has been observed in triple-negative breast cancer (TNBC)/basal-like cancer when collated with other breast cancer subtypes (luminal A, B, and HER2 positive) or normal tissues (Gangrade et al., 2018).

TNBC can be classified into at least six distinct subtypes with differences in clinical behavior and treatment response. TNBC is highly invasive and, due to the non-expression of estrogen receptor, progesterone receptor, or HER-2, it has poor prognosis, high metastatic potential, and is disposed to relapse (Yin et al., 2020). TNBCs are more common in younger and obese women, with premenopausal African American women having the highest prevalence. BRCA1 and BRCA2 gene mutations are identified in approximately 20% of TNBC patients. P53 and Rb1 mutations are also quite common in TNBC tumors (van Barele et al., 2021). Differentially expressed ncRNAs have been found in a variety of human cancers, including breast cancer. Several lncRNA molecules have been linked to tumorigenesis, and their differential expression could constitute a potential new category of biomarkers. The lncRNA HOTAIR (HOX transcript antisense intergenic RNA) was associated with the polycomb repressive complex 2 (PRC2) and the histone demethylation enzyme LSD1 (lysine-specific demethylase 1) in cancer cells, resulting in epigenetic changes that promote tumor development and metastasis (Rodrigues de Bastos and Nagai, 2020). Other circulating lncRNAs, including MALAT1, GAS5, H19, and MEG3, have also been linked to survival and treatment response. LncRNAs have opened up a new field of study for researchers all over the world, and these molecules have been assigned major roles that may have a direct impact on patient survival and therapeutic responsiveness (Gupta et al., 2010). The SPARC gene (secreted protein acidic and rich in cysteine, also known as osteonectin or basement-membrane protein 40) encodes a 32-kDa matricellular glycoprotein involved in a variety of biological activities, including differentiation, proliferation, migration, and adhesion (Zhang et al., 2019).

The canonical Wnt/β-catenin signaling pathway contributes to instigation (**Figure 1**), differentiation, and proliferation of TNBC cells (Zhang et al., 2018), leading to primary tumor formation (Xu et al., 2015), cellular transition of the epithelial-to-mesenchymal (EMT) state, and metastasis (Pohl et al., 2017). Chemoresistance has also been linked with Wnt/β-catenin signaling, with impaired pathways leading to drug-resistant TNBC (Merikhian et al., 2021). This is due to the synergistic contact between the Wnt target gene c-MYC and HIF-1α. This dual gene interaction diminishes cancer cell response to the given drugs. However, knockdown of β-catenin has been reported to cause TNBC cells to respond to doxorubicin or cisplatin (Xu et al., 2015).

This is why the study of this pathway is essential in TNBC, and a comprehensive updated literature review was undertaken to integrate information regarding the Wnt/β-catenin signaling pathway and TNBC.

WNT/Β-CATENIN SIGNALING IN TNBC

The Wnt pathway is tangled with low-density lipoprotein receptor-related protein5/6 (LRP5/6) and frizzled (FZD) receptors for its activation (King et al., 2012). If Wnts are insufficient or non-functional, β-catenin pools with a tetrad of

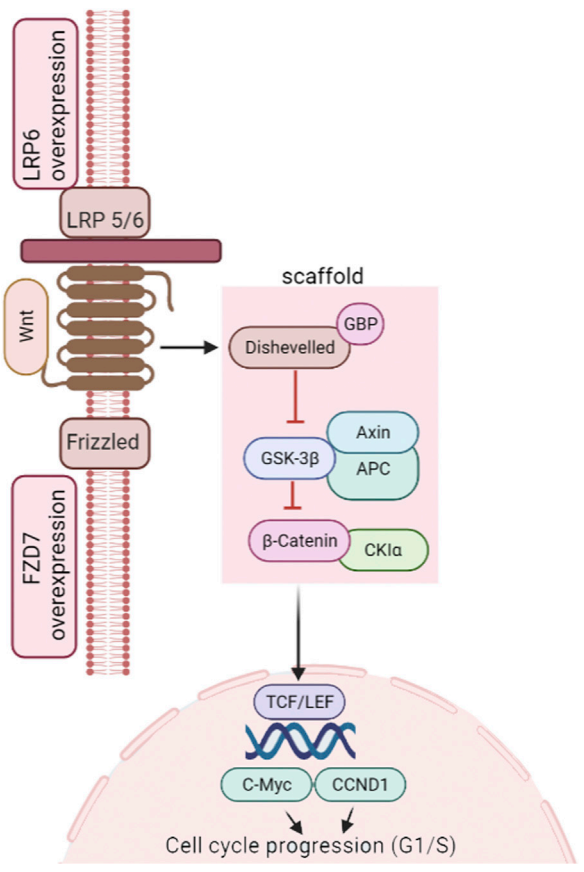

FIGURE 1 | Wnt/β-catenin pathway in TNBC instigation. Figure adapted from the KEGG pathway (https://www.genome.jp/pathway/map05224).

proteins (axin, casein kinase 1 (CK1), adenomatous polyposis *coli* (APC) tumor suppressor, and glycogen synthase kinase-3b (GSK3b). Phosphorylation (by CK1 and GSK3b) is followed by ubiquitination and ultimate degradation of β-catenin (26S proteasome). Conversely, in the presence of the Wnt signal, β-catenin attaches to FZD and LRP5/6 receptors, leading to inhibition of GSK3b and stabilization of cytosolic β-catenin. This β-catenin is then shifted to the nucleus. It associates with T-cell factor/lymphoid-enhancing factor (TCF/LEF) to incite the downstream expression of cell development and cell cycle control genes (MacDonald et al., 2009). Secreted proteins regulate this signaling at the cell surface, where the central modulators are Wnt and R-spondin (Rspo). Inhibitors include Wnt Inhibitory Factor 1 (WIF1), Dickkopf (Dkk), soluble Frizzled-related protein (sFRP), and sclerostin (SOST) (Yao et al., 2011; Danieau et al., 2019). Abnormal Wnt signaling has been implicated in TNBC tumorigenesis (Xu et al., 2015; Mohammadi Yeganeh et al., 2017), stemness, metastasis, and prognosis (Ryu et al., 2020). Dey et al. (2013) identified that patients with dysregulated Wnt/β-catenin signaling had a higher chance of lung and brain metastases. Dysregulation of the canonical pathway is responsible for metastasis in more than half of breast cancer patients as the nucleolar β-catenin level is elevated. However, mutations in the relevant genes are uncommon (Yu et al., 2019). It implies that the role of the β-catenin is indispensable to the Wnt signaling in TNBC advancement, with the dissemination of non-phosphorylated cytoplasmic β-catenin to the nucleus having an imperative role in TNBC metastasis (Breuer et al., 2019; Satriyo et al., 2019).

Green et al. (2013) discovered that Wnt ligands caused enhanced transcription in the majority of TNBC cell lines, while Xu et al. (2015) reported that nuclear accumulation of β-catenin is linked with TNBC characteristics. The role of the key modulators of this pathway in TNBC includes the action of FZD receptors, LRP5/6, Receptor Tyrosine Kinase–Like Orphan Receptors (RORs), and Dead Box Proteins (DDX3 and DDX5) (Pohl et al., 2017). LRP5/6 is crucial in mammary development and is allied with tumorigenesis (Goel et al., 2012). Overexpression of LRP5/6 has been seen in TNBC (Maubant et al., 2018). LRP6 has also been linked with migration and invasion of cells (Ma et al., 2017). Elevated FZD7 expression in TNBC also promotes tumorigenesis (Yang et al., 2011) *via* transformation-related protein 63 (p63) (Chakrabarti et al., 2014), while FZD8-driven Wnt signaling mediated by c-Myc overexpression drives chemoresistance (Yin et al., 2013). Dead box proteins DDX3 and DDX5 have shown increased EMT in TNBC. DDX3 is associated with increased motility and invasiveness. In comparison, DDX5 is linked with tumorigenesis and cancer cell progression (Moore et al., 2010; Wang et al., 2012; Guturi et al., 2014; Pohl et al., 2017).

Wnt/β-Catenin Signaling in TNBC Stem Cells

Cancer stem cells (CSCs) or cancer stem-like cells within the tumor, being less responsive to environmental stop signals, are responsible for cancer progression, metastasis, chemoresistance, and hence, cancer relapse (Clarke, 2019). They differ from other cancer cells as they use mitochondrial reactive oxygen species (ROS) for respiration, which means higher oxygen consumption, increased mitochondrial mass, and high resistance to DNA damage (Peiris-Pagès et al., 2018; Scatena et al., 2018). Abnormal Wnt/β-catenin signaling is linked with CSC formation and, hence, tumorigenesis, stemness, migration, and chemoresistance in TNBC (Alraouji et al., 2020). Upregulation of β-catenin triggers the CSC phenotype of TNBC, and repression by cadherin leads to suppression of this phenotype (Satriyo et al., 2019). Jang et al. (2015) observed that Wnt/β-catenin pathway genes (WNT1, FZD1, TCF4, and LEF1) are upregulated in CSC-enriched mammospheres in breast cancer, while signaling proteins (LEF1, TCF4, and β-catenin) were increased in high CSC activity, depicting cell fraction, compared to that with low CSC activity. However, recently, Brilliant et al. (2019) reported 11% of Wnt signaling expression in high vs. 33% in the low content of cancer stem cells. In contrast, some researchers have reported inhibiting TNBC CSCs by drugs targeting the Wnt/β-catenin pathway (like hydroxytyrosol) (Cruz-Lozano et al., 2019). Others have reported that β-catenin is also responsible for drug resistance (e.g., doxorubicin resistance) in TNBC CSCs (Xu et al., 2015).

lncRNA and miRNAs Entangled With the Wnt/β-Catenin Pathway in TNBC

According to recent research, more than 90% of the transcripts in the human genome may not be able to code for proteins (Wilusz et al., 2009) but regulate the expression of nearby genes (He and Hannon, 2004; Trzybulska et al., 2018). They are categorized according to their size and function (Trzybulska et al., 2018), with microRNAs (miRNAs) being 19–24 nucleotides long and long non-coding RNAs (lncRNAs) being >200 nucleotides in length. There are other non-coding RNAs such as piwiRNAs, free circulating RNAs, and snoRNAs, but our focus will be on miRNAs and lncRNAs in this review. Long non-coding RNAs are abundant in human cells and play critical roles in a range of biological processes, including cell cycle regulation (Lu et al., 2016), genomic expression (Ballantyne et al., 2016), and cell differentiation (Chen et al., 2016). Increasing evidence has recently indicated that abnormal lncRNA expression is linked with various tumor forms (Mendell, 2016). Researchers have proved that miRNA dysregulation also leads to human cancer *via* different mechanisms (Karimzadeh et al., 2021), including altered epigenetics (Arif et al., 2020) and abnormal transcriptional control (Müller et al., 2019; Ali Syeda et al., 2020). Apart from performing as oncogenes, where they support proliferative signaling (Miao et al., 2017), invasion (Chen et al., 2017), repelling cell death, eluding progression suppressors, and metastasis, they act as tumor suppressors too (Hong et al., 2019). This has led to their demarcation as potential biomarkers of cancer (Gai et al., 2018; Dai et al., 2019).

miRNA expression in BCSCs and cancer cells signals that they are crucial for promoting characteristics such as stemness and tumorigenesis. Piasecka et al. (2018) reviewed 121 articles demonstrating the role of miRNAs in TNBC. After scanning a

TABLE 1 | miRNAs impacting TNBC *via* the Wnt/β-Catenin axis.

S. No.	MiRNA	Upregulation or downregulation	Cell line	References
1	miR-142	Upregulated	HEK293T, MCF7, and MDA-MB-231	Isobe et al. (2014)
2	miR221/ 222	Upregulated	MDA-MB-231, MCF7, MDA-MB-468, Hs 578T, HCC1937, and MDA-MB-231, SKBR3, T47D, BT-474, 4T1	Liu et al. (2018)
3	miR-124	Upregulated	BT20, HCC70, 293T	Yang et al. (2020)
4	miR-125b	Upregulated	MDA-MB-468, MDA-MB-231, MCF-10A, and MCF-7	Nie et al. (2019)
5	miR-137	Downregulated	HCC38, MDA-MB-231, and MDA-MB-468	Cheng et al. (2019)
6	miR-29b-1	Downregulated	MB-231, MDA-MB-468, BT20, and HCC-1395	Drago-Ferrante et al. (2017)
7	miR-105	Upregulated	MB-361, MCF-7, BT-483, AU565, SkBR3, MCF-10A, MB-231 (MDA-MB-231), Hs578T, HCC1599, HCC1806, HCC1937, BT-549, DU4475, and HCC70	Li et al. (2017)
8	miR-93	Upregulated	MB-361, MCF-7, BT-483, AU565, SkBR3, MCF-10A, MB-231 (MDA-MB-231), Hs578T, HCC1599, HCC1806, HCC1937, BT-549, DU4475, and HCC70	Li et al. (2017)
9	miR-27a	Upregulated	BT-549, MDA-MB-231, MDA-MB-468, MDA-MB-453, MCF-10A, and DU4475	Wu et al. (2020)
10	miR-130a	Downregulated	MDA-MB-468, MDA-MB-231, and MF-10A	Poodineh et al. (2020)
11	miR-384	Downregulated	MCF-7; MDA-MB-231	Wang et al. (2018a)
12	miR-34a	Downregulated	SUM159PT, mammospheres and Comma-Dβ cells	Bonetti et al. (2019)
13	miR-374a	Downregulated	MCF7, T47D, BT474, MDA-MB-231, MDA-MB-435, MDA-MB-468, and 4T1	Cai et al. (2013)
14	miR-340	Downregulated	MCF-10A, MDA-MB-231, and HEK 293T	Mohammadi Yeganeh et al. (2017)
15	miR-218	Upregulated	MCF-10A, MCF-7, and MDA-MB-231	Taipaleenmäki et al. (2016)

plethora of literature, it was revealed that the miRNAs not only serve as predictive markers of TNBC but also have prognostic clinical utility. They assist in attaining CSC properties in TNBC and EMT. Since these properties are conditions for metastasis, miRNAs play an essential role in cell transition to the metastasis stage. Several miRNAs, which are differently expressed in TNBC cells compared to normal cells and entangled with the Wnt/β-catenin pathway, have also been identified (Avery-Kiejda et al., 2014; Goh et al., 2016; Pohl et al., 2017).

More than 70 miRNAs (previously implicated in BC) have shown differential expression in TNBC, targeting 16 genes from the Wnt pathway, causing their increased expression in TNBC metastasis. The impacting miRNAs comprised the miR-17-92 oncogenic cluster members and the miR-200 family, revealing that most miRNAs are not mainly associated with a cancer subtype (Pohl et al., 2017). miR-340 alters CTNNB1, MYC, and ROCK1 gene expression of the Wnt pathway and causes apoptosis in TNBC (Mohammadi Yeganeh et al., 2017). miR-203 expresses higher methylation and is downregulated in TNBC, along with downregulation of the Wnt pathway (Taube et al., 2013). Telonis and Rigoutsos (2018) have identified miR-200c, miR-21, miR-17/92 cluster, and the miR-183/96/182 cluster to be upregulated in TNBC. Wang et al. (2019) demonstrated that miR-125, MiR, MiR-145, MiR-381, MiR-136, and MiR-4324 are associated with the poorest prognosis in TNBC patients. Thus, miRNAs play a critical role in TNBC and are intertwined with the Wnt pathway gene regulation, making them essential players in Wnt-mediated TNBC progression, prognosis, and other outcomes. Previously reported miRNAs with a role in TNBC regulation have been collected in **Table 1** below:

LncRNAs may support transcription; aid RNA interference; act as a decoy, peptide, and scaffold; or function as a guide/enhancer RNA (Li et al., 2014). They may deactivate miRNAs in cancer *via* the "sponge" effect, that is, act as a competing molecule or decoy to attach to miRNAs and perturb them from their target (Fan et al., 2018; Huang et al., 2019) (**Figure 2**). They have been demarcated as diagnostic and therapeutic targets. Here, we review lncRNA intertwined with the Wnt pathway and TNBC progression, pathogenesis, prognosis, or invasion. LncRNA Lung Cancer–Associated Transcript 1 (LUCAT1) is interlinked with miR-5582 and regulates breast cancer stemness *via* the Wnt/β-catenin pathway (Zheng et al., 2019). UCA1 promotes EMT (Xiao et al., 2016), while actin filament–associated protein 1 antisense RNA1 (AFAP1-AS1) promotes EMT and tumorigenesis (Zhang et al., 2018). and differentiation antagonizing non-protein coding RNA (DANCR) negatively regulates the Wnt pathway (Li and Zhou, 2018) to uplift tumorigenesis (Tao et al., 2019), EMT, and stemness in TNBC (Zhang and Wang, 2020). HOX Antisense Intergenic RNA (HOTAIR) modulates the Wnt pathway (Li et al., 2016) and leads to metastasis (Collina et al., 2019) and poor prognosis in TNBC *via* upregulation by miR-146a (Liang et al., 2019).

Jiang et al. (2020) reported that DiGeorge Syndrome Critical Region Gene 5 (DGCR5) induces tumorigenesis in TNBC. LncRNA associated with poor prognosis of hepatocellular carcinoma (AWPPH) promotes TNBC growth by upregulating the frizzled homolog 7 (FZD7) ligand of the Wnt pathway (Wang et al., 2018b) and decreased manifestation of lncRNA has been reported to increase the malignant spread of TNBCs (Liu et al., 2017). Long intergenic non-protein coding RNA 1234 (LINC01234) modulates TNBC cell growth, invasion, and EMT positively (Xiao et al., 2021). We also mined lncRNAs with a role in TNBC and impacted by Wnt pathway genes (**Table 2**) from the lnc2Cancer 3.0 database (Gao et al., 2021).

In addition, lncRNA–miRNA interactions entwining the Wnt pathway have also been noted in TNBC (Volovat et al., 2020). Among these, intranuclear Metastasis-Related Lung

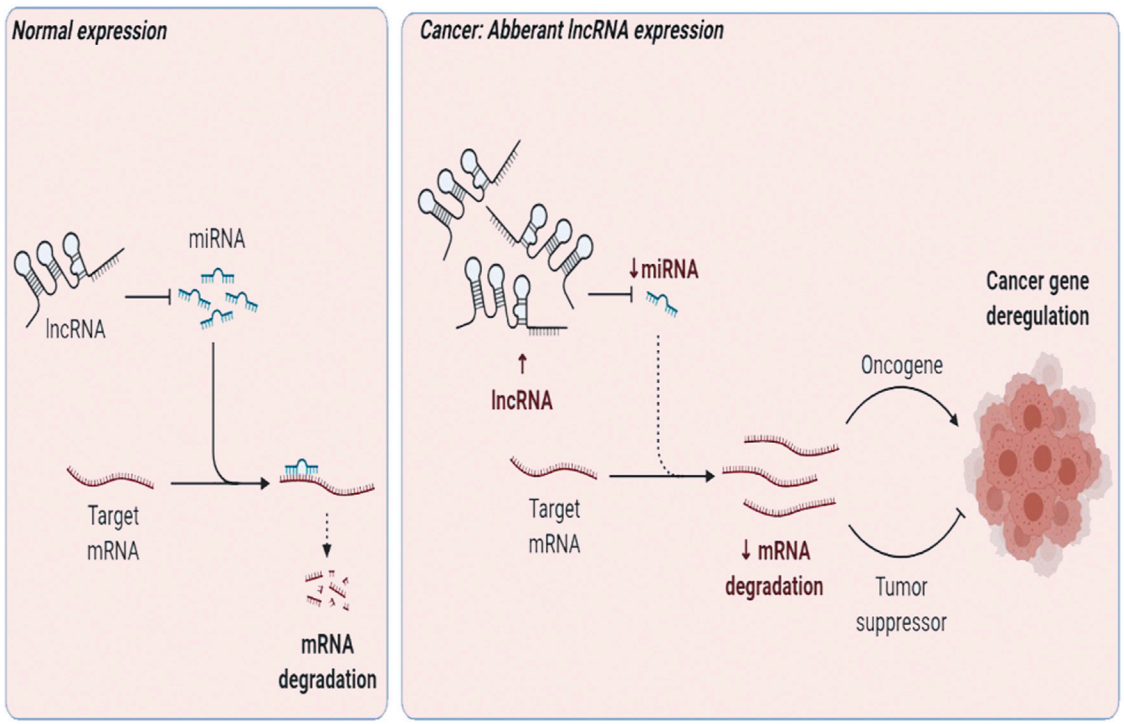

FIGURE 2 | LncRNA–miRNA gene expression regulation in normal vs. cancer phenotype.

TABLE 2 | LncRNAs impacting TNBC *via* the Wnt/β-Catenin axis mined from the lnc2 Cancer database.

S. No.	Name	Method of identification	Expression pattern	References
1	ANRIL	qPCR, luciferase reporter assay, RIP.	Upregulated	Xu et al. (2017)
2	AFAP1-AS1	qPCR, Western blot, *in vitro* knockdown etc.	Upregulated	Zhang et al. (2018)
3	AWPPH	qRT-PCR etc.	Upregulated	Liu et al. (2019)
4	CCAT1	qPCR, luciferase reporter assay, Western blot	Upregulated	Han et al. (2019)
5	DANCR	qPCR, Western blot, *in vitro* knockdown, RIP etc.	Upregulated	Tang et al. (2018a)
6	FAM83H-AS1	qRT-PCR, Western blot	Upregulated	Han et al. (2020)
7	GAS5	qPCR, Western blot, Luciferase reporter assay	Downregulated	Li et al. (2018)
8	H19	qPCR, Western blot, other	Upregulated	Han et al. (2018)
9	HOTAIR	Microarray, qPCR etc.	Upregulated	Chen et al. (2015)
10	LINC00052	Microarray, qPCR etc.	Downregulated	Lv et al. (2016)
11	LINC00115	qRT-PCR, Western blot	Upregulated	Yuan et al. (2020)
12	LINC00152	qPCR, Western blot	Upregulated	Wu et al. (2018)
13	LINC00173	qPCR	Upregulated	Fan et al. (2020)
14	LINC01133	qPCR, Western blot	Upregulated	Tu et al. (2019)
15	LUCAT1	qPCR, Western blot, a luciferase reporter assay, *in vitro* knockdown, RIP	Upregulated	Mou and Wang, (2019)
16	MALAT1	Microarray, qPCR etc.	Upregulated	Chen et al. (2015)
17	NEAT1	qPCR, Western blot	Upregulated	Shin et al. (2019)
18	PCAT6	qPCR, Western blot, luciferase reporter assay, *in vitro* knockdown etc.	Upregulated	Shi et al. (2020)
19	PVT1	qPCR, Western blot, RNAi, other	Upregulated	Wang et al. (2018c)
20	SOX21-AS1	qPCR, Western blot, luciferase reporter assay etc	Upregulated	Liu et al. (2020)
21	XIST	qRT-PCR, Western blot	Downregulated	Li et al. (2020)
22	ZEB1-AS1	qRT-PCR, RIP, dual luciferase reporter assay	Upregulated	Luo et al. (2020)

Adenocarcinoma Transcript 1 (MALAT1) acts as a sponge of miR-129-5p (Dong et al., 2015), and silencing of this non-coding gene causes a decline in cell propagation and movement, illustrating its role in TNBC pathology (Zuo et al., 2017). AWPPH stimulates cell proliferation and contributes to drug therapy resistance when combined with miRNA-21 and is being

exploited as a diagnostic biomarker (Cascione et al., 2013; Liu et al., 2019). At the same time, TUG1 impacts miR-197, prompts NLK expression, and incapacitates the Wnt signaling pathway, making the TNBC cells susceptible to cisplatin therapy (Tang et al., 2018b). AFAP1-AS1 controls miRNA-2110, leading to tumorigenesis and cell invasion (Zuo et al., 2017). The diminished NEF and boosted miRNA-155 levels segregate TNBC patients from controls, suggesting an interlinked modulation prompting enhanced invasion and cell migration in the case of increased miRNA-155 (Song et al., 2019).

CONCLUSION

This narrative review was centered on TNBC association with the Wnt/β-catenin pathway. Moreover, miRNAs and lncRNAs shown to be specific to triple-negative breast carcinoma were listed, both from literature and database searches. Since the Wnt pathway and miRNAs/lncRNAs can modulate TNBC and their intertwinement forms a cascade of complex reactions that impact TNBC and associated processes, their role in TNBC pathology was highlighted concerning molecular processes underlying disease progression. Particular emphasis was put on tumorigenesis, metastasis, and EMT. Apart from cross-talk critical for TNBC tumorigenesis and treatment outcomes, miRNA and lncRNA can serve as predictive or diagnostic markers, so this consolidated information might be of clinical use and offer a consolidated literature resource to scientists working on the Wnt/β-catenin and miRNA/lncRNA axis of TNBC. They also have a role in chemotherapy resistance. However, information on detailed analyses of Wnt/miRNA/lncRNA axis mechanisms in TNBC is still scant, and more work needs to be carried out to infer the pivotal role of these moieties in TNBC. Owing to the heterogeneity of TNBC, recognition of subgroups or their pathologies based on the varied signatures of this axis could be interesting to explore. Furthermore, knock-out or knock-in functional studies in model organisms could be beneficial to understanding the comprehensive role of this axis in TNBC.

AUTHOR CONTRIBUTIONS

Conception/Design: WW, QZ. Collection and assembly of data: XH, WX. Manuscript writing: XH, WW. Final approval of manuscript: WW, QZ, XH, WX.

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