ACTION MECHANISMS OF TRADITIONAL MEDICINAL PLANTS USED TO CONTROL TYPE 2 DIABETES OR CONDITIONS OF METABOLIC SYNDROME

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ACTION MECHANISMS OF TRADITIONAL MEDICINAL PLANTS USED TO CONTROL TYPE 2 DIABETES OR CONDITIONS OF METABOLIC SYNDROME

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EDITORIAL
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Editorial: Mechanisms of Traditional Medicinal Plants Used to Control Type 2 Diabetes or Metabolic Syndrome

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Keywords: mechanisms of action, medicinal plants, metabolic syndrome, diabetes, Treatment

Editorial on the Research Topic

Mechanisms of Traditional Medicinal Plants Used to Control Type 2 Diabetes or Metabolic Syndrome

Type 2 diabetes mellitus (T2DM), the prevalent form of diabetes, a heterogeneous, multifactorial disorder related to diminished insulin secretion, insulin resistance and related factors, such as obesity, sedentary lifestyle, stress and aging. The International Diabetes Federation estimates that without any intervention to slow down the rise in T2DM, there will be at least 700 million people with diabetes by 2045. The changes in lifestyle (lack of exercise, junk food) and increase in life expectancy, human facing more challenges from metabolic disorders (Nazarian-Samani et al., 2018; Xiao and Luo, 2018). Among the metabolic diseases, we have the cluster of conditions (hypertension, hyperglycemia, abdominal obesity, and elevated cholesterol or triglyceride levels) that when occur together are called; metabolic syndrome and T2DM (Saklayen, 2018). Medicinal plants are widely used throughout the world especially in the developing countries to manage the metabolic syndrome and T2DM (Chukwuma et al., 2019). The pathophysiological conditions engage metabolic pathways involve hundreds of enzymes, proteins, co-factors; various cells, tissues and organs especially pancreas, liver, gut, muscle, adipose tissue and kidney. It is important to understand the mechanisms underlying the medicinal plants in controlling the metabolic syndrome and T2DM (Demmers et al., 2017; Ota and Ulrih, 2017; Salehi et al., 2019). However, conceptual and methodological challenges exist. Thus, this Research Topic aims to aid to our understanding of the hypoglycemic actions by medicinal plants and derived phytochemicals. Among 27 submitted manuscripts for this Research Topic, only seven manuscripts have been accepted.

Mao et al. studied the combined use of *Astragalus* polysaccharide (AP) and berberine (BBR) on insulin resistance in IR-HepG2 Cells. Their study demonstrated that AP-BBR attenuated IR in this cell model with a concomitant reduction of H_2O_2 content. The study has also shown that the protein expression of p-FoxO1Ser256 and PEPCK in IR-HepG2 cells was increased while the protein expression of FoxO1 and GLUT2 was decreased. Interestingly, AP-BBR combination was able to reverse the above protein expression changes suggesting that AP-BBR attenuates IR in IR-HepG2 cells probably via the regulation of the gluconeogenesis signaling pathway. While Mata-Torres et al. focused on the study of hepatic glucose output inhibitory activity of some Mexican plants in streptozotocin-induced diabetic rat or *in vitro* assessing their inhibitory effect on glucose-6-phosphatase. The plant extracts of *Ageratina petiolaris*, *Bromelia karatas*, *Equisetum myriochaetum*, *Rhizophora mangle*, and *Smilax moranensis*

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inhibit hepatic glucose output. Besides, Belwal et al. updated the uses of berberine; its phytopharmacology, clinical use, its prevention and treatment of diabetes and other metabolic diseases. Berberine exhibited beneficial effect in animal models of chronic diseases, however, the authors stated that its efficacy in clinical setting has not yet been demonstrated.

In fact, berberine is an isoquinoline alkaloid from *Coptis chinensis* Franch has attracted notable attention for its pharmacological properties. Wang et al. assessed the effect of berberine on special learning memory impairment caused by diabetes. To investigate spatial learning and memory, the authors employed the Morris water maze assay in diabetic rats and the results demonstrated that berberine ameliorated spatial learning memory impairment by attenuation of β-amyloid formation through the activation of the cholinergic anti-inflammatory and insulin signaling pathways in diabetic rats. Through a systematic review and meta-analysis, Wang et al. tried to highlight the capacity of Astragaloside IV (AS-IV), a saponin isolated from *Astragalus membranaceus* (Fisch.) Bunge, which is known for beneficial pharmacological activities to treat renal diseases. Several preclinical studies have demonstrated the renoprotective effect of AS-IV through its antifibrotic, antioxidant, and antiapoptotic actions, resulting in alleviation of endoplasmic reticulum stress, inhibition of mitochondrial fission, and increased autophagic activity. On the other hand, Heydarpour et al. reviewed the pathogenesis of diabetes and concluded that some phytochemicals and plant extracts may act as alternatives to current drugs in diabetes treatment based on their modulation of the autophagy and/or TGF-β signaling pathways.

Park et al. investigated the effect of Taeumjowi-tang (TJT), a traditional Korean sasang remedy on obesity-atopic dermatitis comorbidity. The authors used a high-fat diet (HFD) and 1-fluoro-2,4-dinitrobenzene (DNFB) as obesity and Alzheimer's disease (AD) model, they demonstrated that TJT treatment improved the symptoms related to obesity and AD especially epidermal thickness and eosinophil/mast cell infiltration, reduction in immunoglobulin E, interleukin (IL)-4, IL-6, and tumor necrosis factor-alpha (TNF-α). TJT was shown to suppress HFD/DNFB-associated inflammation-related nuclear factor-kappa beta (NF-κB) and mitogen activated protein kinase. Furthermore, treatment of TJT down-regulated the hypoxia inducible factor-1 alpha protein in the mice model of obesity-atopic dermatitis comorbidity. They suggested that TJT is a potential candidate which could be used to improve clinical symptoms related to obesity-AD comorbidity.

It is valuable to explore the beneficial effects of these plant derived medicines on the metabolic syndrome. We know this may be just the tip of an iceberg, other mechanisms of actions, cellular and molecular action sites remain to be discovered. We are convinced that those promising pre-clinical studies warrant further clinical trials.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Berberine Ameliorates Spatial Learning Memory Impairment and Modulates Cholinergic Anti-Inflammatory Pathway in Diabetic Rats

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Background: Cognitive impairment caused by diabetes has been recognized. Berberine is well known for its resistance to peripheral lesions, but it is rarely used for the treatment of spatial learning and memory caused by diabetes. This study explored the mechanism of berberine to alleviate cognitive impairment *via* the cholinergic anti-inflammatory and insulin signaling pathways.

Methods: Morris water maze was used to appraise spatial learning and memory. Positron-emission tomography (PET) imaging was adopted to detect the transport of glucose, and blood/cerebrospinal fluid (CSF) glucose was checked using commercial blood glucose meter. Insulin level was measured by ELISA kit and β-Amyloid (Aβ) formation was observed by Congo red staining. Western-blot was performed to appraise protein expression.

Results: We found that berberine rectified some aberrant changes in signal molecules concerning inflammation, and cholinergic and insulin signaling pathways in the hippocampus. Furthermore, CSF/blood glucose, inflammatory response or acetyl cholinesterase enzyme (AChE) activity were reduced by berberine. Additionally, acetylcholine levels were enhanced after berberine treatment in diabetic rats. Finally, Aβ formation in diabetic hippocampus was inhibited and spatial learning memory was ameliorated by berberine.

Discussion: In conclusion, berberine clears Aβ deposit and consequently ameliorates spatial learning memory impairment *via* the activation of the cholinergic anti-inflammatory and insulin signaling pathways in diabetic rats.

Keywords: berberine, α7nAChRs, diabetes mellitus, spatial learning and memory, inflammation, Aβ

INTRODUCTION

Diabetes mellitus (DM) is a chronic and widespread metabolic disease characterized as a consequence of both genetic predisposition and dietary indiscretion and has high prevalence rates and mortality worldwide (Bhusal et al., 2014). Accumulating evidence suggests that Alzheimer's disease (AD)-like alteration in DM is a major public health problem (Carvalho et al., 2012). Spatial learning and memory impairment induced by streptozotocin in high-fat diet-induced DM animal models is a consequence of changes in the central nervous system (CNS). Diabetes-induced β-amyloid (Aβ) accumulation promotes cognitive impairment involving several pathogenesis (Ohno et al., 2004; Moreira, 2013; Jayaraman and Pike, 2014). From a neuropathological point of view, the senile plaques caused by Aβ, synapse loss, and apoptosis in the neurons are the principal pathological hallmarks of AD. However, the upstream molecular mechanism of diabetes that leads to Aβ deposit is still worth exploring.

Chronic inflammation infiltration plays an important role in cognitive impairment caused by diabetes (Stranahan et al., 2016). Impaired cholinergic system also leads to cognitive impairment as recently reported by various research groups (Ferreira-Vieira et al., 2016). The alpha-7 nicotinic cholinergic receptor subunit (α7nAChRs), which is derived from nicotinic cholinergic receptor, possesses the potential of anti-inflammation in the periphery and CNS. This action is specifically called "the cholinergic anti-inflammatory pathway (CAP)" (Pavlov and Tracey, 2006; Gallowitsch-Puerta and Pavlov, 2007). α7nAChRs are widely distributed throughout CNS and in multiple inflammatory cells, including mononuclear macrophages (Yi et al., 2015), T lymphocytes (Liu et al., 2014), B lymphocytes (Koval et al., 2018) and natural killer (NK) cells (Zanetti et al., 2016). Additionally, α7nAChRs are also abundantly expressed in astrocytes, which are the key regulators of neuroinflammation in several neurodegenerative diseases (Di Cesare Mannelli et al., 2015; Revathikumar et al., 2016). Activated α7nAChRs blocks NF-κB signaling pathway and reduces neuroinflammation (Patel et al., 2017). In the median prefrontal cortex of diabetic rats, activated astrocytes and inflammatory mediators are remarkably increased (Saravia et al., 2002). Thus, astrocytes were considered as emerging pivotal regulators to CNS inflammatory responses in our model (Sofroniew, 2015). Insulin resistance is caused by the inflammatory response, and inflammation is modulated by the a7nAChR as reported in many experiments (Xu et al., 2012; Li et al., 2016). Moreover, the inflammatory and insulin signaling pathways always intertwine with each other. The mistaken process of Aβ precursor protein (APP) due to insulin resistance has been mentioned in many literatures (Hiltunen et al., 2012). It is possible that inflammatory factor may modify the course of APP to accelerate Aβ deposit *via* insulin resistance promotion.

Berberine, an isoquinoline alkaloid, is purified from *Coptis chinensis* Franch and has attracted notable attention for its powerful capabilities as antioxidant, hypoglycemic, cholesterol-lowering, and acetyl cholinesterase enzyme (AChE) inhibitory effect in the periphery (Hsu et al., 2013; Yu et al., 2018). However, the mechanisms of alleviating spatial learning and memory impairment in CNS, especially in the hippocampus, are still poorly understood. Our previous studies confirmed that berberine (187.75 mg/kg/day) can ameliorate emotional memory decline, but the spatial learning and memory impairment which is induced by diabetes still need more efforts to uncover the veils.

Here, we hypothesized that a7nAChR loss in hippocampus is involved in "CAP" deficit, and excessive inflammatory response may lead to insulin signaling inactivation and cognitive impairment in DM rats. This study aimed to investigate the molecular mechanism of berberine in relieving inflammatory effects and modulating the cholinergic and insulin signaling pathways to improve spatial learning and memory impairment in DM rats.

Abbreviations: DM, diabetes mellitus; AD, Alzheimer's disease; Aβ, β-amyloid; APP, amyloid precursor protein; BACE-1, beta-site amyloid precursor protein cleaving enzyme 1; STZ, streptozocin; HSFD, high sugar and fat diet; OGTT, oral glucose tolerance test; 18F-FDG, 18F-fluorodeoxyglucose; PET, Positron-Emission Tomography; IHC, Immunohistochemical.

MATERIALS AND METHODS

Reagents

Monoclone antibodies IR, PI3K P85, p-NF-κB p65, IKK, BACE-1, APP, α7nAChR and polyclone antibody Aβ were purchased from Abcam (Cambridge, MA, USA). Monoclone antibody p-Akt (Ser473), AKT, NF-κB, p-IKK, p-IRS-1(Ser307), and IRS-1 were purchased from Cell Signaling Technology (Boston, MA, USA). Insulin ELISA kit (EZRMI-13) and PVDF membrane (0.45 μm) were obtained from Millipore (Billerica, MA, USA). The cytokines of IL-1β, IL-18 and TNF-α were purchased from BOSTER (Wuhan, China) and the ACh kits (A105-1: tissue, A105-2: Serum) and the AChE kits (A024) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ladder marker was obtained from Thermo Scientific (Waltham, MA, USA). Finally, the GLU kit was purchased from Shanghai Mind Bioengineering Co., Ltd. (Shanghai, China). Berberine was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (99% pure, Shanghai, China). All other reagents purchased from located market were of analytical grade.

Animal and Experimental Procedure

Male Wistar rats weighting 180–200 g (aged 4–5 weeks) were obtained from Vital River Laboratory Animal Technology co., LTD. (Beijing, China). Animals were raised SPF circumstances with a light/dark cycle of 12/12 h under controlled temperature room. Animal experimental protocols were guided and approved in accordance with all guidelines and regulations of the Animal Care and Use Committee affiliated to Tongji Medical College, Huazhong University of Science and Technology. After 2 weeks of adaptation, except for the normal control group (Nor), high sugar and fat diet (HSFD,

67.5% standard laboratory rat chow, 20% sugar, 10% lard, 2% cholesterol and 0.5% bile salts) was given to the DM group for 4 weeks. Following the previous method in our laboratory (Chen et al., 2017), 25 mg/kg Streptozocin (STZ) was injected through the caudal vein in the HSFD group to form the diabetes animal model. After a week feeding, oral glucose tolerance test (OGTT) was used to appraise whether the model was successfully established. Meanwhile, the rats with a blood glucose levels reaching 11.2 after the meal for 2 h were randomly divided into four experimental groups as follows: DM group (DM), berberine group (BBr, 187.75 mg/kg/day), metformin group (Met, 184 mg/kg/day) and huperzine-A group (Hup, 0.015 mg/kg/day). All drugs were prepared avoiding degradation under conditions used due to long time preservation. The rats in Nor group were given standard rodent diet and water *ad libitum*, and those in the STZ-treated group were fed with HSFD until the time for sacrifice. Behavioral test was conducted before the sacrificed week. At the end of the 17th week, body weight was tracked (**Figure 1C**), and glucose level was detected using the commercial glucometer. A timeline of experimental procedure is presented in **Figure 1A**.

Behavioral Tests

Morris Water Maze (MWM)

To evaluate the cognitive impairment and the therapeutic effect of berberine in DM rats, we adopted the Morris water maze (MWM) assay, which was described 20 years ago as a device to investigate spatial learning and memory, and has become one of the most frequently used laboratory tools

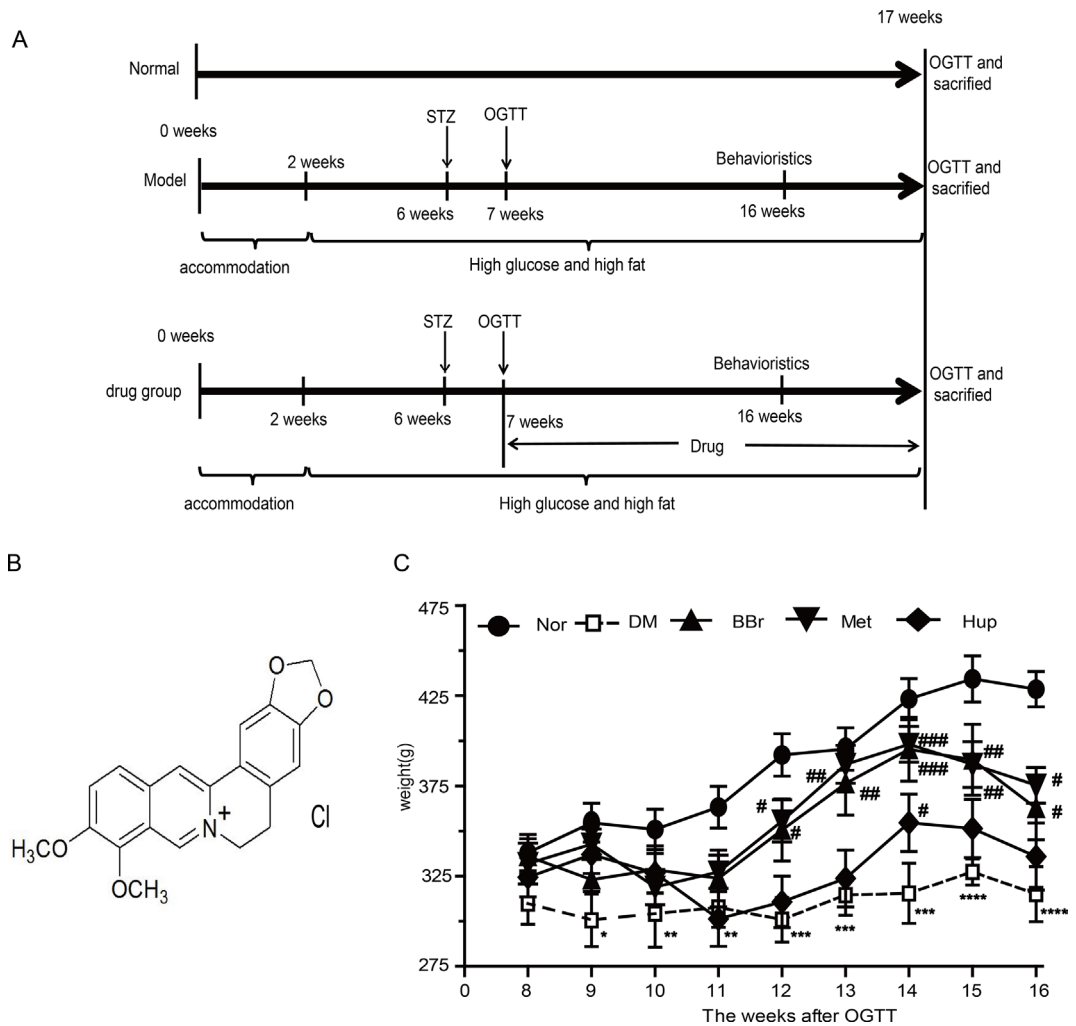

FIGURE 1 | Flow chart of animal experiment design and body weight variation. **(A)** A total of 100 male Wistar rats fed with HSFD diet were assigned to DM model groups, whereas 20 rats fed by standard chow were assigned to control group. After 2 weeks adaptive feeding, DM model group was given HSFD feeding for 4 weeks. Then, 25 mg/kg STZ was injected into caudal vein to form the diabetes animal model in HSFD group, whereas normal group were injected with citrate buffer only. Behavioral evaluation was performed after feeding for 16 weeks. In the 16th week, all rats were sacrificed from which we collected samples and executed follow-up experiment. **(B)** Molecular formula of berberine. **(C)** Body weight variation. $^{*}P < 0.05$ vs. Nor; $^{**}P < 0.01$ vs. Nor; $^{***}P < 0.001$ vs. Nor; $^{****}P < 0.0001$ vs. Nor; $^{\#}P < 0.05$ vs. DM; $^{\#\#}P < 0.01$ vs. DM; $^{\#\#\#}P < 0.01$ vs. DM.

in behavioral neuroscience(D'hooge and De Deyn, 2001). Testing was conducted *via* the platform-relocation protocol (Izco et al., 2014) with minor modification. The experimental apparatus consists of a circular pool (diameter: 160 cm, depth: 60 cm) filled with water (22–25°C). The water was added with black ink to make it opaque. A black circular platform with 11 cm diameter was placed either 1–2 cm above or submerged 1–2 cm below the water surface. Animals were trained in two consecutive daily cue sessions (platform visible) followed by four acquisition sessions (platform submerged). The pool was located in a small obscured room illuminated by a dim light. All the trials were automatically monitored with a camera above 2 m from water level. MWM assay and data analysis were conducted by unwitting observer.

Sample Preparation

Serial Collection of Cerebrospinal Fluid and Serum

Rat was anesthetized through the intraperitoneal injection of phenobarbital sodium and kept at 37 °C during anesthesia induction. Blood was subsequently collected from abdominal aorta, centrifuged at 3,500 rpm for 10 min to obtain serum, and then stored at −80 °C until for further analysis. Next, the rat's head placed on the stereotaxic instrument was handled at the neck and dorsum into roughly 130°, and neck skin was shaved. A sagittal incision of the skin was immediately made as inferior to the occiput, whereas foramen magnum was exposed after subcutaneous tissues and muscles were separated through blunt dissection with forceps. A 100 µl syringe was penetrated into the foramen magnum through the dura mater approximately 2–4 mm, and 100 µl of CSF was collected after carefully drawing the piston handle.

Hippocampus Sample Preparation

Brains were quickly removed after collecting CSF and rinsed in ice-cold saline. Each set of 6 brains was embedding in paraffin after being fixed by 4% paraformaldehyde. After being dissected on a cold plate, a small portion of the hippocampus tissue was used for enzyme activity detection. The remaining hippocampus was used for Western-blot, carefully dissected, flash frozen in liquid nitrogen, and preserved at −80 °C until use.

Biochemical Measurements

Glucose and Insulin Levels

The insulin and glucose levels in serum and CSF were analyzed by the rat's insulin sandwich ELISA kit and glucose detection kit according to the manufacturer's protocol.

TNF-α, IL-1, and IL-18 Measurements

Over-expression of diversified inflammation interleukin has been considered in DM. The levels of TNF-α, IL-1, and IL-18 were measured using ELISA kit as per manufacturer's instructions.

ACh Level and Acetyl Cholinesterase Activity

The cholinergic nerve fibers originating from the forebrain project to the hippocampus is essential to learning and memory. The cholinergic system dysfunction was assessed by measuring the Ach level and AChE activity. According to the commercial biochemical kit, the ACh level and AChE activities in the centrifugal serum and homogenized supernatants coming from hippocampus tissues were measured using a spectrophotometer through a micro plate reader (Synergy 2, USA) as per manufacturer's protocol.

Western Blot

Hippocampus lysate approximately 30–40 µg samples were denatured and loaded onto sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrophoretically transferred to a PVDF membrane (Millipore, 0.45 µm). After blocking for 1 h with 5% BSA, the bands were incubated overnight at 4°C with primary antibodies and then with Odyssey fluorescence secondary antibodies for 1 h, washed, and visualized by scanning *via* the Gene apparatus.

Positron-Emission Tomography (PET) Imaging

PET imaging was performed *via* Trans-PET® BioCaliburn® 700 (Raycan Technology Co., Ltd, Suzhou, China). Rats were fasted overnight and injected with 200 µl 18.5 MBq 18F-FDG (18F-fluorodeoxyglucose) *via* the tail vein. After 60-min uptake periods, the rats were scanned 20 min by a Trans-PET scanner under 2% isoflurane anesthesia. Then, PET images were reconstructed using the 3D OSEM algorithm (1 iteration and 12 subsets). We quantified the 18F-FDG uptake in different brain regions of interest (BROI) through the semi-quantitative analysis. The following equation: $SUV = C_T \cdot \frac{V_T}{W_T} \cdot \frac{1}{D_{Inj}} \cdot W_S$ was adopted to calculate the standard uptake value (SUV) for each BROI. CT is the radioactivity with the unit of mCi/cc tissue. VT and WT express the volume and weight, respectively. Their ratio generates the density of the region. DInj is the dose injected with the unit of mCi. WS is the weight of the rat in the unit of g.

IHC Staining and Congo Red Staining

The slices were immunochemically stained as per previous studies (Wang et al., 2018). Images were obtained using a fluorescence microscope (Olympus).

Congo red staining with minor modification (Labour et al., 2016) was adopted to appraise Aβ deposit. Images were obtained with a fluorescence microscope (Olympus).

Statistical Analysis

Data were collected and analyzed using Graph Pad Prism 5 software and expressed as mean ± SEM. Statistical significance

was tested *via one-way* ANOVA following *Tukey's Post-Test*. *P* values < 0.05 indicated that the levels of significance existed.

RESULTS

Berberine Improves the Glucose Absorption and Metabolism in DM Rats

Glucose intolerance and impairment of insulin secretion are associated with a high risk to develop dementia or AD (Ronnemaa et al., 2008). Insulin resistance in DM is typically characterized by peripheral hyperinsulinemia. Dysfunction of glucose metabolism in the CNS caused by peripheral hyperinsulinemia has always been a mystery. On this basis, we measured the glucose and insulin levels in the peripheral and CSF, respectively. We also probed the potential mechanisms of berberine (the structure is as shown in **Figure 1B**) to improve glucose transport. As shown in **Figure 2 A**, the postprandial blood glucose in peripheral was substantially increased after STZ injection combined with HSFD diet. Berberine or metformin remarkably reduced the glucose level, whereas huperzine-A (an AChE inhibitor) did not have an effect. Although the glucose level was lower in CSF than in serum, the variation tendency of glucose levels was consistent with serum results (**Figure 2B**). Furthermore, the serum insulin level was significantly increased in DM rats but was normalized *via* berberine treatment (**Figure 2C**). However, the insulin level in CSF was remarkably decreased in DM rats, and berberine or huperzine-A can increase its level (**Figure 2D**). Interestingly, metformin reduced insulin level in the serum rather than the CSF in DM rats (**Figures 2C**, **D**). Next, we used PET imaging to detect the brain's ability to metabolize glucose. As shown in **Figures 2E**, **F**, glucose transport ability in hippocampus and cortex were significantly impeded in DM rats. This phenomenon was partly reversed by the administration of berberine and huperzine-A. Metformin slightly increased the transport of glucose. In summary, insulin resistance occurs in the diabetic brain, and berberine may facilitate glucose absorption and utilization in DM rats *via* the amelioration of insulin resistance.

Berberine Alleviates Inflammation in DM Rats

The dysfunction of glucose transport and metabolism in the peripheral or CNS results in systemic or local inflammation, which contributes to DM development (Jorge et al., 2011; Muriach et al., 2014). The inflammatory response in the median prefrontal cortex of diabetic rats impairs the fear memory (Chen et al., 2017). Our results demonstrated that the level of IL-1β, IL-18, and TNF-α were significantly increased in the serum and hippocampus tissue in DM rats, whereas this trend was reversed by treatment with berberine, metformin, and huperzine-A (**Figures 3A–C**). To investigate anti-inflammatory effects of berberine, we examined the phosphorylation levels of NF-κB and IKK in hippocampal tissues. As shown in **Figures 3D–G**, p-IKK and p-NF-κB were significantly increased in the hippocampus in DM rats, suggesting that NF-κB pathway may be activated. This phenomenon can be partly inhibited by treatment with berberine, metformin, and huperzine-A. We used a GFAP marker to identify astrocyte activation in hippocampal tissues and further elucidate the origin of inflammation. The activated astrocytes in DM rats were remarkably attenuated by berberine, metformin, and

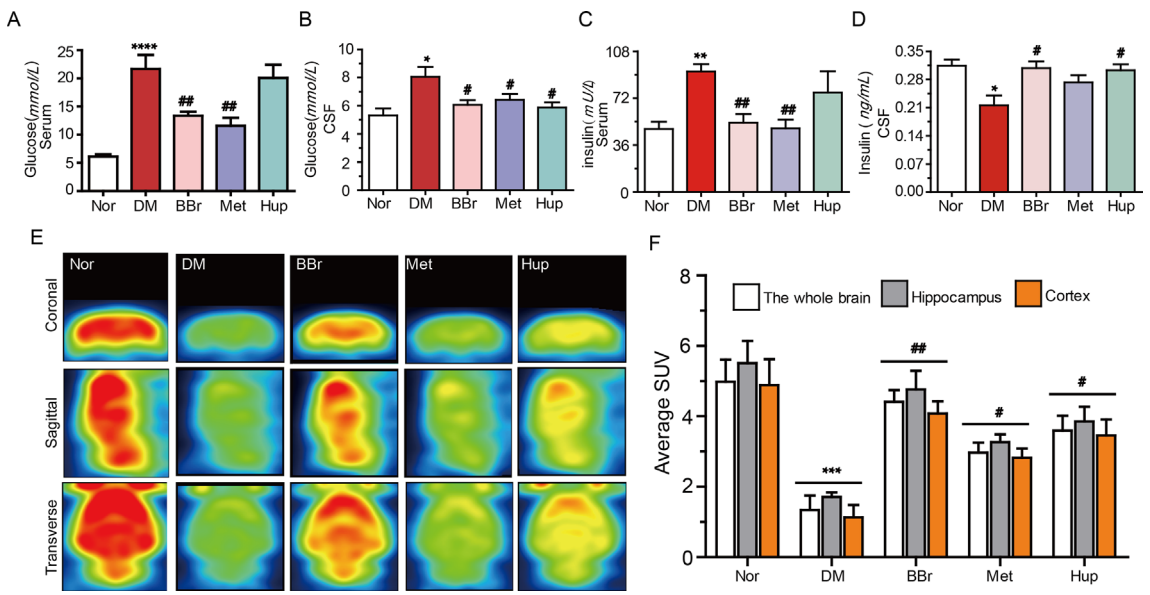

FIGURE 2 | Berberine improves glucose absorption and metabolism in diabetic rats. **(A, B)** Glucose levels in diabetic rat's periphery and CSF, n = 8–12. **(C, D)** The insulin levels in diabetic rat's periphery and CSF, n = 8–12. **(E, F)** The glucose metabolism in the brain were measured by PET imaging and quantified by fluorescence intensity, n = 3. **P* < 0.05 vs. Nor; ***P* < 0.01 vs. Nor; ****P* < 0.001 vs. Nor; *****P* < 0.0001 vs. Nor; #*P* < 0.05 vs. DM; ##*P* < 0.01 vs. DM.

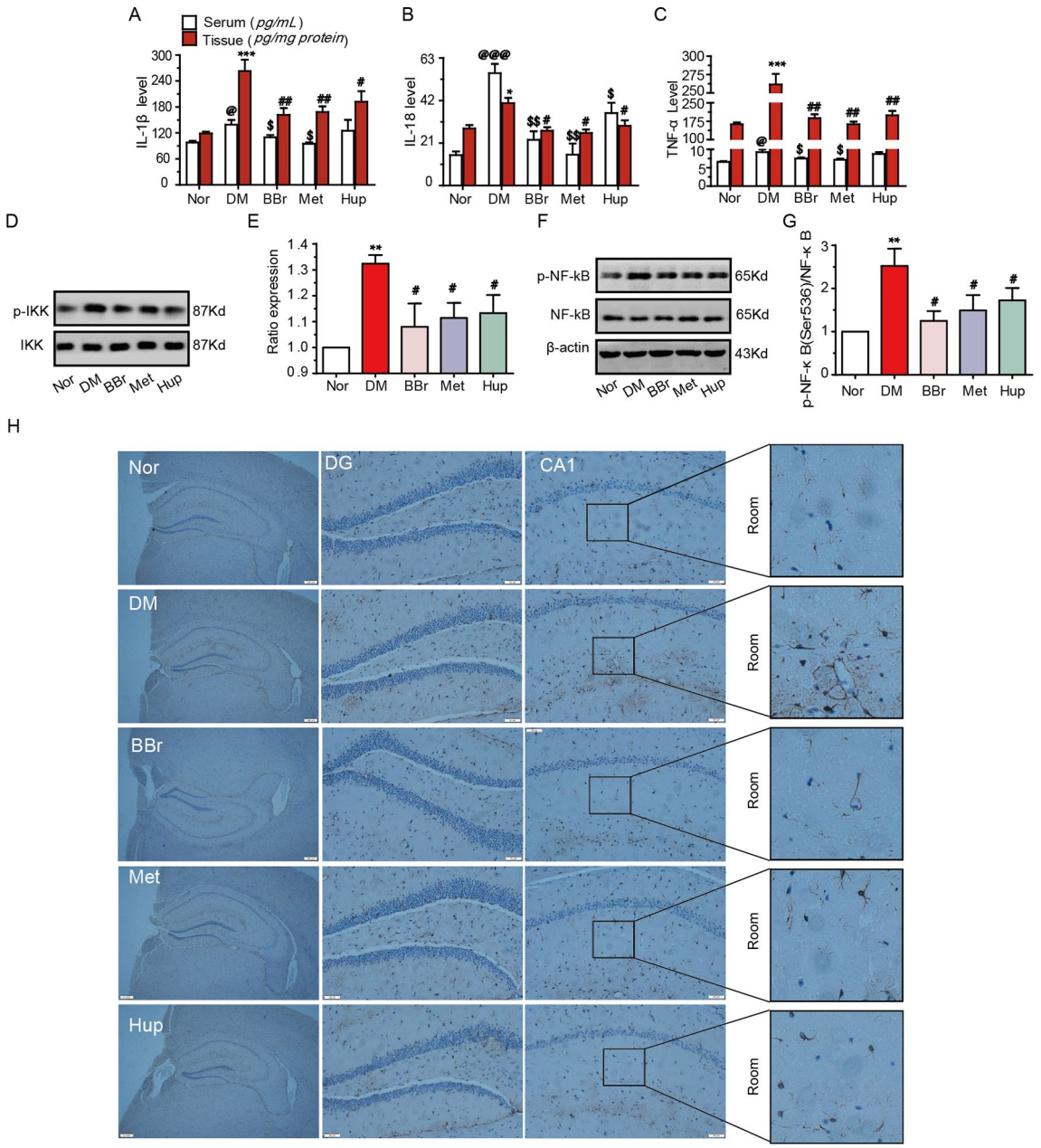

FIGURE 3 | Berberine inhibited inflammatory response in diabetic rats. The IL-1β **(A)**, IL-18 **(B)** and TNF-a **(C)** levels were measured using ELISA kits, n = 8–12. Inflammatory pathway related molecular p-IKK/IKK **(D, E)** and p-NF-kB/NF-Kb **(F, G)** levels were detected by Western-blot, n = 3. The GFAP expression was appraised by IHC **(H)**, n = 3. $^{*}P < 0.05$ vs. Nor; $^{**}P < 0.01$ vs. Nor; $^{***}P < 0.001$ vs. Nor; $^{@}P < 0.05$ vs. Nor; $^{@@@}P < 0.001$vs. Nor; $^{\#}P < 0.05$ vs. DM; $^{\#\#}P < 0.01$ vs. DM; $^{\$}P < 0.05$ vs. DM; $^{\$\$}P < 0.01$ vs. DM.

huperzine-A (**Figure 3H**). Thus, berberine may ameliorate hippocampus inflammation *via* the inhibition of astrocytes activation in DM rats.

Berberine Ameliorates "CAP" Dysfunction in DM Rats

"CAP" plays an important role in the development of systemic and local inflammation. Therefore, we detected the key molecules of the cholinergic signaling pathway within peripheral serum and hippocampus of DM rats. The AChE activity in hippocampal tissues and serum were significantly increased in DM rats, and treatment with berberine, metformin, and huperzine-A decreased the AChE activity (**Figures 4A**, **C**). The ACh levels in DM rats were increased by the treatment with berberine, metformin, and huperzine-A (**Figures 4B**, **D**). We further detected a7nAchR expression to assess the anti-inflammatory role of berberine

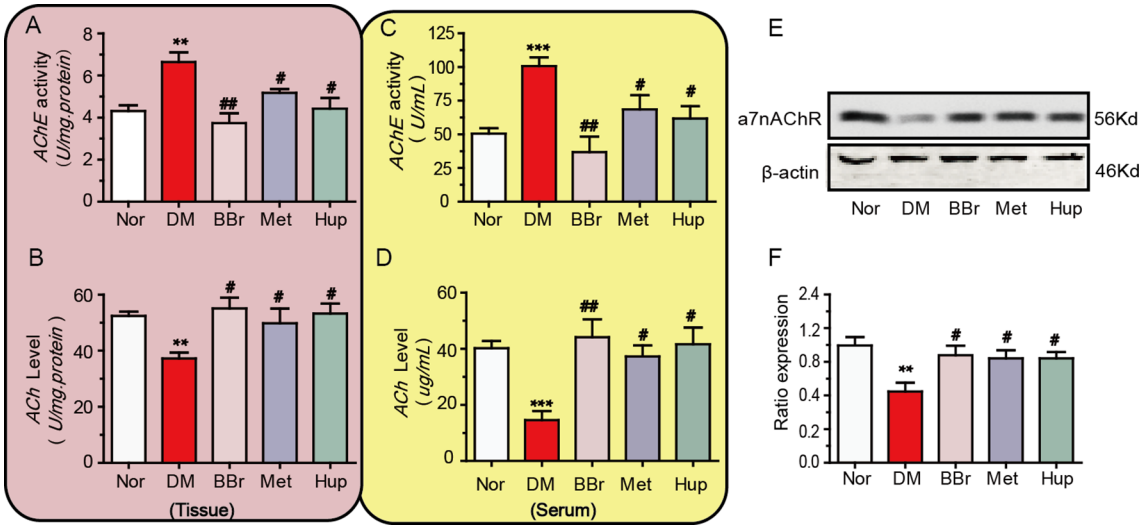

FIGURE 4 | Berberine ameliorates the cholinergic signaling pathway dysfunction. **(A, B)** The AChE activity and ACh levels in diabetic rat's periphery, n = 8–12. **(C, D)** The AChE activity and ACh levels in diabetic rat's hippocampus, n = 8–12. **(E, F)** The a7nAChR expression in diabetic rat's hippocampus was detected by Western-blot and quantified by image J, n = 3. $^{**}P < 0.01$ vs. Nor; $^{***}P < 0.001$ vs. Nor; $^{\#}P < 0.05$ vs. DM; $^{\#\#}P < 0.01$ vs. DM.

via "CAP." Results demonstrated that a7nAchR expression was remarkably down regulated in the hippocampus of DM rats, but was restored *via* the treatment with berberine, metformin, or huperzine-A (**Figures 4E, F**).

Berberine Improves the Disorder of Insulin Signaling Pathway in Diabetic Rat's Hippocampus

Disorders of the inflammatory signaling pathway trigger insulin resistance. We found that the insulin level in the CSF was decreased and the glucose level was increased, suggesting that the insulin signaling pathway may be dysregulated in the hippocampus of DM rats. Thus, we analyzed the changes of insulin signaling pathways in hippocampus. The insulin receptor (IR) expression was significantly down regulated in DM rats, but was recovered by berberine, metformin, and huperzine-A treatment (**Figure 5A**). In addition, the expression of p-IRS-1 at ser307 in DM rats was notably upregulated, whereas it was partly decreased after treatment with berberine, metformin, and huperzine-A (**Figure 5B**). Next, we detected the expression of PI3K/AKT (key downstream proteins in the insulin signaling pathway). Results revealed that PI3K and p-Akt expression were significantly down regulated in DM rats, but were normalized by berberine, metformin, and huperzine-A (**Figures 5C, D**), suggesting that berberine may improve insulin resistance in the hippocampus of DM rats.

Berberine Improves APP Misprocessing in the Hippocampus of Diabetic Rats

Aβ is always tangled with insulin signaling pathway in diabetic encephalopathy and stagnates insulin receptor in the cytosol. We

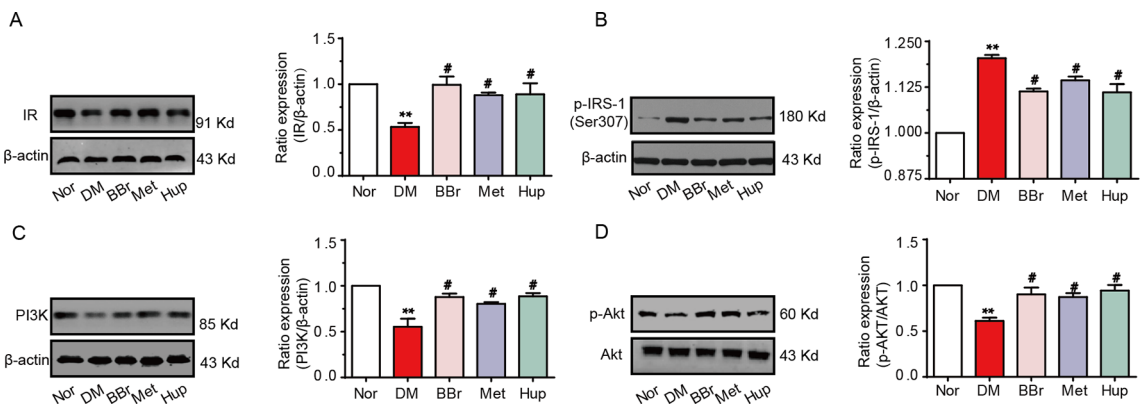

FIGURE 5 | Berberine improves insulin signaling pathway. Western-blot was performed to evaluate the protein expression of IR **(A)**, p-IRS-1 ser307 **(B)**, PI3K **(C)** and p-Akt **(D)** in hippocampus, n = 3. $^{**}P < 0.01$ vs. Nor; $^{\#}P < 0.05$ vs. DM.

confirmed that the increased APP/BACE-1 in the hippocampus of DM rats was significantly inhibited by the three compounds (**Figures 6A–C**). Next, to further detect Aβ deposits in DM hippocampal tissue, Congo red staining was applied to assess Aβ generation. Obviously, dark red particles in the DM group increased, but were significantly improved by berberine, metformin, and huperzine A treatment (**Figure 6D**). The level of Aβ42 in CSF was also significantly increased in DM rats, whereas was strongly reduced after drug treatment (**Figure 6E**).

Berberine Ameliorates the DM-Induced Cognitive Impairment

Cognitive impairment is positively correlation with DM (Luchsinger, 2012). Thus, we analyzed the changes of learning and memory in DM rats using MWM test. Results demonstrated that the escape latency that traveled to the target was significantly retarded in DM rats (**Figures 7A**, **B**). Spatial learning in DM rats was significantly damaged, although the average swimming speed in 6 days was not changed (**Figures 7C**, **D**). Besides, escape latencies within 6 days were remarkably increased compared with those in the control group, suggesting that DM rats exhibited deficiency in memory recall (**Figure 7E**). Fortunately, berberine, metformin, and huperzine-A treatment groups had significantly decreased time to reach the platform compared with the DM rats (**Figure 7E**). Additionally, berberine, metformin, and huperzine-A significantly increased the time spent in target quadrant and the number of platform crossings when compared with the DM rats (**Figures 7F**, **G**). These results suggest that berberine may improve spatial learning and memory impairment in DM rats, so did by metformin and huperzine-A.

DISCUSSION

DM is rapidly growing among the population and patients have higher incidences of cognitive impairment (Ryan et al., 2014; Infante-Garcia et al., 2018). DM-associated cognitive deficit complication is also referred to as DE (diabetic encephalopathy).

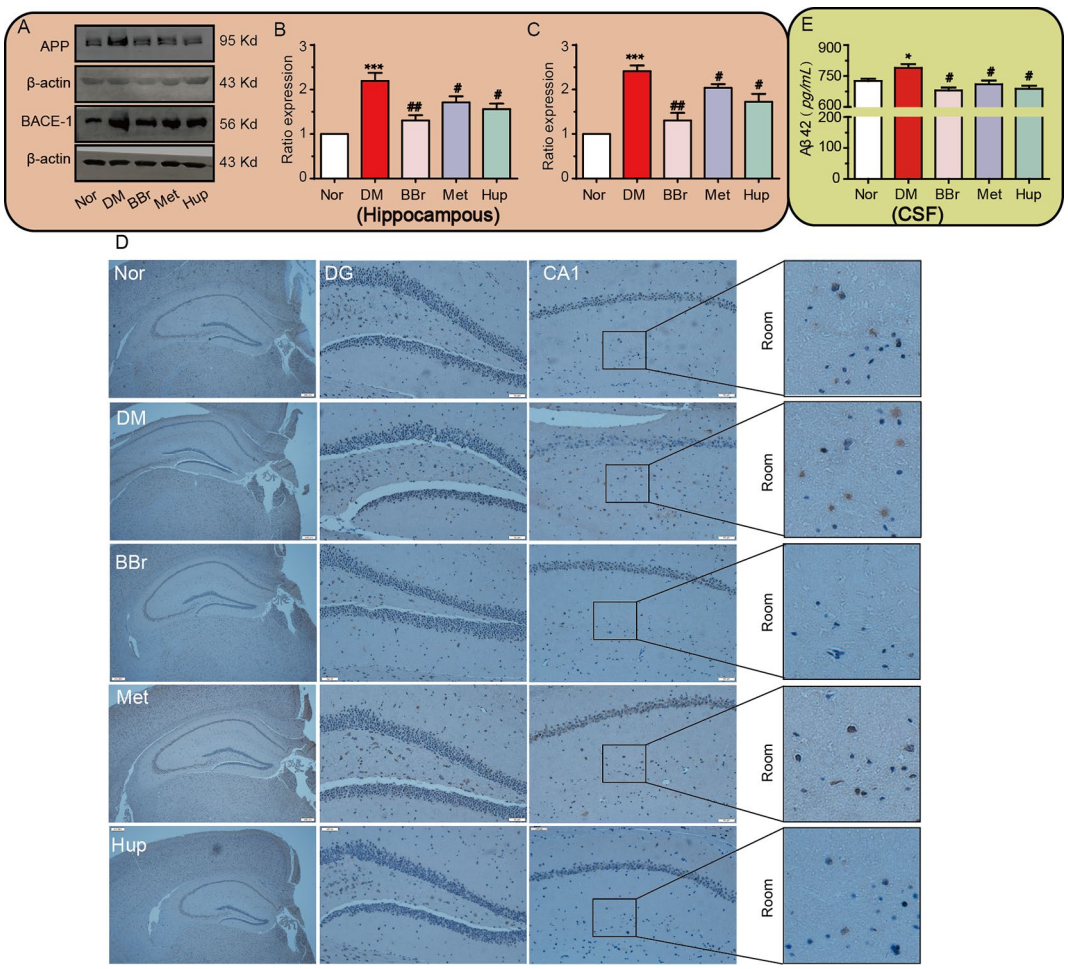

FIGURE 6 | Berberine improves APP misprocessing in the hippocampus. **(A–C)** APP and BACE-1 expressions were assessed by Western-blot, n = 3. The senile plaque formation is evaluated by Congo red staining, n = 3 **(D)**. Aβ 42 levels in CSF were detected using ELISA kits, n = 8–12**(E)**. $^{*}P < 0.05$ vs. Nor; $^{***}P < 0.001$ vs. Nor; $^{\#}P < 0.05$ vs. DM; $^{\#\#}P < 0.01$ vs. DM.

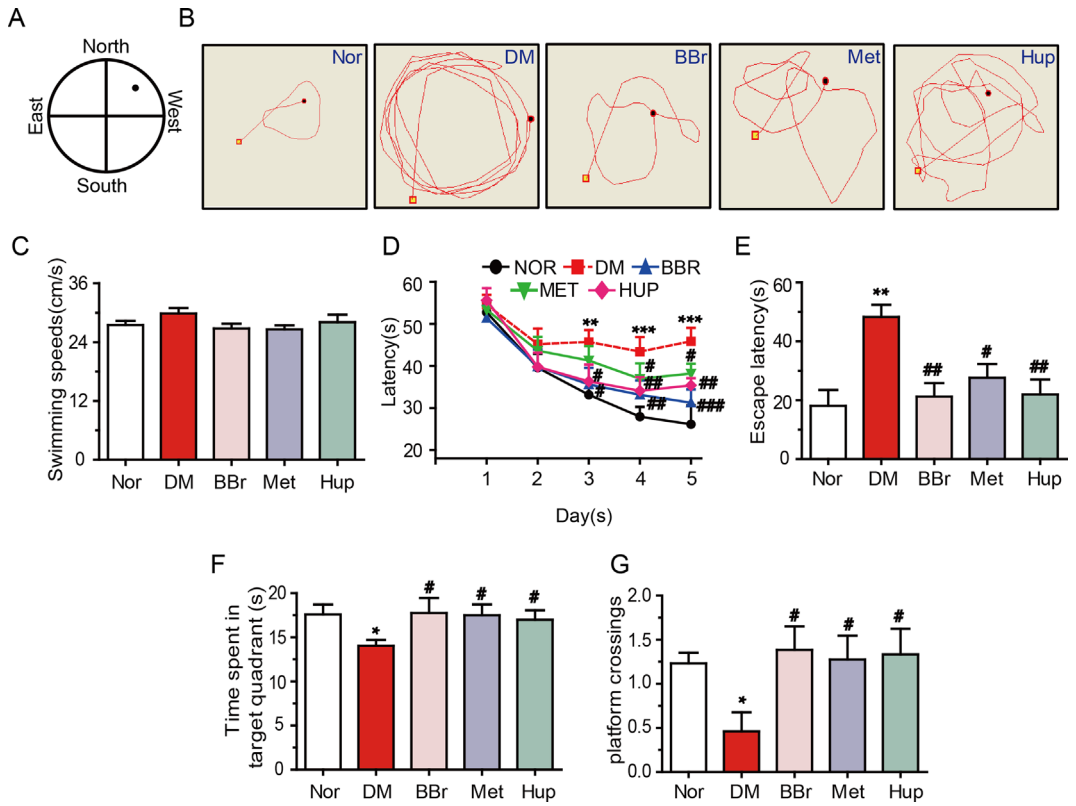

FIGURE 7 | Berberine ameliorates DM-induced cognitive impairment. **(A)** MWM schematic. **(B)** The track of rats in MWM. The swimming speed **(C)** and escape latency analysis in the navigation training trials **(D, E)**. Time spent in target quadrant **(F)** and platform crossing **(G)** in the training trials. n = 8–12. $^{*}P < 0.05$ vs. Nor; $^{**}P < 0.01$ vs. Nor; $^{***}P < 0.001$ vs. Nor; $^{\#}P < 0.05$ vs. DM; $^{\#\#}P < 0.01$ vs. DM; $^{\#\#\#}P < 0.01$ vs. DM.

Meanwhile, AD and DM share several similar molecular processes, which suggests that both may have common pathological characteristics (Mushtaq et al., 2015). Insulin dysfunction, hyperglycemia, impaired cholinergic system, and inflammation in DM may affect synaptic plasticity, learning and memory, and ultimately result in AD. Berberine has versatile functions, such as hypoglycemic, cholesterol-lowing, anti-bacterial, anti-inflammation, clearance of oxygen radical, and a well-documented effect against memory deficit (Kumar et al., 2015; Cicero and Baggioni, 2016). However, the molecular mechanism and the effectiveness of treatment require more exploration studies. Our data showed that berberine mainly ameliorated spatial learning and memory dysfunction *via* alleviating cholinergic neurological disorders in this study.

It has been acknowledged that DM shows hyperglycemia/hyper-insulin in peripheral tissues (Joubert et al., 2018). Thus, we detected the fasting blood glucose/insulin level and found that berberine significantly decreased the elevated peripheral blood glucose/insulin in DM rats. Simultaneously, our results demonstrated that insulin levels in CSF were reduced in DM rats, whereas were normalized by the hypoglycemic agents such as berberine or metformin and the cholinesterase inhibitor huperzine-A. Furthermore, we measured the level of 18F-FDG *in vivo* and found that the transport of glucose from the periphery to the CNS maybe significantly reduction, and which also indicated that the cell activities were inadequate in the brain. The reason for this may be that DM-induced cerebral vascular disease impairs the blood brain barrier (BBB) and glucose application impairment. Many documents have reported chronic hyperglycemia, which resulted in thickening of the capillary basement membrane in the BBB and narrowing of the lumen (Williamson et al., 1988; Carlson et al., 2003). Moreover, lipid metabolism disturbance is a common occurrence in diabetic patients, which results in higher blood viscosity and lower blood flow (Cho et al., 2008). Hence, these may impair saturation transport mechanism of glucose and insulin, and reduce cerebral blood flow. However, long-term effects will inevitably affect the glucose metabolism of neuron (Marioni et al., 2010; Noh et al., 2014; Antunes et al., 2015). Neurons are highly dependent on glucose energy supply. The impairment on glucose metabolism of neuron accelerates disease deterioration and DE formation and studies should adopt this factor to illustrate the detailed mechanism. Fortunately, berberine can attenuate the loss of glucose transport or activate the cell activity, and this effect is similar to those of huperzine-A and metformin. These results demonstrated that berberine may facilitate glucose metabolism to maintain normal brain function.

Neuroinflammation contributes to the initiation and subsequent development of neurodegenerative disorders. In the

brain, two types of glial cells, astroglia and microglia, are major contributors to neuroinflammatory processes (Tribouillard-Tanvier et al., 2012). Thus, microglia has always been considered as professional macrophage or resident immune cell within the brain. Astroglia, which represents most abundant glial cell population, has recently attracted considerable attention for studies on crucial neuroinflammatory processes. Astroglia activation produces numerous cytokines, such as IL-1β, and IL-6 (Wilson et al., 2002; Sofroniew, 2015; Dhanda et al., 2018). Increasing evidence suggests that berberine can inhibit inflammatory response in peripheral tissue of diabetes (Jeong et al., 2009; Zhou et al., 2009; Amasheh et al., 2010), but no reports on the anti-inflammatory activities of berberine in CNS have been documented. Our results exhibit that IL-1β, IL-18 and TNF-α levels in serum and hippocampus were notably increased in DM rats. The phosphorylation of IKK and NF-KB, the downstream molecules of inflammatory signaling pathways, were also upregulated in hippocampus. We also observed that GFAP was significantly increased in the ventral region of the hippocampus, suggesting that astroglia was activated in the hippocampus of DM rats. Berberine reduced the GFAP expression, cytokine levels, IKK and NF-KB phosphorylation, implying that this drug may attenuate inflammation by inhibiting astroglia activation in the hippocampus of DM rats. The "CAP" regulates inflammation mainly through the ACh-α7nAChR interaction. Interestingly, CNS or peripheral inflammation can be inhibited by the activation of a7nAChR (Revathikumar et al., 2016; Lu et al., 2017; Kong et al., 2018). In the peripheral blood and hippocampus, berberine increased ACh level and inhibited AChE activity in DM rats. Meanwhile, we found that berberine also upregulated α7nAChRs expression in the hippocampus of DM rats. These results suggested that berberine may improve the cholinergic signaling pathway, which is equivalent to the cholinesterase inhibitor huperzine A.

Numerous epidemiological and experimental studies showed that individuals with diabetes have a higher risk of developing AD, providing a substantial link between DM and AD (Jimenez-Palomares et al., 2012). Compelling evidence supported that accumulation of Aβ42 can accelerate cognitive impairment in double-transgenic mice (Aso et al., 2015; Guo et al., 2015). Given that AChE induced Aβ fibril formation (Pradhan et al., 2018), as in previous experiments, we have confirmed that berberine reduces the erroneous processing of APP in mPFC. On this basis, we can infer that berberine fulfills its protective effects by lessening Aβ deposition. To clarify the mechanism, we examined the related molecules of Aβ-produced in diabetic hippocampus. Remarkable up-regulation of APP, BACE-1 and Aβ42 were observed in the hippocampus. After treatment with berberine, metformin or huperzine-A, these elevated trends were effectively suppressed. Similarly, Congo red staining was used to further verify that Aβ42 deposit response in the ventral hippocampus of diabetic rats was enhanced. The changes of Aβ42 deposit were attenuated after the drug treatment. Collectively, our findings demonstrated that berberine has the potential of removing Aβ42.

Long-term insulin resistance in brain leads to the formation of Aβ plaque and development of AD, and this phenomenon also appear in DM (Kim and Feldman, 2015). Conversely, impaired cholinergic system (Okamoto and Nishimura, 2015), formation of tau protein hyperphosphorylation (Wang et al., 2018) and pro-inflammatory events (Petersen and Shulman, 2018) were all conducive to insulin signaling deficit. Insulin resistance and massive Aβ appeared in the medial prefrontal cortex of diabetic rats in our previous data, which were ameliorated by berberine (Chen et al., 2017). In our results, reference learning and procedural memory were impairment in DM rats. The escape latency in positioning navigation experiments was significantly prolonged, and the time spent in the target exploration area in the space exploration experiment was significantly shortened. This finding fully explained the impairment of the maintenance of spatial learning and memory in DM rats. Fortunately, berberine effectively improves cognitive impairment. Collectively, current data showed that berberine alleviates the inflammatory, cholinergic and insulin signaling deficits in diabetic hippocampal tissue, and exhibits a protective effect on spatial learning and memory impairment.

It is well known that metformin was used to cure T2DM through the amelioration of insulin resistance. Huperzine-A, an inhibitor of AChE, is usually used to treat AD. Several studies and the present results showed that metformin can reverse diabetes-induced cognitive impairment. In our previous studies, metformin showed anti-inflammation effect, but this function is not through "GAP," which indicates

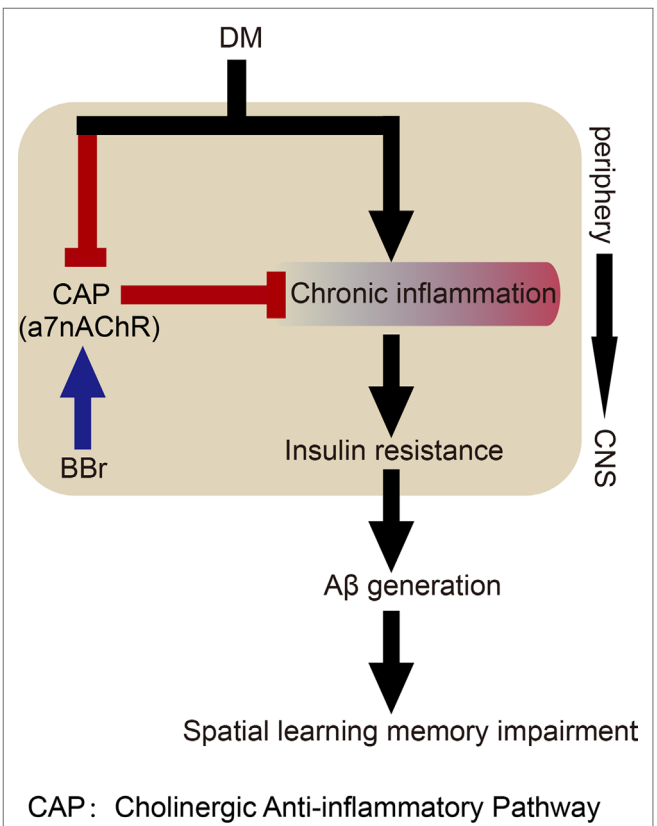

FIGURE 8 | Hypothetical mechanism of berberine to ameliorate impairment of spatial learning and memory in DM rats.

that another signaling pathway participates in the anti-inflammation function. Huperzine-A can activate "GAP" by inhibiting AChE and increasing a7nAChR expression in the CNS. However, Huperzine-A cannot inhibit inflammation generation and have no attenuation to insulin resistance in the periphery. A number of compounds in Chinese herbal medicine exhibit attractive potency involving the modulation of intracellular signaling pathways of inflammation, such as flavonoids (Chen et al., 2018), alkaloids (Chang, 2017), polyphenols (Teng and Chen, 2018), and terpenoids (Teng et al., 2018). It is well known that berberine shows its safety and efficiency in several clinical trials and can be used to treat various metabolic diseases, such as cardiovascular diseases (Feng et al., 2019), nonalcoholic hepatic steatosis (Zhu et al., 2019), nervous systems diseases (Jiang et al., 2015), and kidney disease (Qin et al., 2019). In our studies, berberine can not only activate "GAP" in the CNS, but also activate in the periphery. Besides, berberine alleviates insulin resistance, and modulates glucose metabolism in the CNS and periphery.

In conclusion, our study uncovers that berberine may primarily inhibit astrocyte activation by affecting glucose metabolism and cholinergic disturbance in the periphery and CNS, thereby alleviating inflammatory response and insulin resistance, and finally ameliorate spatial learning and memory impairment (**Figure 8**). The versatility of berberine may help us to elucidate the mechanism in diabetes-induced cognitive impairment through crosstalk between signaling pathways.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

ETHICS STATEMENT

The study protocol was in agreement with Animal Care and Use Committee affiliated to the Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

KW and QC performed most of the experiments. NW and YL participated in data analyses and the discussion of the manuscript. RZ, JW, XZ, and DG collected the samples. CL and JC participated in its design and coordination. All authors read and approved the final manuscript.

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Taeumjowi-tang, a Traditional Korean Sasang Remedy, Improves Obesity-Atopic Dermatitis Comorbidity by Regulating Hypoxia-Inducible Factor 1 Alpha

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Atopic dermatitis (AD) is an inflammatory disease of the skin, resulting from an immune dysfunction, that often occurs as a comorbidity of obesity. This investigation evaluated the capacity of Taeumjowi-tang (TJT), a Korean herbal formulation from the Sasang medical tradition to influence prognostic features of AD and obesity in a mouse model. Here, obesity and AD were induced by a high-fat diet (HFD) and 1-fluoro-2,4-dinitrobenzene (DNFB). Following an 8-week HFD regimen and 4 weeks of DNFB administration, the comorbid (CO) group manifested increased body weight and AD-like lesions, as compared to normal control (NC) mice, while TJT administration diminished these symptoms of obesity and AD. Specifically, TJT treatment reduced epidermal thickness and eosinophil/mast cell infiltration, along with reduction in immunoglobulin E, interleukin (IL)-4, IL-6, and tumor necrosis factor-alpha (TNF-α). It was additionally demonstrated that TJT suppresses HFD/DNFB-associated increase of the inflammation-related nuclear factor-kappa beta (NF-κB) and mitogen activated protein kinase. Moreover, significantly increased levels of hypoxia inducible factor-1 alpha (HIF-1α) protein was observed in CO group versus controls, an increase significantly down-regulated by TJT-treatment. These outcomes suggest that TJT may prove useful in clinical management of obesity-AD comorbidity treatment, an effect that may be due to regulation of HIF-1α expression.

Keywords: comorbidity, atopic dermatitis, obesity, Taeumjowi-tang, hypoxia-inducible factor 1 alpha

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease, characterized by continuously relapsing eczematous lesions (Tollefson and Bruckner, 2014). Over 20% of children in industrialized countries are afflicted with AD and global prevalence of the disorder is on the increase at the time of this writing, especially in developing nations (Flohr and Mann, 2014). AD is a known co-morbidity of

obesity, which is defined as a condition of 'over-nutrition', in which energy intake through diet, exceeds energy expenditure, resulting in storage of surplus energy in adipocytes which accumulates as fat deposits (Park et al., 2013a). Obesity has emerged as a public health challenge, particularly in affluent nations in which sedentary lifestyles combined with overnutrition exacerbate the problem. According to a report by the World Health Organization, over 1.4 billion individuals aged 20 years or older worldwide are overweight (WHO, 2018).

AD and obesity are two totally different diseases clinically. However, the prevalence of the comorbidity of these two diseases is reported to be quite high. According to the National Health and Nutrition Examination Survey of 2005 to 2006, overweight children and adolescents had a higher incidence of AD than that of normal weight children (Visness et al., 2009). Relevant to this, the National Survey of Children's Health in 2007–2008 reported that the prevalence of overweight and obesity were increased in adolescents with AD compared to adolescents without AD (Silverberg and Simpson, 2014). However, despite the epidemiological relevance of these two diseases, the underlying mechanism is not fully understood. Some studies indeed attempted to search for the link between obesity and AD. While Hooper and Hooper suggest heat shock proteins as the main factor (Hooper and Hooper, 2009), Jeong et al. offer adipokines have a key role (Jeong et al., 2015). On the other hand, Savetsky and colleagues suggest the impaired lymphatic function is the main cause (Savetsky et al., 2015). Although most researchers agree that inflammation is the main mechanism which links the two diseases, the detailed mechanism has not been elucidated clearly.

In Sasang constitutional medicine, a unique field of Traditional Korean Medicine, people are categorized into four types based on their structural and functional variations: Taeyang, Taeum, Soyang, and Soeum (Lee et al., 2009a). Among them, a Taeum type person theoretically show higher vulnerability to obesity-allergy comorbidity than other three types. Taeumjowi-tang (TJT) is an herbal medication introduced by Lee Je-ma in Donguisusebowon. TJT, consists of eight herbs and originally is prescribed to treat stomach-related symptoms in Taeum type persons (Park et al., 2012). However, theoretical possibility of the application of this remedy to obesity and AD leads to clinical use of TJT nowadays. Several studies have reported that TJT showed anti-obese effects in rodents (Park and Cho, 2004; Lee et al., 2009b), and a 12-week trial of 102 participants revealed its potential as a possible safe anti-obesity treatment (Park et al., 2013b). TJT may also be prescribed for AD in Taeum type patients, and Sun et al. have even shown some positive results from a follow-up study (Sun et al., 2004). However, its effect on obesity-AD comorbidity has not been reported to date.

Because we expected TJT to be an efficient treatment for obesity-AD comorbidity, we established a basic level obesity-AD comorbidity mouse model by feeding high fat diet (HFD) while administrating 1-fluoro-2,4-dinitrobenzene (DNFB), an immune sensitizing agent widely used to study AD-like contact dermatitis (Galli and Tsai, 2012). Then, the effect of TJT was confirmed by assessing the histomorphological changes, mast cell infiltration, and also the AD-related markers in the serum and skin. Furthermore, we evaluated the role of hypoxia-inducible factor-1 alpha (HIF-1α) in obesity-AD comorbidity, and then investigated whether it is regulated by TJT treatment.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols involving the use of animals conform to the NIH guidelines (Guide for the Care and Use of Laboratory Animals, 8th edition). Animal Care and Use Committee of the Institutional Review Board of Kyung Hee University (confirmation number: KHUASP(SE)-12-036) has approved all the animal experiments.

Chemical Reagents

TJT was purchased from I-World Pharm. Co. (Incheon, Republic of Korea). The herbal constituents of TJT are shown in **Table 1**. The antibodies for p38 (sc-7149), NF-κB (sc-372), p-IκB (sc-8404), histone H3 (sc-10809), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p-p38 (#4511), p-ERK (#4695), p-JNK (#9255), and JNK (#9252) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ERK antibody (AP0484) was obtained from Bioworld Technology (St. louis Park, MN, USA), and anti-HIF-1α antibody (610958) was from BD Bioscience (San Jose, CA, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium penicillin/streptomycin/glutamine and bovine serum (BS) were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Control siRNA (sc-37007) and siHIF1A (sc-35561) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and LPS was from Sigma Aldrich Inc. (St Louis, MO, USA).

TABLE 1 | Herbal constituents of TJT (of total 25 mg).

Herbal constituent	Taxonomic name	Dose
Coicis semen	*Coix lacryma-jobi* var. *ma-yuen (Rom.Caill.) Stapf*	3.75 mg
Castaneae semen	*Castanea crenata Siebold & Zucc.*	3.75 mg
Raphani semen	*Raphanus raphanistrum subsp. sativus (L.) Domin*	2.5 mg
Schisandrae fructus	*Schisandra chinensis (Turcz.) Baill.*	1.25 mg
Platycodi radix	*Platycodon grandiflorus (Jacq.) A.DC.*	1.25 mg
Acori graminei rhizoma	*Acorus gramineus Aiton*	1.25 mg
Ephedra herba	*Ephedra sinica Stapf*	1.25 mg
Liriopis tuber	*Liriope muscari (Decne.) L.H.Bailey*	1.25 mg

Ultra Performance Liquid Chromatography (UPLC) Analysis

Liquid chromatography-mass spectrometry system consisted of a Thermo Scientific Vanquish UHPLC system (ThermoFisher Scientific, CA, USA) with Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 μm) column (Agilent) and a triple ToF 5600+ mass spectrometer system (Triple ToF MS) (SCIEX, Foster City, CA, USA). Triple TOF MS, equipped with a DuospraTM ion source, was used to complete the high resolution experiment.

Animal Experiments

Four-week-old male C57BL/6J mice weighing 17–18 g were purchased from the Dae-Han Experimental Animal Center (Eumsung, Republic of Korea). The mice were maintained for 1 week prior to the experiments in a 12-h light/dark cycle at a humidity of 70% and a constant temperature of 23 ± 2°C. After acclimation, the animals were divided into five groups (n = 7 per group): (a) a normal control (NC) group fed normal chow diet (CJ Feed Co., Ltd., Seoul, Republic of Korea); (B) an AD (DNFB) group fed normal chow diet and applied with DNFB 3 times/week, starting on week 5; (C) and obesity (HFD) group fed with 60% high-fat diet (HFD) (Rodent diet D12492, Research diet, New Brunswick, NJ, USA) for 8 weeks; (D) an obesity-AD comorbidity (CO) group fed a HFD for 8 weeks with a 4-week-DNFB application starting on week 5; and (E) a TJT group which were fed a HFD for 4 weeks to induce obesity, and then fed for 4 additional weeks with HFD plus TJT mixed in diet (125 mg/kg/day) while DNFB was applied starting on week 5, same as the CO group. DNFB sensitization was induced by repeated application of 150 μl of 0.35% DNFB in acetone-olive oil combination (3:1) on the shaved dorsal skin three times a week. The body weight and food intake amount were recorded every other day. At the end of the experiment, the animals were fasted overnight, anesthetized with CO_2 asphyxiation, and the dorsal skin was removed and divided in half; one half was fixed in 10% formalin and embedded in paraffin for histomorphological assays, and the other half was stored at -80°C for further assays.

Serum Analysis

Serum was separated by centrifugation at 4,000×*g* for 30 min immediately after blood collection *via* cardiac puncture. Total cholesterol, low density lipoprotein (LDL) cholesterol, triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine levels were assessed using enzymatic colorimetric methods performed by Seoul Medical Science Institute (Seoul Clinical Laboratories, Seoul, Korea).

Dermatitis Score

Severity of AD-like lesions was evaluated according to the SCORAD (SCORing Atopic Dermatitis) index (Oranje et al, 2007). This scoring was based on the severity of erythema, edema/papulation, oozing/crusts, excoriations, and lichenification each on the scale from 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe). Overall score was determined by summing all individual scores. The SCORAD assessment was performed after group blinding.

Hematoxylin and Eosin (H&E) Staining, Toluidine Blue Staining, and Immunofluorescence (IF) Assay

The dorsal skin specimens were prepared in 5-μm-thick formalin-fixed, paraffin-embedded tissue sections as previously described (Youn et al., 2017). Five slides per mouse were prepared for the analyses. The sections were deparaffinized in xylene and rehydrated in serial alcohol. After treatment of 150 μl of a 0.1% trypsin working solution (consisting of trypsin 0.4 mL, calcium 0.01 g, and chloride 0.01 g in D.W. 7 mL) for 15 min, the sections were blocked using fetal bovine serum (FBS). For H&E staining, the sections were stained in hematoxylin for 5 min, and then washed with water for 5 min, followed by 30 s of eosin staining. For toluidine blue staining, the sections were stained in toluidine blue for 5 h, and then washed with water. For the IF assay, sections were incubated in 4°C overnight with a 1:50 dilution of the primary antibody and then incubated at room temperature for 30 min with a 1:500 dilution of the Alexa Fluor 633 conjugate (Pierce Thermo Scientific, Rockford, IL, USA). Then, the sections were dehydrated and mounted by routine methods. Five slides for each group were randomly chosen and analyzed by a researcher who was blinded from the experiment. The slides were examined using the Olympus IX71 Research Inverted Phase microscope (Olympus Co., Tokyo, Japan), and the density was measured with the ImageJ 1.47v software (National Institute of Health, Bethesda, MD, USA).

RNA Extraction and Real-Time RT-PCR

RNA extraction of dorsal skin was performed using the GeneAllR RiboEx total RNA extraction kit (GeneAll Biotechnology, Seoul, South Korea), and Real-time RT-PCR was performed with the Power cDNA synthesis kit (iNtRON Biotechnology, Seongnam, Kyunggi, South Korea), SYBR Green Power Master Mix (Applied Biosystems, Foster City, CA, USA) and the Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions as previously described (Lim et al., 2016). The primers used in this study are shown in **Table 2**.

TABLE 2 | Primer information used in this study.

Gene name	Sequence	Product size	Accession no.
mTnfa	Sense: 5′-CTGTGAAGGGAATGGGTGTT-3′ Antisense: 5′-GGTCACTGTCCCAGCATCTT-3′	180 bp	U69613.1
mll4	Sense: 5′-ACAGGAGAAGGGACGCCAT-3′ Antisense: 5′-GAAGCCCTACAGACGAGCTCA-3′	74 bp	NM_021283.2
mll6	Sense: 5′-GAGGATACCACTCCCAACAGACC-3′ Antisense: 5′-AAGTGCATCATCGTTGTTCATACA-3′	117 bp	NM_031168.2
mGapdh	Sense: 5′-CCAGGTTGTCTCCTGCGACT-3′ Antisense: 5′-ATACCAGGAAATGAGCTTGACAAAGT3′	80 bp	NM_001289726.1
mHif1a	Sense: 5′-AGCTTCTGTTATGAGGCTCACC-3′ Antisense: 5′-TGACTTGATGTTCATCGTCCTC-3′	369 bp	NC_000078.6

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assay was performed under modifications of methods by Kim et al. (2017). Skin tissue was homogenized in 1.5 mL extraction buffer (containing 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) per gram of tissue, centrifuged at 13,000×*g* for 10 min at 4°C, and the supernatant was used for ELISA analysis. Serum IgE was measured with a mouse IgE ELISA kit (Abcam Inc., Cambridge, MA, USA) from total serum samples (n = 7 per group), and TNF-α and IL-6 expressions of the dorsal skin tissues were measured with a mouse TNF-α ELISA kit (Pierce Thermo Scientific, Rockford, IL, USA) and a mouse IL-6 ELISA kit (BD Bioscience, San Jose, CA, USA). Color development was measured by a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

Western Blot Analysis

Western blot analyses were performed as described previously (Lee et al., 2016). Briefly, prepared skin tissues were cut into pieces and homogenized with the Bullet Blender homogenization kit (Next Advance Inc., Averill Park, NY, USA). Homogenized tissues were lysed in ice-cold RIPA buffer. Nuclear extracts for NF-κB evaluation were prepared using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Pierce Thermo Scientific, Rockford, IL, USA). After the protein concentration determination, equal amounts of total protein were resolved by 6–12% SDS polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane. After an overnight incubation with the primary antibodies (NF-κB, p-ERK, ERK, p-JNK, JNK, p-p38, p38, and HIF-1α, the blots were then incubated with proper horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) for 1 h at RT. The chemiluminescent intensities of the protein signals were quantified with the ImageJ 1.47v software (National Institute of Health, Bethesda, MD, USA).

Adipocyte Cell Culture, Differentiation, and Conditioned Medium (CM) Preparation

Murine 3T3-L1 mouse embryo fibroblasts (American Type Culture Collection, Rockville, MD, USA) were cultured in 10% BS plus DMEM and differentiated in 10% FBS plus DMEM containing 0.5 mM IBMX, 1 μM dexamethasone and 1 μg/mL insulin (MDI) at 37°C, 5% CO2 as in a previous report (Lim et al., 2016). After 8 day of full differentiation into mature adipocytes, the cells were discarded, and culture media was saved for CM application.

Establishment of Obesity-AD Comorbidity *In Vitro* Model and siRNA Transfection

Human keratinocytes HaCaT cells were purchased from CLS CellLines Service (Eppelheim, Baden-Württemberg, Germany). HaCaT cells were cultured in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin in a humidified atmosphere of 37°C, 5% CO_2 as previously described (Kee et al., 2017) 59.5 μg/mL of LPS was treated for 48 h to HaCaT cells cultured in 50% adipocyte CM in fresh culture medium to mimic an obesity-AD condition. TJT was treated for 24 h, starting at 24 h after CM supplementation. siHIF1A was applied for 24 h prior to TJT treatment following the manufacturer's instructions.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM). Significant differences between the groups were determined with the Student's t-test and one-way ANOVA followed by post-hoc Tukey's multiple comparisons tests. All statistical analyses were performed with SPSS statistical analysis software version 11.5 (SPSS Inc., Chicago, IL, USA). A probability value of $P < 0.05$ was considered as statistical significant.

RESULTS

Chromatographic Analysis of TJT

First, we used the UPLC method to analyze components of TJT. As a result, we could identify several components including ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine, sinapine, platycodin D, schizandrin, schizandrin A, wuweizisu B (**Figure 1**, **Table 3** and **Supplementary Table S1**).

TJT Ameliorates Obesity in HFD/DNFB-Induced Comorbidity Mice

Based on previous publications (Kimber et al., 2001; Gould et al., 2003; Katagiri et al., 2007), we established an obesity-AD

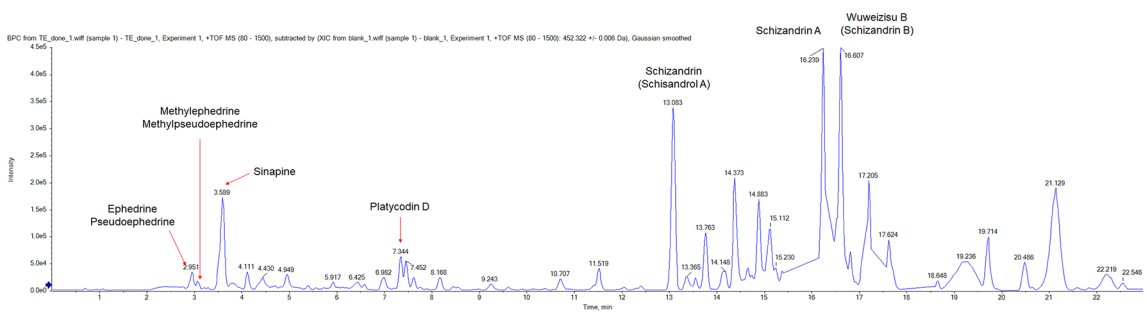

FIGURE 1 | The UPLC analysis of TJT. The representative chromatogram of TJT and identification of its components. Ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine, sinapine, platycodin D, schizandrin, schizandrin A, and wuweizisu were identified.

TABLE 3 | Identified components of TJT.

No.	Retention time (min)	Characteristic ion	Herbal constituent	Proposed structure
1	2.951	[M+H]+	*Ephedra sinica Stapf*	(Pseudo) Ephedrine
2	3.094	[M+H]+		Methyle (pseudo) ephedrine
3	3.589	[M+H]+	*Raphanus raphanistrum subsp. sativus (L.) Domin*	Sinapine
4	7.344	[M+H]+	*Platycodon grandiflorus (Jacq.) A.DC.*	Platycodin D
5	13.083	[M+H]+	*Schisandra chinensis (Turcz.) Baill.*	Schisandrol A
6	16.239	[M+H]+		Schizandrin A
7	16.607	[M+H]+		Schizandrin B

comorbidity mouse model by administrating 60% kcal HFD and 0.35% DNFB (**Figure 2A**). As seen in **Figure 2B**, 4 weeks of HFD administration resulted in a significant body weight difference between the normal control and HFD-treated mice (24.87 ± 0.43 g vs. 30.52 ± 1.18 g, respectively). At the start of week 5, the HFD-treated mice were randomly divided into 3 groups (n = 7 per group), and 2 groups were fed HFD, 1 group was fed HFD + TJT, and DNFB application was applied three times a week. After 4 weeks of additional HFD/DNFB administration, the body weight difference among the 3 groups were as follows: the NC group, 26.77 ± 1.31 g; CO group, 34.35 ± 0.86 g, and the TJT group, 32.27 ± 1.07 g (**Figures 2B, C**). Furthermore, TJT treatment reduced the weight of inguinal adipose tissues (**Figure 2D**) and several serum parameters such as triglyceride (**Figure 2F**), total cholesterol

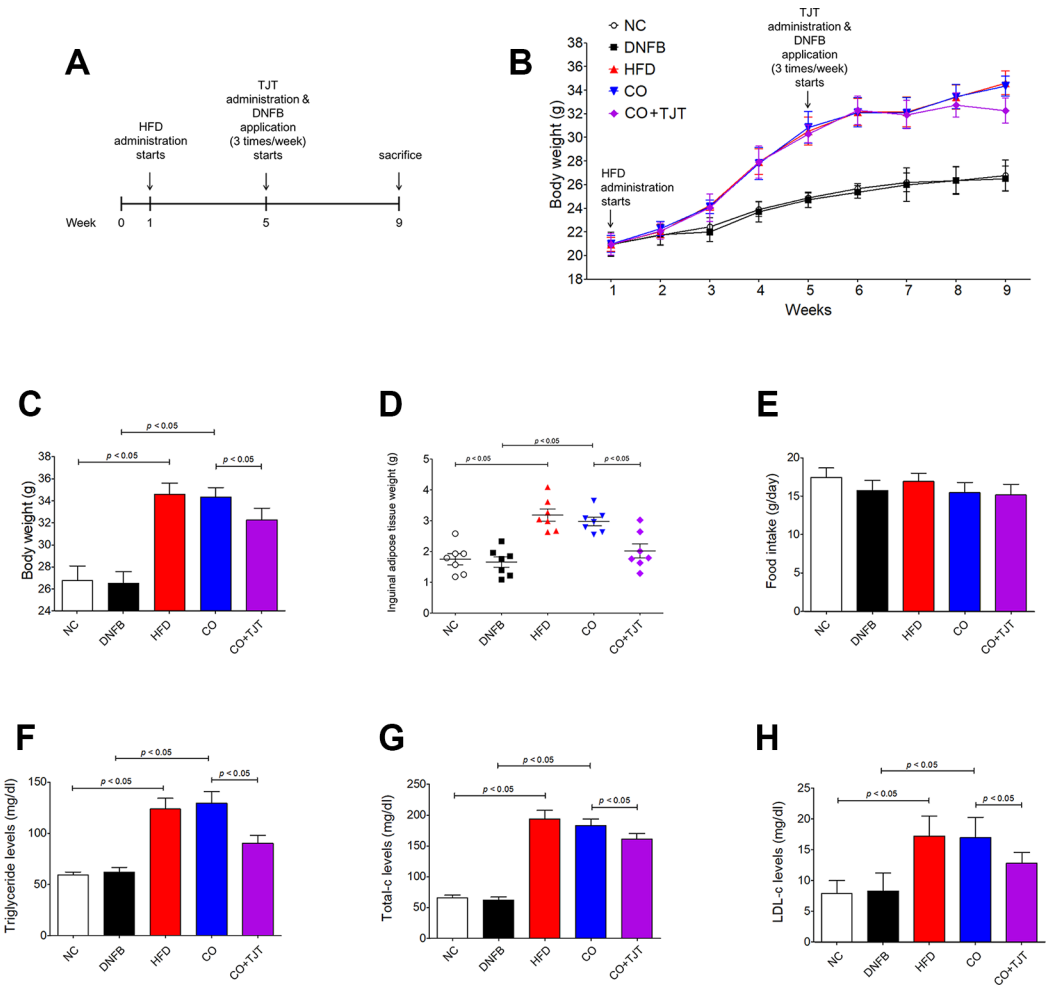

FIGURE 2 | TJT ameliorates obesity-related symptoms in the HFD/DNFB-induced obesity-AD comorbidity mouse model. **(A)** The mice (n = 7 per group) were administered 60% kcal HFD for a total of 8 weeks, while TJT treatment and 0.35% DNFB application (3 times/week) started on week 5. **(B, C)** The body weight changes of the mice were measured every week. **(D)** Inguinal adipose tissue weight of mice was compared. **(E)** Food intake was measured. Plasma levels of **(F)** triglyceride, **(G)** total cholesterol, and **(H)** low density lipoprotein cholesterol were measured. The data are represented as the mean ± SEM. NC, normal control group; DNFB, DNFB-induced AD group; HFD, HFD-induced obesity group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

(**Figure 2G**), and LDL cholesterol (**Figure 2H**), without affecting food intake (**Figure 2E**). On the other hand, to assess whether TJT induced any toxicity in the liver or kidney, we measured serum levels of ALT, AST, and creatinine. As in **Supplementary Figure S1**, TJT did not alter any of these parameters. Thus, we were able to confirm that TJT administration can improve obesity-related symptoms induced by HFD, without displaying neither hepatotoxicity nor nephrotoxicity.

TJT Improves AD-Related Clinical Symptoms in HFD/DNFB-Induced Comorbidity Mice

When visually observed, HFD/DNFB administration increased AD-like symptoms compared to the DNFB only group (**Figure 3A**). Number of scratching was slightly increased. The DNFB group showed 133.57 ± 9.44 times of scratching in 20 min, While the obesity-AD comorbidity group showed 158.86 ± 7.46 times of scratching during the same time length. The SCORAD scores were also increased significantly (12.77 ± 0.67 Vs. 13.62 ± 0.79) as well (**Figures 3B, C**). However, as shown in **Figure 3A**, TJT remarkably ameliorated the clinical symptoms of the HFD/DNFB-Induced AD. the skin lesions were improved by the TJT treatment (**Figure 3A**), While the number of scratching in 20 min and the SCORAD index was significantly decreased in the TJT group when compared to the CO group (scratching, 158.86 ± 7.46 to 119.43 ± 5.48 times per 20 min; SCORAD index, 8.76 ± 0.98 to 13.62 ± 0.79) (**Figure 3C**).

TJT Reduces Serum Immunoglobulin E (IgE) in HFD/DNFB-Induced Comorbidity Mice

Because IgE is one of the most important factors in the pathology of AD (Sismanopoulos et al., 2012), the IgE level in total serum was evaluated. As shown in **Figure 3D**, the CO group showed a noticeable increase in the serum IgE level (1,139.22 ± 145.94 ng/mL) when compared with the NC group (367.28 ± 83.14 ng/mL). This IgE level of the CO group was also significantly higher than that of the AD group (900.97 ± 30.26 ng/mL) as well. The difference of IgE level and AD-related clinical symptoms between the DNFB group and CO group showed basic proof to our first hypothesis: obesity aggravates AD. However, the increased IgE was attenuated by TJT (813.79 ± 96.97 ng/mL), showing its potential as a treatment for obesity-AD comorbidity.

TJT Alleviates Histological Changes in HFD/DNFB-Induced Comorbidity Mice

In order to confirm the protective effect of TJT on obesity-AD comorbidity, we performed an H&E assay. As shown in **Figure 4A** and **Supplementary Figure S2a**, the average epidermal thickness was greater in the CO group than in the NC group (293.94 ± 49.18 μm vs. 73.46 ± 14.15 μm). However, the epidermal hyperplasia was significantly reduced by TJT treatment (115.98 ± 18.70 μm). As mast cell and eosinophil infiltrations are also commonly observed in AD (Stone et al., 1976; Galli and Tsai, 2012; Sismanopoulos et al., 2012; Desai et al., 2013), we next counted the number of eosinophils infiltrated into the skin lesions of mice. The number of infiltrated eosinophils was highly suppressed in the TJT group (213.20 ± 32.04 cells vs. 309.80 ± 32.06 cells in the CO group). Then, we performed a toluidine blue staining assay to evaluate mast cell infiltration in the dermis. Mast cells are closely linked to allergic reactions because they possess a variety of inflammatory mediators such as histamines, which have important roles in AD (Stone et al., 1976; Huber et al., 2002). The increased number of infiltrated mast cells by HFD/DNFB administration was suppressed in the TJT group (**Figure 4B** and **Supplementary Figure S2b**).

TJT Decreases Pro-Inflammatory Cytokine Levels in HFD/DNFB-Induced Comorbidity Mice

Besides elevated IgE several inflammatory mediators such as nuclear factor-κB (NF- κB) (Huber et al., 2002), interleukins (IL)-4 (Furue et al., 1989) and 6 (Toshitani et al., 1993), and mitogen-activated protein kinases (MAPKs) (Pastore et al., 2005) are also known to participate in the pathology of AD. Therefore, we did further experiments on cytokine expressions in the dorsal skin. The levels of IL-4 and 6 and tumor necrosis factor-alpha (TNFα) were evaluated with an ELISA assay. As seen in **Figures 5A–C**, IL-4 and IL-6 and TNFα were highly up-regulated by HFD/DNFB administration (1.94 ± 0.16 pg/mL, 2.11 ± 0.12 pg/mL, 3.71 ± 0.25 pg/mL, respectively) compared to those in the NC group (0.98 ± 0.18 pg/mL, 1.23 ± 0.18 pg/mL, 1.30 ± 0.15 pg/mL, respectively), while TJT treatment successfully suppressed the levels of IL-4 and TNFα (1.46 ± 0.12 pg/mL, 2.97 ± 0.10 pg/mL, respectively) although it could not decrease the level of IL-6 (P = 0.06, 1.72 ± 0.18 pg/mL). In addition, the mRNA levels of these cytokines were decreased by TJT treatment (Tnfa, 0.63-fold; Il4, 0.72-fold; Il6, 0.33-fold) (**Figure 5D**).

TJT Down-Regulates Protein Expressions of NF-κB and MAPKS in HFD/DNFB-Induced Comorbidity Mice

The CO group showed 3.50-fold higher expressions of nuclear NF-κB than that of the NC group. However, in the TJT group, this key inflammatory factor was suppressed down to 54.2% of that of CO mice (**Figure 6A**). In addition, phosphorylation of the MAPKs was also reduced by TJT (**Figure 6B–D**). While ERK, JNK, and p38 MAPKs were highly activated in the CO group when compared to the NC group, however, in the TJT group, phosphorylation levels of these three MAPKs were reduced (p-ERK/ERK, 0.67-fold; p-JNK/JNK, 0.57-fold; p-p38/p38, 0.54-fold), implying suppressed inflammation by TJT treatment.

TJT Decreases HIF-1α Expression in HFD/DNFB-Induced Comorbidity Mice

HIF-1α, a transcription complex that has a key role in hypoxic conditions, can also be expressed in normoxic inflammation (Zhou et al., 2003). Several studies report this factor has a crucial role in inflammatory skin diseases as well (Simonetti et al., 2006; Viemann et al., 2007; Kim et al., 2011a). We therefore evaluated the HIF-1α expression in the dorsal skin lesions. As shown in **Figure 7A**, H*if1a*, the gene which transcripts HIF-1α, was

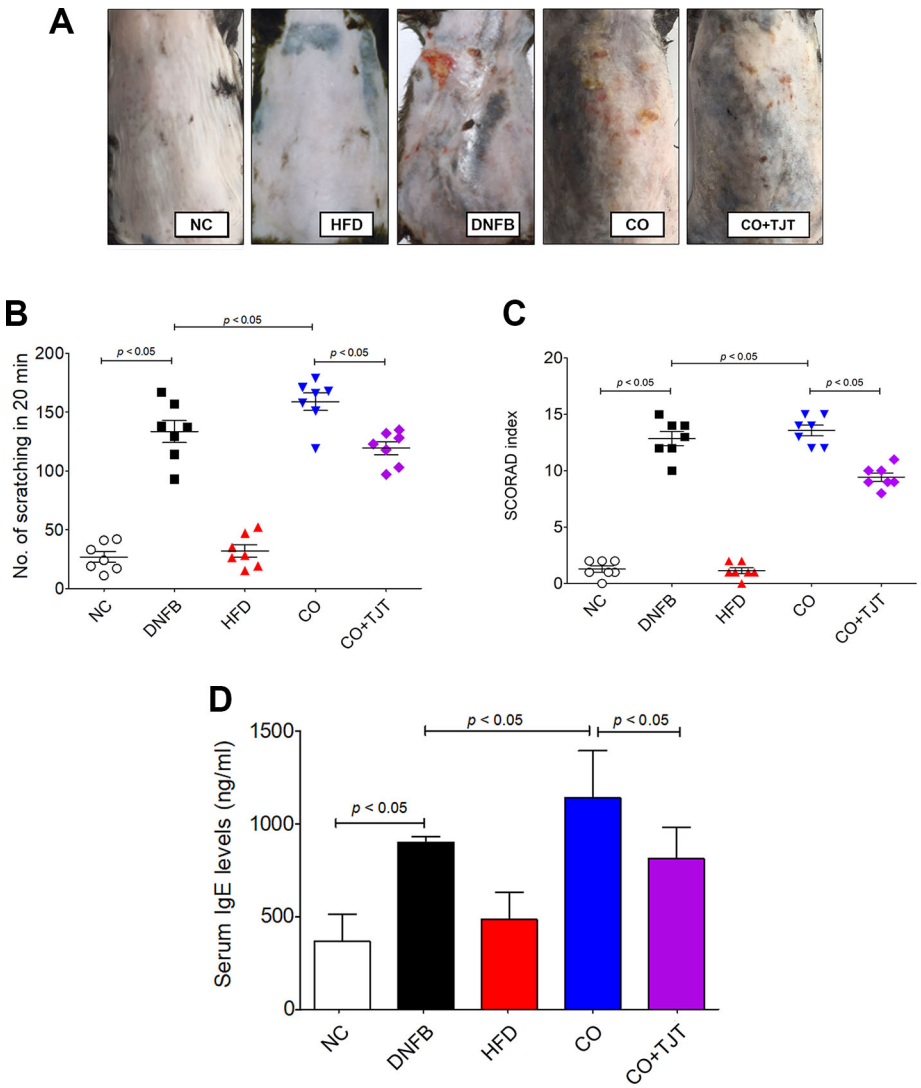

FIGURE 3 | TJT improves AD-related symptoms in the HFD/DNFB-induced obesity-AD comorbidity mouse model. **(A)** The AD-like lesions of the dorsal skin were observed macroscopically, **(B)** the 20-min-scratching behavior and **(C)** the SCORAD index of mice (n = 7 per group) was evaluated. An ELISA assay was performed to evaluate the level of **(D)** serum IgE. The data are represented as the mean ± SEM. NC, normal control group; DNFB, DNFB-induced AD group; HFD, HFD-induced obesity group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

markedly increased in the CO group. This elevation of *Hif1a* was suppressed by TJT treatment. Similar results were observed in western blot assays. The protein expression of HIF-1α was significantly altered in the skin tissues of CO group than in those of DNFB group (2.02-fold increase), which already showed elevated HIF-1α levels when compared to the NC group (**Figure 7B**). Therefore, we assumed HIF-1α might be the crucial factor which worsens the AD-like condition in obesity-AD comorbidity. While protein expression of HIF-1α was up-regulated by HFD/DNFB administration as expected, this increase was suppressed in the TJT group shown by western blot assay. Next, we performed an IF assay to examine changes in HIF-1α in the dorsal skin tissue. As seen in **Figure 7C**, the IF assay showed that HIF-1α expression, mostly located in the cytosol close to the nucleus, was highly increased in the CO group. The elevated HIF-1α was reduced by TJT administration.

TJT Ameliorates Obesity-AD Comorbidity by Regulating HIF-1α

To elucidate the role of HIF-1α in the pathogenesis of obesity-AD comorbidity, we established a comorbidity *in vitro* model. To mimic the comorbid condition of obesity and AD, HaCaT human keratinocytes were cultured in adipocyte conditioned media (CM) and inflammatory response was induced by treating lipopolysaccharide (LPS). As shown in **Figure 8A**, LPS-induced elevation of HIF-1α expression was further increased when the cells

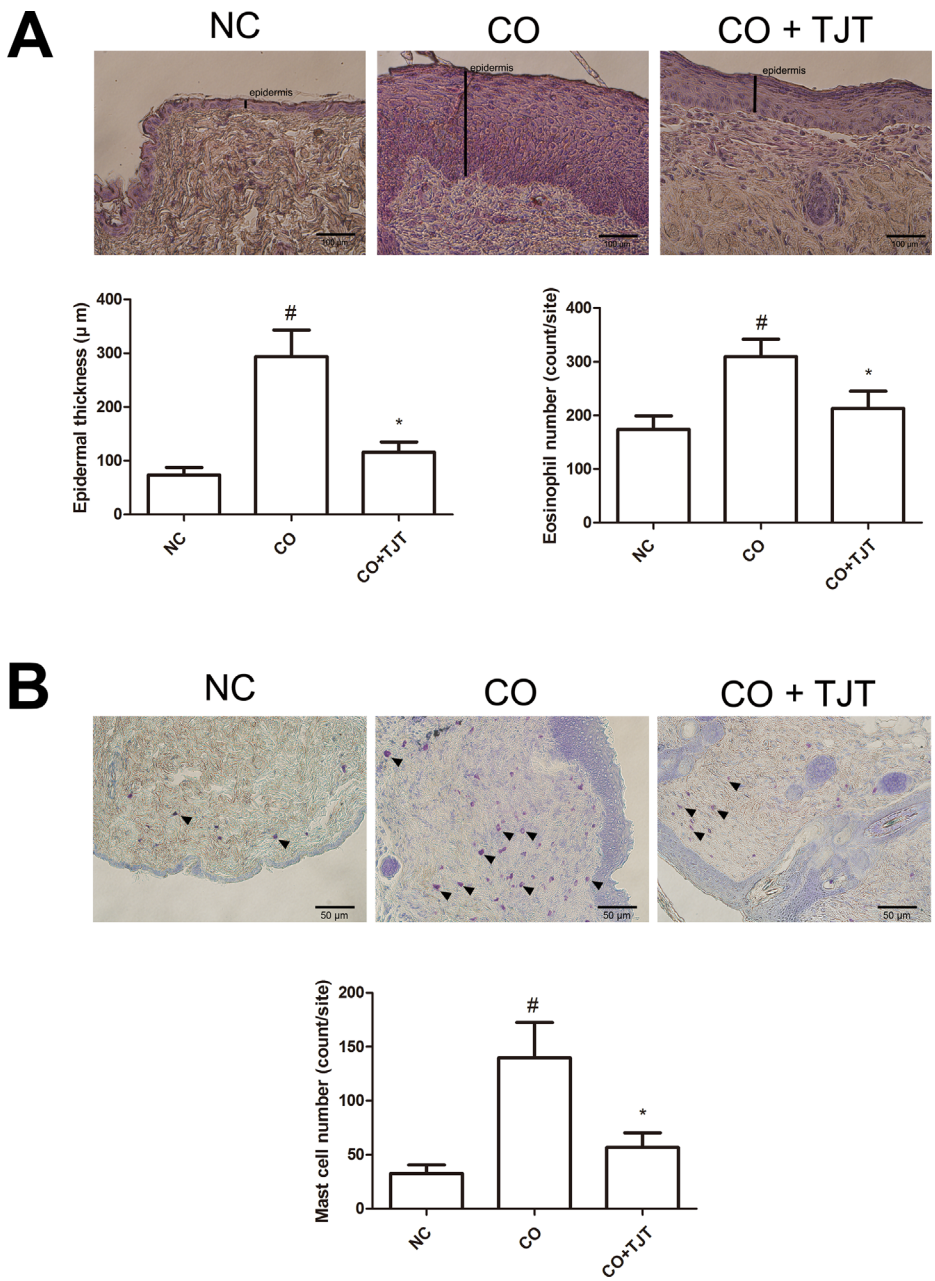

FIGURE 4 | TJT decreases epidermal hyperplasia, eosinophil infiltration, and mast cell infiltration in the skin lesions of the HFD/DNFB-induced obesity-AD comorbidity mouse model. **(A)** An H&E assay was performed and microscopically observed at 400× magnification, and the epidermal thickness and eosinophil number were measured. **(B)** A toluidine blue staining assay was performed and microscopically observed at 200× magnification, and the infiltrated mast cell number was counted. Epidermal thickness, eosinophil number, and mast cell number were evaluated in five random slides per group. The data are represented as the mean ± SEM. $^{\#}P < 0.05$ when compared to NC; $^{*}P < 0.05$ when compared to CO. NC, normal control group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

were cultured in CM, implying the impact of adipocytes on the inflammatory response of keratinocytes. An IF staining assay revealed that CM treatment induced the localization of NF-κB into the nucleus of HaCaT cells, which was inhibited in TJT-treated cells (**Figure 8B**). TJT treatment regulated NF-κB-mediated inflammatory signaling by suppressing HIF-1α in the vitro model of obesity-AD comorbidity in a dose-dependent manner.

Next, we used the small interfering RNA (siRNA) method to confirm the role of HIF-1α during the action mechanism of TJT in ameliorating obesity-AD comorbidity. When *HIF1A* gene was knocked down by *siHIF1A*, TJT failed to decrease inflammatory markers such as nuclear NF-κB and p-IκB (**Figure 8B**). These results suggest the improving of TJT on obesity-AD comorbidity is dependent, at least partially, on HIF-1α pathway.

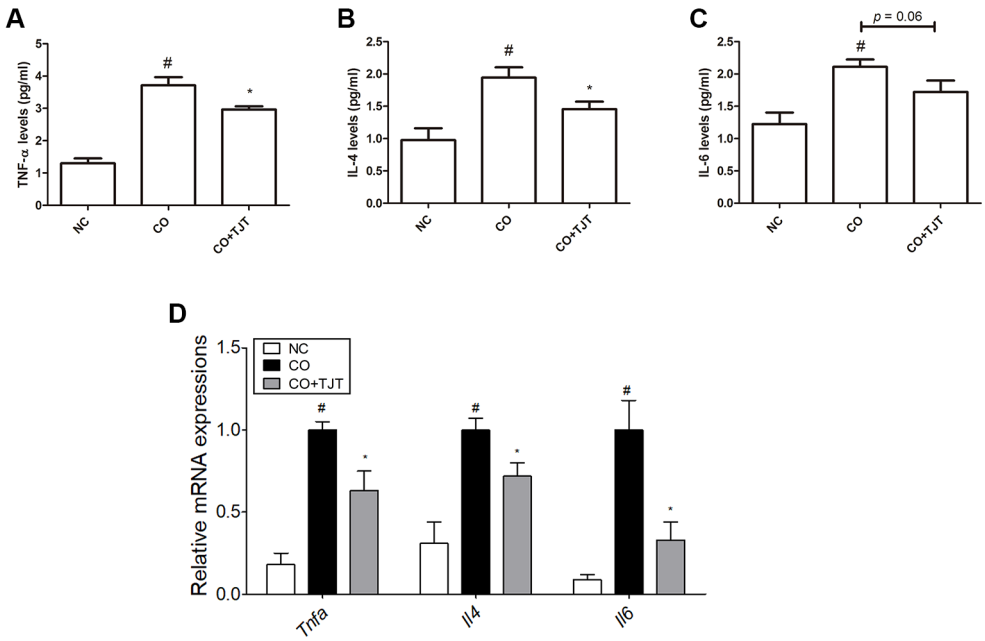

FIGURE 5 | TJT reduces pro-inflammatory cytokines in the HFD/DNFB-induced obesity-AD comorbidity mouse model. An ELISA assay was performed to evaluate the levels of dorsal skin tissue-expressions of **(A)** TNFα, **(B)** IL-4, and **(C)** IL-6. **(D)** A Real-Time RT-PCR assay was performed to evaluate the mRNA levels of *Tnfa*, *Il4* and *Il6* in the dorsal skin tissue of mice (n = 7 per group). The data are represented as the mean ± SEM. $^{\#}P < 0.05$ when compared to NC; $^{*}P < 0.05$ when compared to CO. NC, normal control group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

DISCUSSION

Obesity and AD are two separate diseases both etiologically and pathologically. While obesity is a metabolic disorder with excess accumulation of energy, AD is a chronic inflammatory illness of the skin. However, these two diseases often occur together. In addition to the prevalence, the severeness of the problem is quite affected by each other (Visness et al., 2009; Silverberg and Simpson, 2014). However, the exact correlation mechanism of the two is yet to be discovered. Katagiri et al. tried to provide evidence for the effect of obesity on inflammatory responses by the administration of trinitrochlorobenzene (TNCB) or ovalbumin in diet induced obese mice (2007). Based on this experiment model, we established four different obesity-AD comorbidity mouse models. HFD-fed and 0.15% DNFB-applied BALB/c mice did not show any weight gain. On the other hand, obesity was certainly induced in C57BL/6J mice, but 0.15% DNFB administration failed to show AD-like dermatitis. By application of 0.5% DNFB, unexpected weight loss in the mice was occurred. Finally, with C57BL/6J mice and administration of HFD plus 0.35% of DNFB, we successfully induced both obesity and AD. The results showed that HFD/DNFB application induced body weight gain and AD-like skin lesions. In addition, serum parameters and clinical AD symptoms was increased in the comorbidity model.

TJT is an herbal remedy which originally is used to treat 'exterior-cold disease by a cold in the esophagus of Taeum type patients (Lee et al., 2009). However, several experimental and clinical reports support the use of TJT as treatment for both obesity (Park and Cho, 2004; Kim et al., 2011b; Park, S., et al., 2013) and AD (Sun et al., 2004). Therefore, we expected TJT to be a potential treatment for obesity-AD comorbidity management. As in our hypothesis, TJT treatment for 4 weeks showed remarkable improvement on HFD/DNFB-induced obesity and AD-like skin lesions. The positive effects of TJT also influenced other changes caused by HFD and DNFB. The epidermal thickness was reduced; eosinophil/mast cell infiltration was decreased, and IgE, TNF-α, IL-4, IL-6, NF-κB, and MAPK phosphorylation were all suppressed. Furthermore, HIF-1α was highly down-regulated by TJT treatment.

Identified first in the 1960s (Johansson and Bennich, 1967), IgE was recognized to be a key factor in several allergic diseases due to its high level in such patients (Johansson, 1969). AD also is not an exception because allergens are taken up by dendritic cells, followed by binding of the allergen-specific IgE to the receptors (FcεRI) in mast cells, which are then stimulated to degranulate and release pro-inflammatory molecules (Cookson, 2004). This important regulator in AD is surprisingly increased in obesity as well, a disease that seemed to be non-relevant to inflammation or allergy (Visness et al., 2009). This fact leaves us a hint for the correlation between obesity and AD. Supporting this clinical phenomenon, some studies have shown the relationship of IgE and obesity in *in vivo* and *in vitro* experiments (Nagai et al., 2012; Ramalho et al., 2012). In the same context, our newly established obesity-AD comorbidity model showed elevated IgE levels by HFD/DNFB application. TJT treatment was able to ameliorate the changed IgE levels and also the other related factors such as TNF-α, IL-4, 6, and NF-κB. IL-4, a pro-inflammatory cytokine, is secreted from the T helper 2 cells

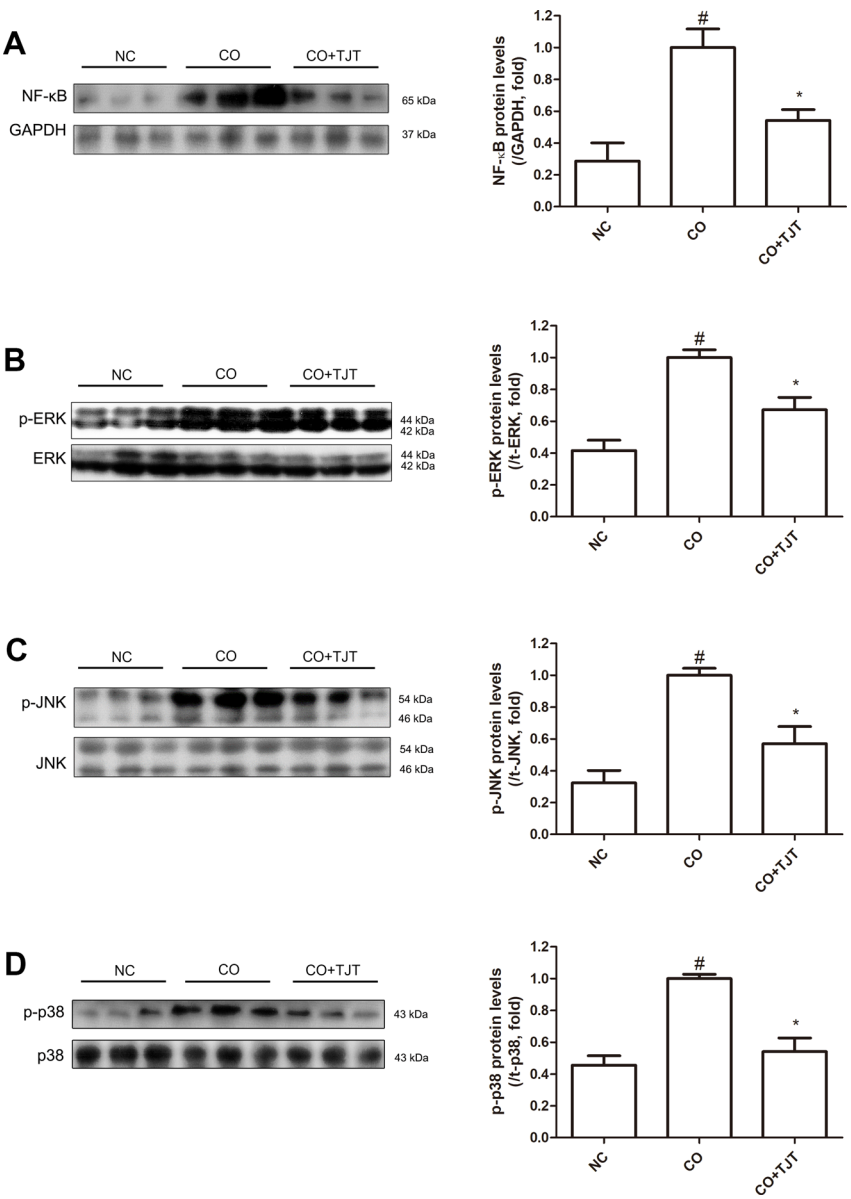

FIGURE 6 | TJT suppresses activation of nuclear NF-κB and MAPKs in the HFD/DNFB-induced obesity-AD comorbidity mouse model. Western blot analyses were done to evaluate the levels of **(A)** nuclear NF-κB **(A)**, **(B)** p-ERK, **(C)** p-JNK, **(D)**, and p-p38. The data are represented as the mean ± SEM of three or more experiments. $^{\#}P < 0.05$ when compared to NC; $^{*}P < 0.05$ when compared to CO. NC, normal control group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

as an inflammatory response resulting in IgE production, which leads to degranulation of mast cells (Friedmann, 2006). IL-6 is a cytokine released by various immune-related cells, including macrophages, T cells, and B cells (Brandt and Sivaprasad, 2011), while TNF-α is a cytokine which is produced mostly by macrophages and involved closely in inflammation, especially in the acute phase reaction (Gahring et al., 1996).

NF-κB and MAPKs were also up-regulated in the obesity-AD comorbid mice. NF-κB, a transcription factor which controls cytokine production and cell survival, is one of the key factors in the immune response process (Tak and Firestein, 2001; Lawrence, 2009). MAPKs are involved in the pathology of AD as they are regulated by IgE and can induce histamine release or HIF-1α accumulation (Sumbayev et al., 2010). In this study, NF-κB and MAPKs were highly marked in the HFD/DNFB-treated group, suggesting severe comorbidity of obesity and AD. In the TJT group, the activation of these factors was decreased close to the levels in the NC group.

HIF-1 was originally recognized as an oxygen homeostasis factor with the known purpose to regulate oxygen delivery in mammals (Semenza, 1999). Among its biological functions, recent perspectives suggest the role of HIF-1α in inflammation

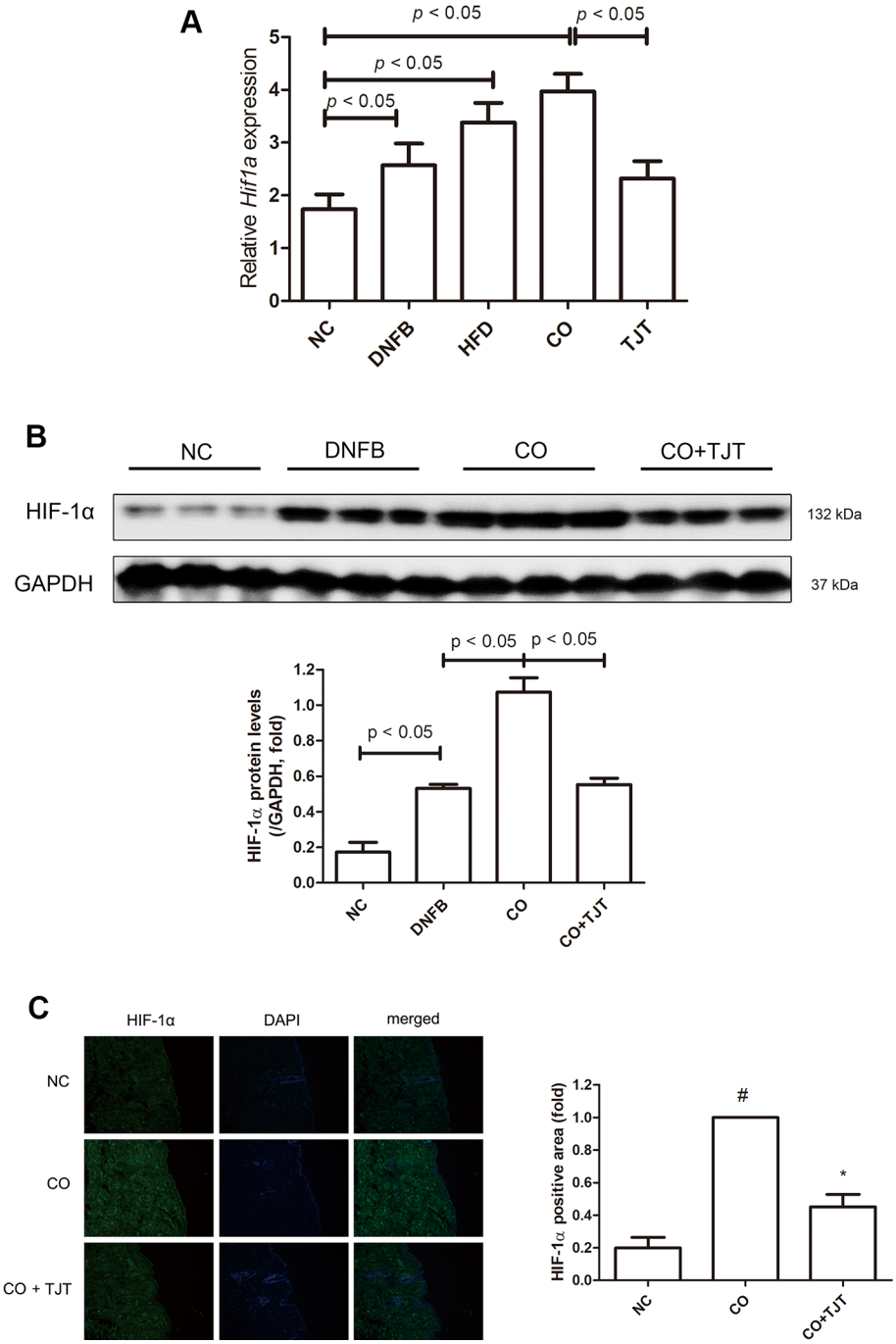

FIGURE 7 | TJT decreases HIF-1α expression in the HFD/DNFB-induced obesity-AD comorbidity mouse model. **(A)** A western blot assay and **(B)** immunofluorescence assay were done to examine HIF-1α expressions (IF magnification ×400). HIF-1α positive area was evaluated in five random slides per group using Image J. The data are represented as the mean ± SEM of three or more experiments. $^{\#}P < 0.05$ when compared to NC; $^{*}P < 0.05$ when compared to CO. NC, normal control group; DNFB, DNFB-induced AD group; CO, HFD/DNFB-induced obesity-AD comorbidity group; CO+TJT, TJT-treated comorbidity group.

to be important. As IgE activates the IgE receptor FcεRI in basophils, two main members of the MAPK chain, ERK and p38, start to induce the protein accumulation of HIF-1α (Sumbayev et al., 2009). Generally, the role of HIF-1α in AD is not widely known; however, considering its response to IgE and roles in inflammation, HIF-1α could be a participant. Other studies also imply the possibility that it is a key regulator in AD. HIF-1α is known a key regulator in immune responses (Rezvani et al., 2007; Jang e t al., 2013; Leire et al., 2013). Such previous reports cannot reveal the exact role of HIF-1α in AD; however, they briefly

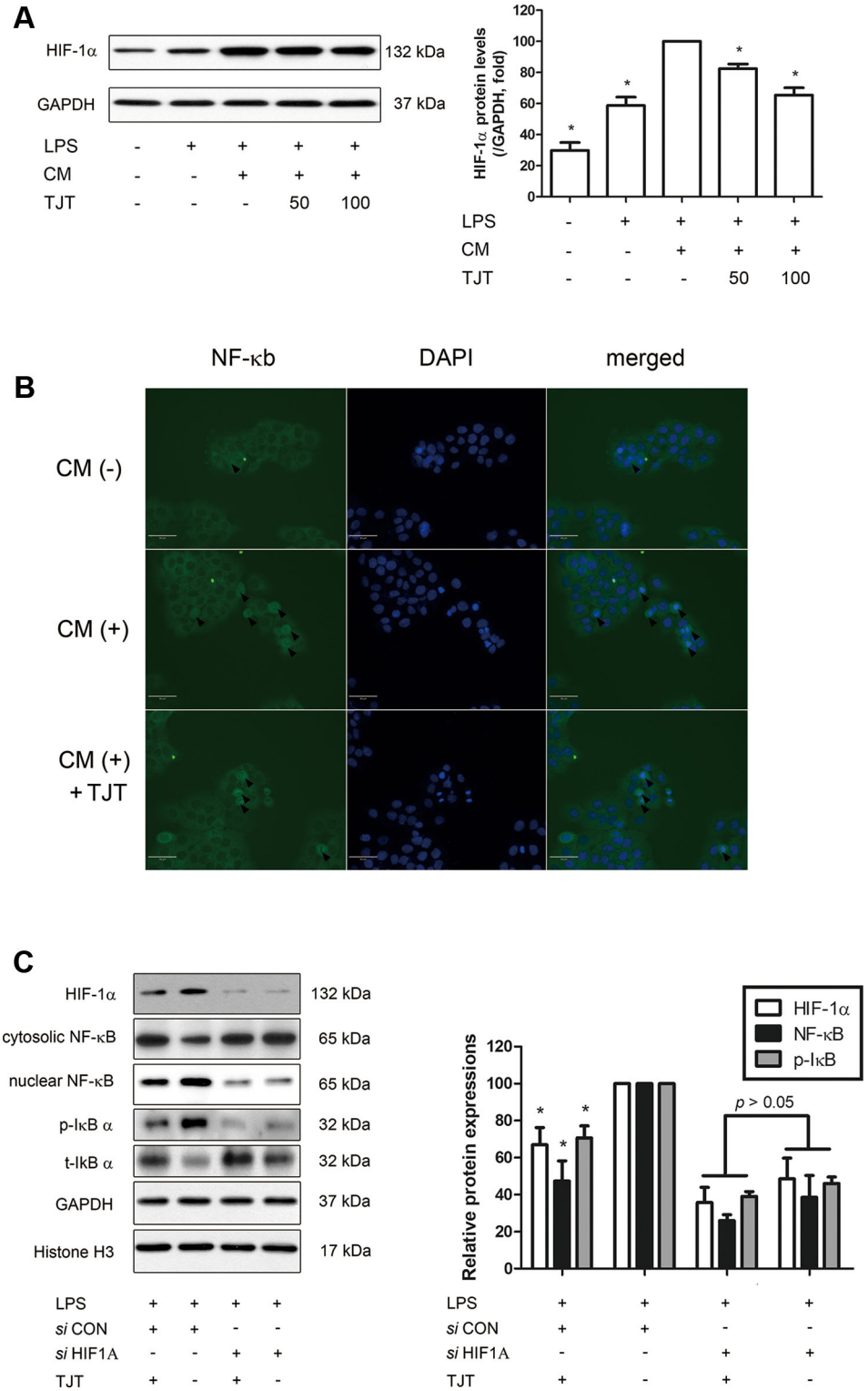

FIGURE 8 | TJT improves obesity-AD comorbidity *via* HIF-1α regulation. **(A)** A western blot assay was performed to examine HIF-1α expression in an obesity-AD comorbidity vitro model of LPS-activated, adipocyte CM-treated HaCaT cells. **(B)** Localization of NF-κB is shown by IF staining of adipocyte CM-treated HaCaT cells. **(C)** Expression levels of HIF-1α, cytosolic and nuclear NF-κB and p-IκB were determined by western blot assays. The data are represented as the mean ± SEM of three or more experiments. HIF-1α was normalized to GAPDH, p-IκB was normalized to t-IκB, and nuclear NF-κB was normalized to histone H3. *$P < 0.05$ when compared to LPS and CM-treated HaCaT cells in **(A)**; *$P < 0.05$ when compared to LPS and control siRNA-treated HaCaT cells in **(B)**.

suggest the possibility of its correlation. Our study showed remarkably high expression of HIF-1α in the HFD/DNFB-induced obesity-AD comorbidity mice model, supporting its involvement in this certain status. And by TJT treatment, HIF-1α was attenuated to a similar level as in the normal mice. These results indicate the role of HIF-1α in obesity-AD comorbidity, and TJT can successfully improve the comorbidity disease by regulating HIF-1α.

Nevertheless, our study only provides a hint; the actual role of HIF-1α in obesity-AD comorbidity remains unclear. Some suggestive studies may support the idea that HIF-1α links obesity and AD. Several reports introduce the connection between HIF-1α and cytokines such as IL-4, IL-5, IL-13 (Crotty Alexander et al., 2013; Mo et al., 2014), and other studies show that these cytokines are elevated in obese individuals (El-Wakkad et al., 2013; Schmidt et al., 2015). Furthermore, a study by Desai et al. reports that IL-5 is increased in obese individuals with severe asthma (2013). Regarding the pathological similarity between asthma and AD, the role of HIF-1α and cytokines, especially IL-5 in particular, may give an answer to the unrevealed mechanism of obesity-AD comorbidity. Considering the role of HIF-1α in necrosis suggest another possibility. In necrotic conditions of skin tissue, impaired O_2 supply promote HIF-1α stabilization (Lee et al., 2007). Infiltrated immune cells, such as mast cells or eosinophils may also contribute to HIF-1α in skin lesions (Walczak-Drzewiecka et al., 2008; Mo et al., 2014). In these cases, the reduction of HIF-1α expressions by TJT could be a secondary effect resulting from alleviation of inflammation. Our results from an *in vitro* model of obesity-AD comorbidity-mimicking conditions suggested the possible role of HIF-1α in the action mechanism of TJT is crucial (**Figure 9**), but still further intense studies, perhaps using HIF-1α knock-out mice, must be proceeded in order to investigate the underlying mechanism of TJT on HIF-1α.

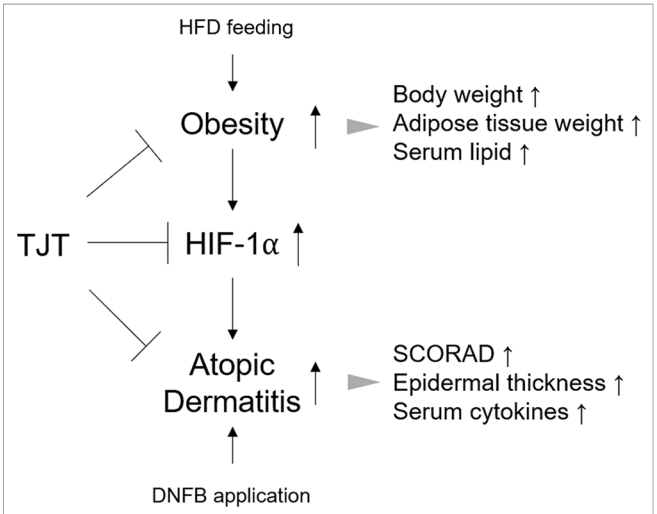

FIGURE 9 | TJT alleviates obesity-AD comorbidity via regulation of HIF-1a.

CONCLUSION

Obesity and AD are separate diseases with different clinical symptoms, but closely linked epidemiologically. Despite the prevalence of obesity-AD comorbidity, its exact pathological mechanism remains to be elucidated. In our study, we propose a basic *in vivo* model of obesity-AD comorbidity through HFD/DNFB administration, and discovered HIF-1α as one of the possible links between the two diseases. Although we showed TJT could successfully improve the symptoms of obesity-AD comorbidity by regulating HIF-1α, the specific role of HIF-1α in the pathology of obesity-AD and the detailed regulatory mechanism of TJT require further investigation. Taken all together, TJT might be useful in obesity-AD comorbidity treatment because it improves clinical symptoms, and this may be due to its HIF-1α regulating effect.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experimental protocols involving the use of animals conform to the NIH guidelines (Guide for the Care and Use of Laboratory Animals, 8th edition). Animal Care and Use Committee of the Institutional Review Board of Kyung Hee University (confirmation number: KHUASP(SE)-12-036) has approved all the animal experiments.

AUTHOR CONTRIBUTIONS

JP and J-YU designed the protocol and prepared the manuscript. JP, D-HY, and JK performed the experiments. JP, D-HY, and JK curated data. HJK and KSA provided technical and material support. J-YU was in charge of conducting the whole experiment and proofreading the manuscript. All authors approved the final version to be published.

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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.2019.01458/full#supplementary-material

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Combined Use of Astragalus Polysaccharide and Berberine Attenuates Insulin Resistance in IR-HepG2 Cells *via* Regulation of the Gluconeogenesis Signaling Pathway

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Insulin resistance (IR) is likely to induce metabolic syndrome and type 2 diabetes mellitus (T2DM). Gluconeogenesis (GNG) is a complex metabolic process that may result in glucose generation from certain non-carbohydrate substrates. Chinese herbal medicine astragalus polysaccharides and berberine have been documented to ameliorate IR, and combined use of astragalus polysaccharide (AP) and berberine (BBR) are reported to synergistically produce an even better effect. However, what change may occur in the GNG signaling pathway of IR-HepG2 cells in this synergistic effect and whether AP-BBR attenuates IR by regulating the GNG signaling pathway remain unclear. For the first time, we discovered in this study that the optimal time of IR-HepG2 cell model formation was 48 h after insulin intervention. AP-BBR attenuated IR in HepG2 cells and the optimal concentration was 10 mg. AP-BBR reduced the intracellular H_2O_2 content with no significant effect on apoptosis of IR-HepG2 cells. In addition, a rapid change was observed in intracellular calcium current of the IR-HepG2 cell model, and AP-BBR intervention attenuated this change markedly. The gene sequencing results showed that the GNG signaling pathway was one of the signaling pathways of AP-BBR to attenuate IR in IR-Hepg2 cells. The expression of p-FoxO1^{Ser256} and PEPCK protein was increased, and the expression of GLUT2 protein was decreased significantly in the IR-HepG2 cell model, and both of these effects could be reversed by AP-BBR intervention. AP-BBR attenuated IR in IR-HepG2 cells, probably by regulating the GNG signaling Pathway.

Keywords: astragalus polysaccharide, berberine, insulin resistance, IR-HepG2 cell model, intracellular calcium flux, gluconeogenesis signaling pathway

Abbreviations: IR, Insulin resistance; T2DM, type 2 diabetes mellitus; GNG, Gluconeogenesis; IR-HepG2, Insulin resistance HepG2 cells; AP, Astragalus polysaccharide; BBR, Berberine; AP-BBR, Astragalus polysaccharide plus berberine; ROS, reactive oxygen species; FoxO1, Forkhead box protein O1; PEPCK, Phosphoenolpyruvate carboxykinase; GLUT2, Glucose transporte; 2PPAR, Peroxisome proliferators-activated receptor; IRS-2, Insulin receptor substrate-2; GLUT4, Glucose transporter 4; UPR, Unfolded protein reaction.

INTRODUCTION

Diabetes mellitus is classified as type 1 (T1DM) and type 2 (T2DM). Clinically, more than 90% DM patients belong to T2DM. Insulin resistance (IR) is a pathological condition in which a target cell or a target organ fails to respond normally to insulin, so that insulin is unable to stimulate glucose disposal, which is a serious risk factor leading to the onset of T2DM. Other than T2DM, IR is forerunner for the development of many metabolic diseases, such as hypertension, obesity, and atherosclerosis. Therefore, ameliorating the IR status is an effective approach for the treatment of T2DM (Rodriguez-Gutierrez et al., 2018). IR is a state in which the response of target organs to insulin is decreased, or the normal dose of insulin secreted by pancreatic islet β cells produces less than normal biological effects. The IR theory has been generally accepted as the theoretical foundation for the study of T2DM by the medical community.

Our previous study (Zhu-jun et al., 2017)showed that combined use of astragalus polysaccharide (AP) and berberine (BBR) could synergistically produce a better therapeutic effect than either of them alone by promoting the basic secretion ability of IR-INS-1 cells, and this research achievement is on the way of applying for a patent in China (patent application no.: CN201810266347.9; publication/announcement no.: CN108478591A).

The liver is the main organ of glucose and lipid metabolism and plays an important role in energy metabolism of various tissues and organs including muscles and adipose tissues through regulation of insulin, glucagon, epinephrine, growth hormone, and other hormones (von Horn and Minor, 2019). The construction of an IR cell model is an important way to explore the pathological mechanism and drug development of IR-related diseases.

HepG2 cells originating from human hepatic embryonal tumor cells are a phenotype very similar to hepatocytes. It was reported that the number of insulin receptors on the surface of HepG2 cells decreased by 58% under the high-level insulin condition, and the remaining receptors then exhibited a 50% decrease in insulin internalization and degradation on a per receptor basis (Williams and Olefsky, 1990). The degree of such a decrease was positively correlated with the insulin level and the duration of stimulation, indicating that HepG2 is a good cell model to study IR pathogenesis and the mechanism of hypoglycemic drugs *in vitro*.

The results of gene sequencing in a previous study (Tsujimoto et al., 2015) showed that there were significant differences in glycolysis/gluconeogenesis pathway between the control and model groups, and between the model and drug administration groups. Gluconeogenesis (GNG) is a metabolic process of converting non-carbohydrates, such as amino acids, pyruvate, and glycerin, into glycogen or glucose, which is important for maintaining blood glucose levels within the physiological range.

Under normal physiological conditions, the liver is the main organ of GNG. It has rapid adaptation to metabolic shift between fed and fasting states through a reciprocal mechanism (Wahren and Ekberg, 2007): Hepatic gluconeogenic activity is suppressed in response to postprandial insulin secretion so as to limit glucose production. Conversely, hepatic GNG is stimulated in response to reduced insulin action and elevates glucagon secretion during fasting. The renal glycogenic ability is only 1/10 of the liver (Edgerton et al., 2009).GNG change is not obvious under normal dietary and metabolic conditions, but can undergo significant change in prolonged fasting or exercise and under low-carbohydrate diet conditions, or when there is a significant decrease in glycogen storage in the liver. GNG is an energy-consuming process that starts with two molecules of pyruvate and ends with the synthesis of one molecule of glucose, which requires six molecules of ATP/GTP. It produces a net 2ATP compared to glycolysis. In T2DM patients, the main source of endogenous glucose production is from GNG rather from glycogende composition. Increased hepatic GNG is a hallmark of T2DM, so maintaining the normal rate of GNG is the key to avoiding T2DM (Matsumoto et al., 2007).

However, what change may occur in the GNG signaling pathway of IR-HepG2 cells, and whether AP-BBR can attenuate IR by regulating the GNG signaling pathway remain unclear.

In the present study, we established a model of IR- HepG2 cells by insulin induction *in vitro* to determine the concentration and timing of stable insulin induction, and detect glucose uptake, reactive oxygen species (ROS) and apoptosis, CCK8 cell viability, the H_2O_2 concentration, and intracellular calcium ion in HepG2 cells to see whether AP-BBR attenuated IR by regulating the GNG signaling pathway.

MATERIALS AND METHODS

Determination of Glucose Content in HepG2 Cells

HepG2 Cell Culture

HepG2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in 1640 medium (Hyclone, Beijing, China) containing 10% fetal bovine serum (FBS, Hyclone, Beijing, China) and 1× streptomycin in a 37°C 5% CO_2 saturated humidity incubator. The normally cultured HepG2 cell lines in log phase were centrifuged at 100 grpm for 5 min, and 20000 cells/well were placed in a 96-well plate and incubated at 37°C.

Grouping and Drug Administration

The experiment was performed in 24-h control group, 24-h model group, 36-h control group, 36-h model group, 48-h control group, 48-h model group, 72-h control group and 72-h model group.

Insulin (Gibco, NY, USA) was diluted to a final concentration of 10^{-6} mol/L in complete medium. 200-μl insulin preparation was added into each well for the model group and an equal amount of complete medium was added into each well for the control group. Culture was performed in a 37°C 5% CO_2 and saturated humidity incubator. The supernatant of the corresponding medium was

collected according to the time point by centrifugation at 3000 r/min for 5 min and stored at −80°C for use.

Determination of the Glucose Content

The reagent for determining the glucose content (RSBIO, Shanghai, China) was balanced and configured at room temperature. 20-ml R1 reagent and 20-ml R2 reagent were mixed well. The EP tubes were marked as a blank tube, a calibration tube, a quality control tube, and a sample tube accordingly. 1000 μl working fluid was added to each tube; 10 μl distilled water was added to the blank tube; 10 μl calibration product was added to each tube; 10 μl quality control product was added to each tube of quality control tube; and 10 μl sample was added to each sample tube. Six samples were added in each group. After full mixing, the EP tubes were placed in 37°C water bath for 15 min. The 200-μl sample from each tube was transferred to a 96-well plate, and the absorbance was measured at 505 nm.

Detection of HepG2 Cell Viability by CCK8

HepG2 cell culture was conducted in the following groups: a normal control group; an insulin model group; a 1-mg AP-BBR group; a 5-mg AP-BBR group, a 10-mg AP-BBR group; a 20-mg AP-BBR group; and a 40-mg AP-BBR group. Astragalus polysaccharide (AP) used in this study was provided by Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China; Lot No. B20562; AP is a kind of macromolecular active substance extracted from the dried roots of the leguminous plant Astragalus membranaceus or Astragalus membranaceus. It is mainly composed of 75.19% glucose and a small amount of fructose, galactose, arabinose, and xylose. AP is a neutral polysaccharide that makes the iodine liquid blue and has a melting point above 200°C.), and BBR was provided by Beijing Century Aoko Biotechnology Co., Ltd (Beijing, China; Lot No. BWB50136; Molecular formula: C20H18NO4; Molecular weight: 336.37; Melting point: 145°C; boiling point: 354.2°C at 760 mm Hg). AP-BBR was administered at a 1:1 mass ratio of AP: BBR. The above ratio was set up according to our previous studies on AP and BBR (Zitai et al., 2018; Zhu-jun et al., 2018). After successful establishment of the IR model, different concentrations of AP-BBR were added. The system of each pore was 200 μl. The experiment was repeated three times independently. After different time points of drug action, 10 μl CCK8 detection solution (UNOCI, Hangzhou, China) was added to each pore, and the reaction time was 2 h at 37°C. A blank control group was set up and treated in the same way without anything added. Optical density (OD) was measured at 450 and 650 nm. The results were calculated using the following equation: Cell survival rate (%) = OD value of experimental group/OD value of non-drug group × 100%.

Detection of Apoptosis and ROS of HepG2 Cells by Flow Cytometry

Grouping

HepG2 cells were divided into three groups: a normal control group, a model group, and a 10-mg AP-BBR group. The original culture medium was retained in the wells to which 100 μl of the respective insulin concentration was added and treated for 48 h.

ROS Probe Incubation

The cry preserved tube containing 1.5-ml cells was taken out from the liquid nitrogen tank and quickly placed in water bath at 37°C for about 2 min. The cell suspension in the tube was transferred into a 15-ml centrifuge tube, to which 5 ml complete medium was added, and centrifuged at 300×g for 5 min at room temperature. After removing the supernatant, cells were re-suspended with a moderate amount of complete medium heavy precipitation, inoculated in a 10-cm petri dish, added with complete medium to 10 ml, and cultured again in 37°C and 5% CO_2, saturated humidity. 1 μl ROS probe (Beyotime, Shanghai, China) was added to the resuscitated cells in the proportion of 1:1000, mixed, incubated at 37°C for 20 min, and oscillated several times per 5 min. After 5 centrifugations, cell precipitation was collected, 1 ml PBS was resuscitated, and centrifuged at 500 × g for 5 min. 1 ml PBS was re-suspended. A negative control group was set up and treated in the same way without adding the probe.

Apoptosis Probe Incubation

The prepared cells were precipitated; 0.1 ml banding buffer was resuscitated; FITC probe 5 μl and PI probe 10 μl were added, mixed, incubated at room temperature for 5 min, and finally tested after addition of the banding buffer to 0.5 ml. The negative control group was treated in the same way without anything added. The single staining group was treated in the same way with addition of the FITC probe or PI probe only.

Detection of ROS and the Apoptosis Rate by Flow Cytometry

The ROS signal was detected by flow cytometry (BIO-RAD, USA) and FL-1A channel. The proportion of ROS-positive cells and the mean fluorescence intensity of cells were recorded. FITC signal was detected by flow cytometry and FL-1A channel, PI signal was detected by FL-2A channel, and the apoptosis rate was recorded.

Determination of the Intracellular H_2O_2 Concentration in HepG2 Cells

Cells in each group were cultured according to "Methods 1.1". The Krebs-Ringer buffer was prepared with double steamed water, and the pH was 7.5. The final concentration of Amplex Red was 100 mg, and the final concentration of HRP was 0.25 U/ml with 1× buffer. H_2O_2 standard was diluted by gradient, and the standard curve was drawn. After removing the original liquid from the 96-well plate and washing PBS, 50-μl working fluid was added to each cell and incubated away from light at 37°C for 30 min. The fluorescence intensity was measured at the excitation wavelength of 530 nm and the emission wavelength of 590 nm.

Detection of Intracellular Ca^{2+} in HepG2 Cells by Laser Confocal Technique

Hepg2 Cells Were Cultured and an IR-HepG2 Model Was Established

Fluo-4 probe loading was performed as follows: the prepared cell confocal dish was washed with 1 ml PBS buffer without Ca^{2+}

and Mg^{2+}; after addition of the Fluo-4 probe at 1:1 000 and thorough mixing, cells were incubated at 37°C for 30 min, and then washed with PBS without Ca^{2+} and Mg^{2+} 3 times before testing.

Administration and Detection of Intracellular Ca^{2+} by Laser Confocal Microscopy

The working module of living cells was opened in advance, and the conditions were set as follows: 37°C, 5% CO_2, saturated humidity. The incubated cell confocal dish samples were placed in the workbench, the control samples and the model samples were put immediately under 100 times the laser confocal microscope x 100 (OLYMPUS FV1000, Japan). The blue light channel (excitation wavelength 488 nm and emission wavelength 526 nm) was scanned by laser every 5 s, and the fluorescence intensity was recorded. The control group and model samples were taken from the blue light channel. After dripping AP-BBR with the final concentration of 10 mg, the fluorescence intensity was recorded, and the control group and the model sample were taken from the blue light channel (488 nm excitation wavelength and 526 nm emission wavelength). Immediately, the blue light channel (488 nm excitation wavelength and 526 nm emission wavelength) was scanned by laser every 5 s, and the fluorescence intensity was recorded. Cells were photographed continuously for 0 to 1,000 s.

Transcriptome Sequencing and Pathway Enrichment Analysis

RNA isolation, purification, and quantification: Total RNA was isolated and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The RNA amount and purity of each sample were quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Agilent 2100 with RIN number >7.0.

cDNA Library Construction: Poly(A) RNA was purified from total RNA (5 μg) using poly-T oligo-attached magnetic beads using two rounds of purification. Then, the poly(A) RNA was fragmented into small pieces using divalent cations under high temperature. Then the cleaved RNA fragments were reverse-transcribed to create the cDNA, which was subsequently used to synthesize U-labeled second-stranded DNAs with *Escherichia coli* DNA polymerase I, RNase H, and dUTP. An A-base was then added to the blunt ends of each strand, which were prepared for ligation to the indexed adapters. Each adapter contained a T-base overhang for ligating the adapter to the A-tailed fragmented DNA. Single- or dual-index adapters were ligated to the fragments, and size selection was performed with AMPureXP beads. After the heat-labile UDG enzyme treatment of the U-labeled second-stranded DNAs, the ligated products were amplified by PCR under the following conditions: initial denaturation at 95°C for 3 min, 8 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 15 s, extension at 72°C for 30 s, and then final extension at 72°C for 5 min. The mean insert size for the final cDNA library was 300 bp (±50 bp). Finally, 150-bp paired-end sequencing was performed on an Illumina Hiseq 4000 (LC Bio, China) following the vendor's recommended protocol.

Pathway enrichment analysis: Using the DAVID database and mouse genome as background control, the differentially expressed genes were analyzed by gene ontology under "FunctionalAnnotation Chart" functional module. The differentially expressed genes were divided into three categories according to their functions: the biological process, the cell component, and the molecular function. Pathway analysis was carried out by KEGG analysis function.

Analysis of Protein Expression

HepG2 cells were lysed in NP 40 lysis buffer and centrifuged at 12,000×*g* for 20 min at 4°C. The protein concentration was quantitated, and the protein lysate (20 μg proteins) was subjected to SDS-PAGE on 10% polyacrylamide gels and then electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then blocked by 5% fat-free milk, and incubated respectively with FoxO1 antibody (78 kDa; 1:1000), Phospho-FoxO1 (Ser256) antibody (78 kDa; 1:1000), PEPCK (PCK2) antibody (71 kDa; 1:1000), GLUT2 antibody (53 kDa; 1:1000), and anti-GAPDH (36 kDa; 1:2000) (CST, Cambridge, MA, U.S.) at 4°C overnight. Horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit or mouse) were used to detect bound antibodies, which were visualized by enhanced chemiluminescence (ECL, Millipore, Billerica, MA, USA) on a VersaDoc 4000 MP (BIO-RAD, USA) workstation. Using the ImageJ software, densitometric analysis was performed to determine the relative expression level of the target protein, which was standardized to GAPDH (Bethesda, Maryland. USA).

Statistical Analysis

Data concerning miRNA expression were analyzed with independent sample t-test. All the other data were analyzed with one-way ANOVA followed by LSD when equal variances assumed, and Dunnett's-T3 when equal variances not assumed. The results are expressed as the means ± SEM. All statistical analyses were performed by using SPSS23. $p < 0.05$ was considered statistically significant. Figures were drawn by GraphPad Prism 6.02 and Adobe Illustrator CS6.

RESULTS

Determination of Glucose Content in HepG2 Cells

The results of intracellular glucose content determination in HepG2 cells are shown in **Figure 1A**. The best time for the HepG2 insulin model was at 48 h of insulin intervention.

Result of the Cell Survival Rate of HepG2 Cells

CCK8 assay was performed to detect the cell survival rate of HepG2 cells. As shown in **Figure 1B**, the survival rate of IR-HepG2 cells was increased after AP-BBR administration. The optimal dosage of AP-BBR was 10 and 20 mg. For convenience, 10 mg was used in the subsequent experiments.

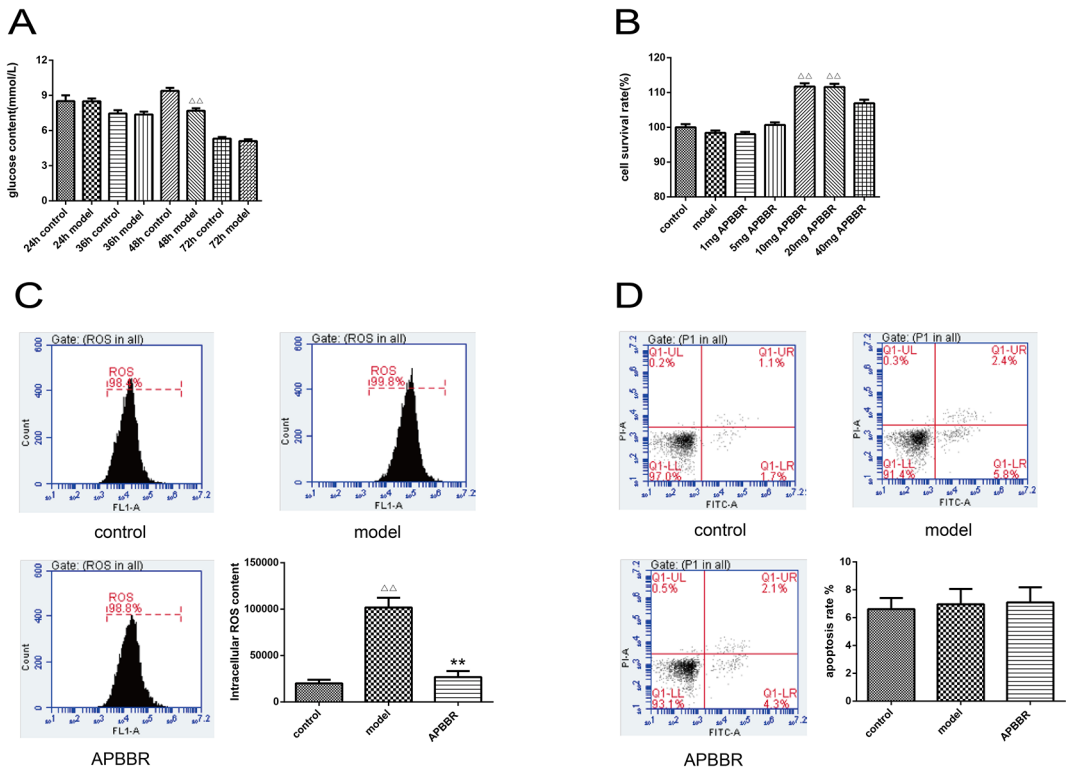

FIGURE1 | The glucose concentration of HepG2 cells **(A)**. Data are shown by mean ± SEM (n = 3), 48 h control group *vs.* 48 h model group, $^{\Delta\Delta}P < 0.01$. Proliferation activity of HepG2 cells **(B)**, AP-BBR group *vs.* model group, $^{**}P < 0.01$. The ROS content in HepG2 cells **(C)**, model group *vs.* control group, $^{\Delta\Delta}P < 0.01$, AP-BBR group *vs.* model group, $^{**}P < 0.01$. Apoptosis rate of HepG2 cells **(D)**, model group *vs.* control group, $P > 0.05$, AP-BBR group *vs.* model group, $P > 0.05$.

Determination of ROS Content in HepG2 Cells

ROS content in HepG2 cells was detected by flow cytometry (**Figure 1C**). The results showed that the level of ROS in IR-HepG2 cells increased significantly, and the level of ROS decreased significantly in AP-BBR group. The results showed that the level of ROS in IR-HepG2 cells increased significantly, and AP-BBR decreased the level of ROS in the IR-HepG2 cell model.

The Apoptosis Rate of HepG2 Cells

The effect of AP-BBR on apoptosis of IR-HepG2 cells was detected by flow cytometry, and the results are shown in **Figure 1D**. The results showed that the IR model and AP-BBR treatment had no significant effect on apoptosis of HepG2 cells.

H_2O_2 Concentration in HepG2 Cells

The effect of AP-BBR on the concentration of H_2O_2 in IR-HepG2 cells was detected. As shown in **Figure 2**, the H_2O_2 content of IR-HepG2 cells was significantly increased, and the H_2O_2 content of IR-HepG2 cells was significantly decreased in AP-BBR group.

Change in Intracellular Ca^{2+} in HepG2 Cells

The photos of each HepG2 cell group under the laser confocal microscope are shown in **Figure 3A**.

The slope of intracellular Ca^{2+} fluorescence intensity dynamic (0–150 s) curve fitting is shown in **Figure 3B**. The results suggest that change in intracellular calcium flow became stronger and faster after modeling, and slower and weaker after AP-BBR administration.

The experimental results of laser confocal detection of intracellular Ca^{2+} change in HepG2 cells are shown in **Figure 3C** (the raw data is shown in **Supplementary Table 1**). These results suggest that the intracellular calcium flow changed rapidly in IR-HepG2 cells, and the intracellular calcium flow change was decreased after 10 mg AP-BBR administration.

Results of Pathway Enrichment Analysis

The statistical enrichment of differentially expressed genes in KEGG pathway was analyzed by local perl script and R software package ggplot2. Red indicates a significant difference, and blue indicates no significant difference. The size of the circle reflects the number of genes. Among the top 20 rich factors, pathways related to IR included the PPAR pathway, glucose metabolism pathway, and glycolysis/gluconeogenesis pathway. The results of significant differential expression analysis showed significant differences in the effect of glycolysis/GNG and other pathways on IR (**Figure 4A**). After AP-BBR intervention, there was significant difference expression in Glycolysis/GNG pathway (**Figure 4B**).

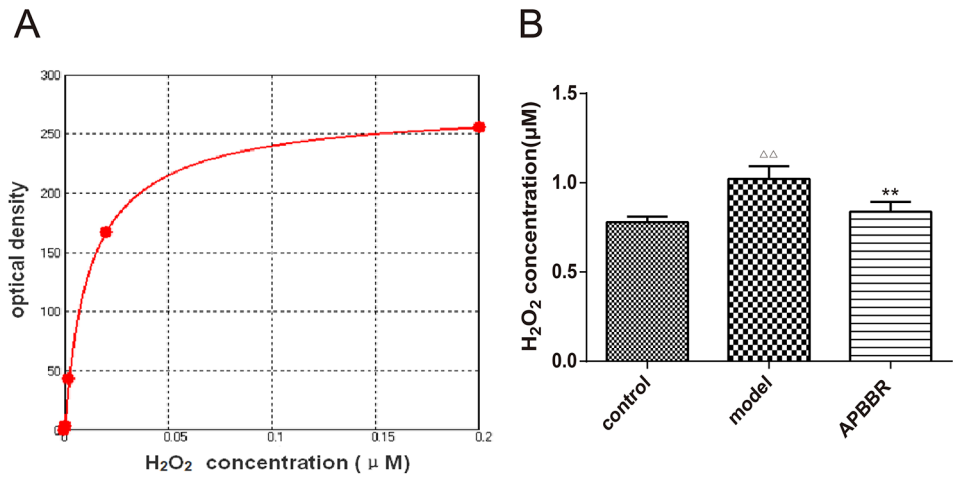

FIGURE 2 | H_2O_2 standard curve **(A)**. X axis: concentration (mM); Y axis: fluorescence OD value. Four-parameter Logistic curve fitting, equation: y = (A -D)/[1 + (x/C) ^B] + D.A = 274.48194; B = -0.92136; C = 0.01225; D = -1.30186; r^2 = 0.99990. H_2O_2 concentration in HepG2 cells **(B)**. Data are shown by mean ± SEM (n = 3). $^{\Delta\Delta}P < 0.01$ showing a significant difference as compared with control group; $^{**}P < 0.01$ showing a critical difference as compared with model group (n = 3).

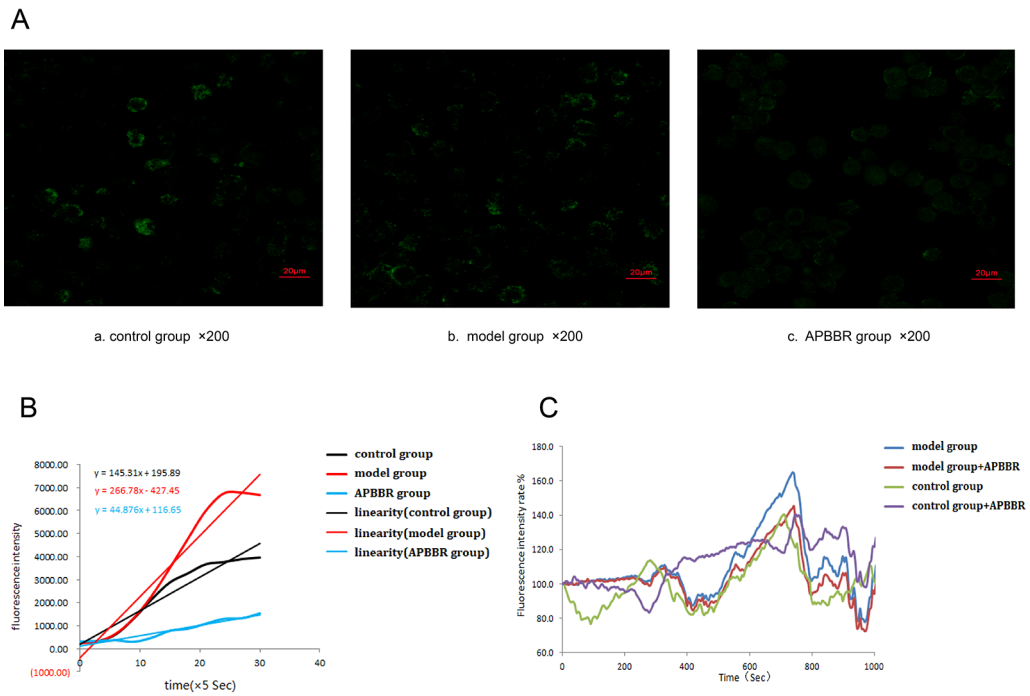

FIGURE 3 | The photos of each HepG2 cells group under laser confocal microscope (×400) **(A)**. Calculation of slope by curve fitting of intracellular Ca^{2+} fluorescence intensity dynamics (0–150 s) **(B)**. Changes of intracellular Ca^{2+} fluorescence intensity in HepG2 cells(0–1000 s) **(C)**.

Protein Expressions

The results of Western blot in each HepG2 cell group are shown in **Figure 5**. Compared with the control group, the protein expression of p-FoxO1^{Ser256} and PEPCK in the model group was significantly increased, while the protein expression of FoxO1 and GLUT2 was significantly decreased (n = 3). Compared with the model group, the protein expression of p-FoxO1^{Ser256} and PEPCK in AP-BBR group was significantly decreased, while the protein expression of FoxO1 and GLUT2 was significantly increased (n = 3).

DISCUSSION

Deficiency of insulin signaling and IR in the liver has a great impact on energy balance and metabolism. Liver IR can lead to a

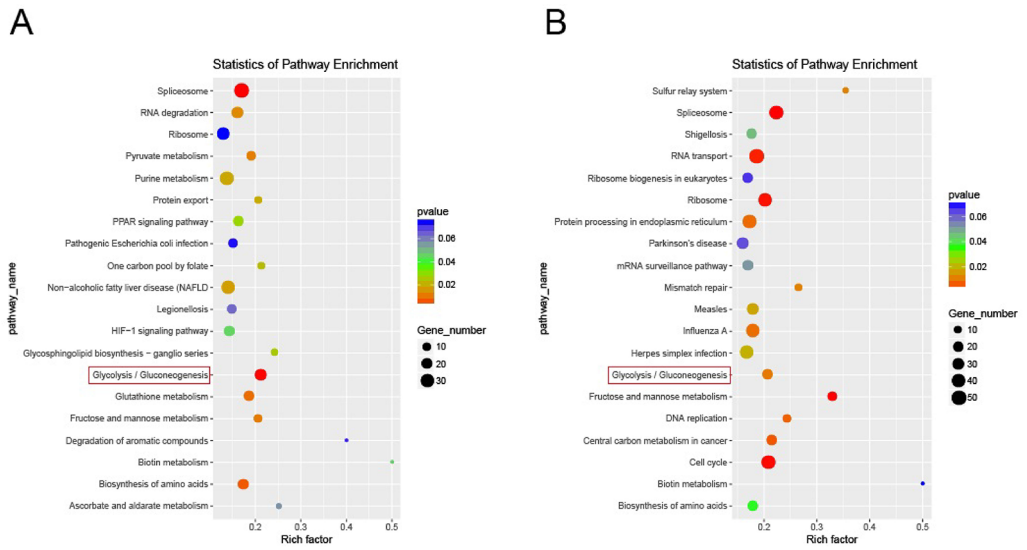

FIGURE 4 | Enrichment of differentially expressed genes in KEGG pathway. Model group vs. control group **(A)**. AP-BBR group vs. model group **(B)**.

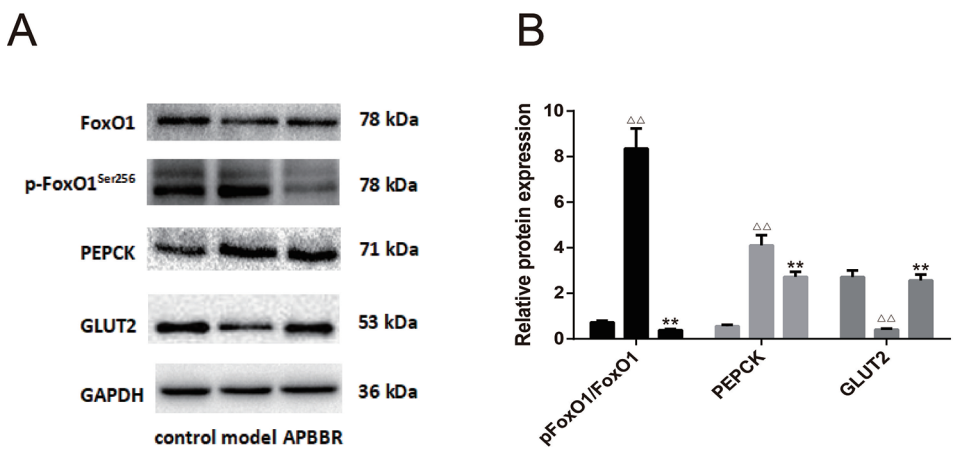

FIGURE 5 | The protein expression in HepG2 cells. The protein expressions of FoxO1, p-FoxO1^{Ser256}, PEPCK, and GLUT2 were detected by Western blot. The figure represents one of three experiments with similar results **(A)**. In **(B)**, $^{\Delta\Delta}P < 0.01$ *vs.* control group, $^{**}P < 0.01$, *vs.* model group (n = 3).

series of metabolic abnormalities, such as hyperglycemia, dyslipidemia, and increased secretion of inflammatory factors. The inhibitory effect of insulin on hepatic glucose production is impaired during liver IR. When IR progresses to T2DM, the lack of insulin signaling molecules is common as hyperglycemia. Insulin signaling can impact T2DM by regulating glucolipid metabolism and energy homeostasis *via* action on the liver, skeletal muscle, and adipose tissue. It is reported that disruption of insulin signaling in the moue liver, skeletal muscle or adipose tissue can lead to diabetes (Boucher et al., 2014). Mice lacking insulin receptor substrate-2 (IRS-2) in the liver developed severe IR and hyperglycemia (Williams and Olefsky, 1990). All in all, according to the data of human and animal research, liver IR leads to the development of hyperglycemia and T2DM.

Astragalus membranaceus is one of the most widely used traditional Chinese herbal medicines. It can be used as an antidiabetic agent. The main component of Astragalus membranaceus is AP, which can improve overall IR (Sun et al., 2017). Coptis chinensis has been used as an anti-diabetic drug in Traditional Chinese Medicine (TCM) for centuries, and BBR is the main bioactive alkaloid of Coptis chinensis and can moderate T2DM by alleviating IR (Abd et al., 2013).

AP attenuated IR mainly by decreasing the phosphorylation of protein kinase and inhibiting GLUT4 translocation in KKAy mice (Zhang and Pugliese, 2015). AP also promoted the expression of phosphorylated PI3Kp85 and GLUT4 by inhibiting the expression of JNK and phosphorylating insulin receptor substrate-1 (Jiang et al., 2015). In addition, AP decreased serum TNF-α, MCP-1, and

ICAM-1 and inhibited the expression of NF- κ B mediated inflammation gene (Gui et al., 2013).

BBR attenuated IR mainly by inhibiting the Rho GTPase signaling pathway to reduce renal inflammation in diabetic rats (Xie et al., 2013), activating various human cell lines and their promoter-dependent protein kinase C-(PKC-), and increasing insulin receptor mRNA and protein expression (Pang et al., 2015). It also inhibited liver GNG of the LKB1-AMPK-TORC2 signaling pathway and improved LPS-induced β-cell damage through LPS4-dependent JNK/NF-kB signaling pathway (Jiang et al., 2015). In addition, BBR exerted its anti-inflammatory effect through insulin sensitization, reduced cytokine production and serine phosphorylation, and increased insulin-mediated tyrosine phosphorylation (Lou et al., 2011).

The liver is the central site of glucose synthesis and metabolism, and also the main target organ for IR (Reinke and Asher, 2016). Therefore, it is very important to establish a stable and reliable IR hepatocyte model for studying the molecular mechanism of IR *in vitro* and screening drugs for prevention and treatment of IR. HepG2 cells originating from human hepatic embryonal tumor cells have similar morphological and biological functions to normal human hepatocytes, and therefore, are most commonly used for studying hepatic IR. Insulin has been widely recognized as an inducer to establish IR cell models.

In the present study, we explored the conditions in establishing the IR- HepG2 cell model and the effect of AP-BBR on IR. The glucose content in the supernatant of HepG2 cells treated with insulin for 24, 36, 48, and 72 h was measured. It was found that the optimal time for modeling was at 48 h of insulin intervention. CCK8 was used to detect the effect of AP-BBR on the viability of IR-HepG2 cells after 48 h. The results showed that AP-BBR attenuated IR in HepG2 cells.

Oxidative stress in hepatocytes refers to the pathological process of hepatocyte injury caused by excessive or/and endogenous antioxidant capacity of ·OH, H_2O_2, and other active ROS, and disturbed balance between the oxidant and antioxidant systems (Cortes-Rojo and Rodriguez-Orozco, 2011). Oxidative stress causes lipid peroxidation damage, DNA oxidative damage, and abnormal protein expression in the liver by producing excessive ROS. It is involved in the pathogenesis of viral hepatitis, non-alcoholic/alcoholic hepatitis, drug-induced liver disease, and other liver diseases (Souli et al., 2015; Yu et al., 2015). When oxides or alkylates enter cells, they induce the production of a large amount of ROS. If ROS is not cleared in time, free radicals, the intermediate products of ROS, can directly act on nucleic acid, resulting in base modification and DNA strand breaks, resulting in oxidative DNA damage (Cadet et al., 2011; Rinna et al., 2015). Alterations in ROS and apoptosis of HepG2 cells were detected by flow cytometry. The results showed that the level of ROS increased significantly in IR-HepG2 cells and decreased after AP-BBR treatment. However, AP-BBR treatment had no significant effect on apoptosis of HepG2 cells.

Ca^{2+} is an important second messenger in cells. There is a close interaction between the ROS signaling system and the calcium signaling system in the intracellular environment. On the one hand, ROS can change the amplitude and temporal and spatial characteristics of local and whole-cell calcium signals by modifying the key protein components of the calcium signaling system, such as voltage-dependent calcium and intracellular calcium release channels. On the other hand, Ca^{2+} can accelerate mitochondrial metabolism by regulating the activities of dehydrogenase in tricarboxylic acid cycle (Rizzuto and Pozzan, 2006), ATP synthase, and adenine nucleoside transporter so as to accelerate mitochondrial metabolism and increase ROS production. $[Ca^{2+}]i$ can adjust mitochondrial proton driving force ($\Delta\psi m$ and ΔPH) and affect mitochondrial energy conversion and ROS production. Ca^{2+} is also involved in the regulation of cell cycle, cell differentiation, muscle contraction, cell movement, exocytosis, endocytosis, chemical chemotaxis, synaptic transmission and synaptic plasticity, and apoptosis (Liu and O'Rourke, 2009). $[Ca^{2+}]i$ is both a survival signal and a death signal. $[Ca^{2+}]i$ in cells is harmful to cells when it is too high or too low, so cell $[Ca^{2+}]i$ is under very strict regulation and control. The dynamic change of cell $[Ca^{2+}]i$ is the core link in cell signal transduction and regulatory network (Feske et al., 2001). Physiological levels of ROS and Ca^{2+} to adapt to the changes in energy requirements continue to adjust adaptively, so as to regulate the dynamic balance of mitochondrial morphology and distribution.

When the regulation of Ca^{2+} and ROS at physiological level fails to adapt to the change of energy demand, the abnormal level of Ca^{2+} and ROS leads to mitochondrial fusion and division, thus disrupting the balance of dynamic distribution, causing dysfunction of mitochondrial oxidative phosphorylation function and further affecting the cell function. The body is in a pathological state, and at the same time, mitochondrial oxidative phosphorylation dysfunction further leads to abnormal levels of Ca^{2+} and ROS, forming a vicious circle. ROS plays an important role in regulating cell proliferation, differentiation, apoptosis and cell senescence. Low concentrations of H_2O_2 can be used as intracellular signaling molecules to initiate gene transcription, thus inducing cell growth. High concentrations of ROS oxidized lipid, protein, and DNA can cause damage to cell integrity (Scherz-Shouval and Elazar, 2007).

Apoptosis induced by H_2O_2 is closely related to calcium overload. The molecular mechanism of apoptosis induced by oxidative stress is very complex and involves many signal transduction pathways, including the classical mitochondrial pathway (Chiara et al., 2012), death receptor pathway (Gomez-Quiroz et al., 2008) and endoplasmic reticulum pathway (Ip et al., 2011). Oxidative stress damage of cells leads to the release of a large amount of Ca^{2+} from the endoplasmic reticulum accompanied by endoplasmic reticulum stress, leading to unfolded protein reaction (UPR), which is used to reconstruct the normal function of endoplasmic reticulum (Greotti et al., 2019). If the intracellular Ca^{2+} concentration continues to increase and endoplasmic reticulum stress lasts for a prolonged time or severely, Ca^{2+}-dependent kinases and phosphatase, such as calpain, caspase-12, and caspase-3 cascade reactions, will be activated, eventually leading to apoptosis (Das et al., 2006). In our experiment, change in the intracellular H_2O_2 concentration in IR-HepG2 cells was detected after AP-BBR treatment. The results showed that the H_2O_2 content of IR-HepG2 cells increased after modeling and decreased after AP-

BBR treatment. The change in intracellular calcium flow in IR-HepG2 cells was rapid and reduced after AP-BBR treatment. These results suggest that Ca^{2+} was activated in the IR HepG2 cell model, and AP-BBR could inhibit the activation of Ca^{2+} to a certain extent.

The IR-INS-1 cell model in our previous study (Zhu-jun et al., 2017) showed that combined use of AP and BBR could promote basic secretion of IR-INS-1 cells significantly and showed a better therapeutic effect than that when either of them was used alone. The results showed that the compatibility of the two drugs had an optimal synergistic effect, and that AP-BBR could promote the proliferation of IR-HepG2 cells and reduce intracellular calcium flow, though the underlying mechanism remains unclear at present.

Insulin is a key hormone that suppresses GNG. It plays a vital role in the regulation of hepatic glucose output. Insulin regulates hepatic GNG by transcriptional modulation and activation of the relative signaling pathways (Hatting et al., 2018). Even an indirect effect of insulin on extra hepatic tissues can be sufficient to maintain normal glucose metabolism (O-Sullivan et al., 2015). T2DM is characterized by excess glucagon and IR. In T2DM patients, insulin could not suppress glucagon secretion or hepatic glucose production, which underscores the importance of determining the GNG rate in the potential treatment for T2DM.

Forkhead box protein O1 (FoxO1) is a member of the forehead box transcription factor class O (FOXO) family, and a direct transcriptional regulator of GNG. It is regulated by insulin reciprocally and plays a major role in regulating insulin sensitivity (Tikhanovich et al., 2013). The activity of FOXO1 as a regulator of blood glucose is modulated by several mechanisms including phosphorylation, acetylation, and deacetylation balance, as well as some other more novel mechanisms (Zhou et al., 2011).

In the high blood glucose state, insulin is released from the pancreas into the blood stream. Insulin signaling causes the activation of PI3kinase and the subsequent production of PIP3 phosphorylates Akt. Akt then phosphorylates FoxO1 to its nuclear exclusion, and is ubiquitinated and degraded by the proteosome (Matsuzaki et al., 2003). That is to say, FOXO1 is a counter of insulin-induced Akt activation in glycogen synthesis-GNG balance, which can subsequently suppress GNG during the fed state (Daitoku and Fukamizu, 2007).

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme in the lyase family used in the metabolic pathway of GNG. It converts oxaloacetate into phosphoenolpyruvate and carbon dioxide (Mendez-Lucas et al., 2014).

The transcriptional induction of gluconeogenic enzyme PEPCK-C is an irreversible step of GNG, which therefore is of great importance in glucose homeostasis, as evidenced by the overexpression of PEPCK-C in T2DM laboratory mice. The role that PEPCK-C plays in GNG may be mediated by the citric acid cycle, the activity of which was found to be directly related to PEPCK-C abundance. Previous studies suggested that PEPCK-C level alone was not highly correlated with GNG in the mouse liver (Marandel et al., 2019). While the mouse liver almost exclusively expresses PEPCK-C, humans equally present a mitochondrial isozyme (PEPCK-M). Therefore, the role of PEPCK-C and PEPCK-M in GNG may be more complex and involve more factors than was previously believed.

Glucose metabolism depends on the uptake of glucose by cells. However, glucose is not free to enter cells through the lipid bilayer structure of the cell membrane. Glucose uptake by cells requires the help of glucose transporter (glucose transporters) on the cell membrane, which is known as the glucose transporter (GLUT) transport function.

GLUTs facilitate the movement of glucose from the plasma membrane across cell membranes. Member 2 (SLC2A2) of Glucose transporter 2 (GLUT2) is a principal transmembrane transporter for transfer of glucose between the liver and blood (Gould et al., 1991). It is of great significance for hepatic glucose uptake. Unlike GLUT4, GLUT2 does not rely on insulin for facilitation of glucose diffusion.

GLUT2 has low affinity (high Km, ca. 15-20 mM) for glucose, but it has high capacity for glucose. What's more, GLUT2 isoform is found abundant in the liver and pancreatic β-cells, so it has been suggested to function as a glucose sensor (Jung et al., 2006). GLUT2 may play a minor role in human β-cells, and can be an efficient glucose carrier so as to improve glucose and insulin metabolism.

In our study, the protein expression of p-FoxO1^{Ser256} and PEPCK in IR-HepG2 cells was increased, but the protein expression of FoxO1 and GLUT2 was decreased, and AP-BBR could reverse the above protein expression changes. The results suggest that AP-BBR attenuates IR in IR-HepG2 cells probably *via* the regulation of the GNG signaling Pathway.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI GEO https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139929.

AUTHOR CONTRIBUTIONS

Z-JM generated the data, wrote the manuscript, and approved its final version. Z-JM and L-PQ designed the study. Z-JM, ML, and XZ carried out the experiments. All authors have read and approved the final 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.2019.01508/full#supplementary-material

SUPPLEMENTARY TABLE 1 | The raw data of intracellular Ca^{2+} fluorescence intensity.

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REVIEW
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Phytopharmacology and Clinical Updates of *Berberis* Species Against Diabetes and Other Metabolic Diseases

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The incidences of diabetic mellitus and other metabolic diseases such as hypertension and hyperlipidemia are increasing worldwide; however, the current treatment is not able to control the rapidly increasing trend in diabetes mortality and morbidity. Studies related to the effectiveness of extracts and pure compounds obtained from plants have shown promising responses in preclinical and clinical studies related to these metabolic diseases. Plants belonging to the genus *Berberis* (Family: Berberidaceae) are widely distributed with nearly 550 species worldwide. Extracts and compounds obtained from *Berberis* species, especially Berberine alkaloid, showed effectiveness in the management of diabetes and other metabolic diseases. Various pharmacological experiments have been performed to evaluate the effects of *Berberis* extracts, berberine, and its natural and chemically synthesized derivatives against various cell and animal disease models with promising results. Various clinical trials conducted so far also showed preventive effects of *Berberis* extracts and berberine against metabolic diseases. The present review focuses on i) research updates on traditional uses, ii) phytopharmacology and clinical studies on *Berberis* species, and iii) active metabolites in the prevention and treatment of diabetes and other metabolic diseases with a detailed mechanism of action. Furthermore, the review critically analyzes current research gaps in the therapeutic use of *Berberis* species and berberine and provides future recommendations.

Keywords: *Berberis*, berberine, diabetes, metabolic diseases, pharmacology, clinical studies

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that is characterized by an abnormal long-term increase in plasma glucose levels. Diabetes is mainly classified into four types, i.e., type I diabetes (T1DM), type II diabetes (T2DM), gestational diabetes, and specific types of diabetes due to other causes (American Diabetes Association, 2019). Many factors, such as insulin deficiency or resistance as well as altered carbohydrate, protein, and fat metabolisms, are usually the reasons for high blood glucose levels leading to DM. Chronic hyperglycemia related to diabetes is often associated with many other complications, such as cardiovascular, dermatological, neurological, renal, retinal, and nerve diseases. Diabetes is one of the most common chronic disease, and it has shown an increasing rate of occurrence over the past decade (Bullard et al., 2018). According to the World Health Organization (WHO), the total number of people with diabetes worldwide substantially increased from 108 million in 1980 to 422 million in 2014 (World Health Organization, 2016). Along with diabetes, the incidence of other metabolic diseases, such as hyperlipidemia, is also increasing rapidly (Karr, 2017).

Metabolic syndrome (MS) is associated with a group of disease conditions that occur together, and it is composed of central adiposity, hyperglycemia, hypertriglyceridemia, low high-density lipoproteins (HDL)-cholesterol, and hypertension. This disease cluster of diabetes and cardiovascular diseases is also known as "The Deadly Quartet", "Syndrome X", and "The Insulin Resistance Syndrome" (Alberti, 2005). Various treatment options are available to mitigate MS, including the diabetic condition and related disorders (Deedwania and Volkova, 2005). As MS is manifested by the cluster of diseases, use of a single drug candidate might not be able to provide necessary therapeutic effects. Plant extracts and isolated compounds can be possible options as adjuvants in such cases. Traditionally, various medicinal plants and their products (extracts and isolated compounds) have been used in the treatment of diabetes and hypertension (Oyedemi et al., 2009; Tabassum and Ahmad, 2011; Rizvi and Mishra, 2013; Ezuruike and Prieto, 2014). Various research showed the protective/curative effect of plant extracts as a whole and/or an individual bioactive compound against diabetes and other metabolic diseases (Tabatabaei-Malazy et al., 2015; Waltenberger et al., 2016).

Plants belonging to the genus *Berberis* (Family: Berberidaceae) are widely distributed worldwide with nearly 550 species. A decoction prepared from the roots of *Berberis* plants is one of the common traditional recipes for the treatment of diabetes (Neag et al., 2018). Various studies have reported the traditional uses *Berberis* plants for the treatment of metabolic diseases (e.g., diabetes and hyperlipidemia) in many countries, including India, Pakistan, China, and Iran (Hamayun et al., 2006; Uniyal et al., 2006; Rahimi Madiseh et al., 2014; Rana et al., 2019). Various bioactive compounds, such as alkaloids, polyphenols, flavonoids, anthocyanins, etc., have been found in *Berberis* species along with various vitamins and mineral components (Andola et al., 2010; Srivastava et al., 2015; Belwal et al., 2016; Belwal et al., 2017). Berberine (BBR), a quaternary ammonium salt belonging to a group of benzylisoquinoline alkaloids, is the most active compound reported from *Berberis* species, and it is considered to be highly effective against diabetes and other metabolic diseases (Dong et al., 2012; Lan et al., 2015; Wang H. et al., 2018). BBR is also distributed in various plant species of other genera such as *Coptis*, *Hydrastis*, *Mahonia*, *Tinospora*, *Xanthorhiza,* and many others (Neag et al., 2018). In the genus *Berberis*, the distribution of BBR and other alkaloids is mostly in its root part, followed by the stem bark and the stem itself (Andola et al., 2010). In addition, its presence in trace amounts has been reported from leaves and berries. Various studies have been conducted to evaluate the effectiveness of *Berberis* extract or bioactive alkaloidal compounds against diabetes and other MS with promising results (Gulfraz et al., 2008; Meliani et al., 2011; Imenshahidi and Hosseinzadeh, 2016; Mirhadi et al., 2018). Moreover, various clinical trials were also conducted on testing their effectiveness against diabetes and other metabolic diseases and showed variable effects (Zhang et al., 2010; Pérez-Rubio et al., 2013).

Considering the *Berberis* species and their active alkaloidal components, the present review specifically focuses on their

Abbreviations: 2h-PPG, 2-hour postprandial plasma glucose; A-FABP, Adipocyte fatty acid–binding protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMPK, AMP-activated protein kinase; BBA, berbamine; BBD, benign breast disease; BBR, berberine; BFFAL, blood free fatty acids level; BF, berberine fumarate; BGL, blood glucose levels; BJ, Berberis juice; b.i.d., twice daily; BMI, body mass index; BP, blood pressure; BW, body weight; BWG, body weight gain; CAT, catalase; C/EBPα, CCAAT enhancer binding protein alpha; CMS, cardio metabolic syndrome; COX2, Cyclooxygenase-2; CPK, serum creatine phosphokinase; DAG, diacylglycerol; DBP, diastolic blood pressure; DVIS, diabetic vascular insulin sensitivity; DPP-IV, dipeptidyl-peptidase IV; DN, Diabetic nephropathy; eNOS, endothelial nitric oxide synthase; EZE, ezetimibe; FA, fructosamine; FASN, fatty acid synthase; FBS, Fasting Blood Sugar; FBGL, fasting blood glucose levels; FOP, fibrodysplasia ossificans progressive; FPG, fasting plasma glucose; FPI, fasting plasma Insulin; FSIL, fasting serum insulin level; GHb, glycosylated hemoglobin; GLP-1, glucagon-like peptide-1; GLUT4, Glucose transporter type 4; GPx, glutathione peroxidase; GR, glutathione reductase; HbA1c, glycated haemoglobin; HDL-C, high density lipoprotein HDL-cholesterol; HFD, High Fat Diet; HOMA-R, Homeostatic Model Assessment; HOMA-IR, and HOMA-β%; IC, insulin concentration; IFG, Impaired fasting glycemia; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthase; INSR-mRNA, insulin receptor gene messenger RNA; InsR, Insulin resistance; InsS, insulin sensitivity; LDL-C, low density lipoprotein cholesterol; LDLR, Low density lipoprotein receptor; LEL, liver enzyme levels; Lp, lipid profile; MALA, metformin-associated lactic acidosis; MAPK, Mitogen activated protein kinase; MDA, Malondialdehyde; MMP, mitochondrial membrane potential; MS, metabolic syndrome; RCT: randomized, controlled trial; OXPHOS, impaired oxidative phosphorylation; PAB, prooxidant-antioxidant balance; pBBR, pseudoberberine; PG, plasma glucose; PGs, prostaglandins; P-gp, P-glycoprotein; pi3k, phosphoinositol 3 kinase; PKC, protein kinase C; PMBG, post-meal blood glucose; PON1, Paraoxonase-1; PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma; PPBG, postprandial blood glucose; SBP, systolic blood pressure; SOD, superoxide dismutase; SREBP-1, sterol regulatory element-binding protein 1; STZ, streptozotocin; T1DM: type-1 diabetes mellitus; T2DM, type-2 diabetes mellitus; TAG, triacylglycerol; TC, total cholesterol; TG, triglycerides; TIC, total insulin consumption; TIS, total insulin secretion; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha; ULK1, Unc-51-like autophagy-activating kinase 1.

effectiveness against diabetes and other metabolic diseases. This review discusses various traditional uses of *Berberis* against metabolic diseases, along with its cell- and animal-model studies. The pharmacological effects of *Berberis* extracts and alkaloids against diabetes and other metabolic diseases are also discussed along with the molecular mechanism of action. Furthermore, based on the present studies of *Berberis* species against diabetes and metabolic diseases, research gaps were highlighted, and future recommendations were made.

METHODOLOGY

The scattered scientific information on *Berberis* species and isolated compounds used to counteract metabolic diseases was collected and documented. The synonyms of the various species were crosschecked with the plant name database The Plant List (www.theplantlist.org, Retrieved on November 22, 2019). Afterwards, the available articles on respective species were retrieved using popular search engines and various databases, such as SciFinder, ScienceDirect, PubMed, Scopus, Mendeley, JOAP, Microsoft academic, and Google Scholar. The keywords used were *Berberis*, berberine, diabetes, metabolic diseases, metabolic syndrome, ethnopharmacology, ethnobotany, chemical constituents, alkaloids, *in vitro*, *in vivo*, clinical study, and clinical trials. The data were congregated through the Boolean information retrieval method by using a plant name along with an "AND" operator followed by diabetes and metabolic syndrome. No prerequisite limitations on publications, i.e., language, year, and publication type (original contribution, review article, or key editorial note), were taken into consideration.

TAXONOMY AND ECOLOGY OF GENUS *BERBERIS*

According to The Plant List database (www.theplantlist.org, retrieved on September 20, 2019), the family Berberidaceae consists of a total of 19 genera. The members of the genus *Berberis* are reported to be difficult to identify taxonomically due to their extreme morphological variation in relation to the environmental factors and natural hybridization (Ahrendt, 1961; Rao et al., 1998). Various overlapping morphological characters, such as flowers, leaves, stems, and berries—which also depend upon the season—and plant age also make it difficult to identify during field tasks (Rao and Hajra, 1993; Rao et al., 1998; Tiwari and Singh Adhikari, 2011). *Berberis* species are widely cultivated around the world due to their high medicinal and ornamental value. Most members of the genus *Berberis* are reported to be tolerant to shade, resistant to drought, and widely distributed in open and wooded habitats and wetlands. These plants are also studied as indicators of habitat degradation in the temperate region due traditionally to their thorny stem and unpalatable shoots (Champion and Seth, 1968). Representative photographs of some *Berberis* species from the Indian Himalayan Region (IHR) are shown in **Figure 1**, and their major plant parts used to extract berberine and other bioactive alkaloids are shown in **Figure 2**.

ETHNOPHARMACOLOGY OF *BERBERIS* SPP. AGAINST DIABETES AND OTHER METABOLIC DISEASES

A literature review revealed that the ethnopharmacological uses of *Berberis* species have been documented from different parts of the world for the treatment of diabetes, hypertension, and obesity, and some of them also revealed the formulation methods. A majority of *Berberis* species were found to be used in the Himalayan region of India and Pakistan.

B. lycium Royle has been used traditionally for the treatment of diabetes mellitus and other diseases, particularly by the local inhabitants of the Himalayan region (Hamayun et al., 2006). Apart from diabetes, *B. lycium* is also used to treat bone fractures, diarrhoea, fever, intestinal colic, internal wounds, jaundice, menorrhagia, ophthalmic disorders, piles, rheumatism, sun blindness, and throat pain (Jabeen et al., 2015; Adhikari et al., 2019). Fruits and leaves of *B. lycium* are also reported to be used for the treatment of diabetes mellitus in south-west of Iran (Rahimi Madiseh et al., 2014) and Pakistan (Zain-Ul-Abidin et al., 2018). The water extract obtained by soaking the root bark in water is used for the treatment of diabetes (Ahmed et al., 2004). The whole plant is used to treat diabetes in Chamba district of Himachal Pradesh, West Himalaya, India (Rana et al., 2019). The Bhotiya tribal community of the Central Himalayan region of India used *B. lycium* roots with water for the treatment of diabetes (Phondani et al., 2010).

The stem of *B. aristata* DC. is widely used in Indian traditional medicine for the treatment of diabetes (Upwar et al., 2011), which is also reported in Ayurvedic Pharmacopoeia. The decoction (5–10 mL) of roots or stems of this species prepared with water was taken twice a day for 1–2 weeks to treat diabetes in Uttarakhand region (Kumar et al., 2019). It is also used by Uttarakhand people for the treatment of hypertension (Singh et al., 2019). The root, stem, and fruit also have been used to treat obesity (Chandrasekaran et al., 2018). *B. asiatica* is also used for the treatment of diabetes by the tribal communities of Chhota Bhangal, Western Himalaya, India. The decoction prepared from the roots is concentrated and dried in shade and then used with the sap of bitter guard for the treatment of diabetes (Uniyal et al., 2006).

In Iranian traditional medicine, *B. vulgaris* L. is extensively used to treat diabetes and hypertension (Rahimi-Madiseh et al., 2017). Local people use a decoction from the fruits and roots of *B. vulgaris* to treat hypertension (Baharvand-Ahmadi et al., 2016). The fruits are most frequently used in traditional and modern medicine (Rahimi Madiseh et al., 2014). Dried roots of *B. crateagina* DC. were recorded to be used as anti-diabetic

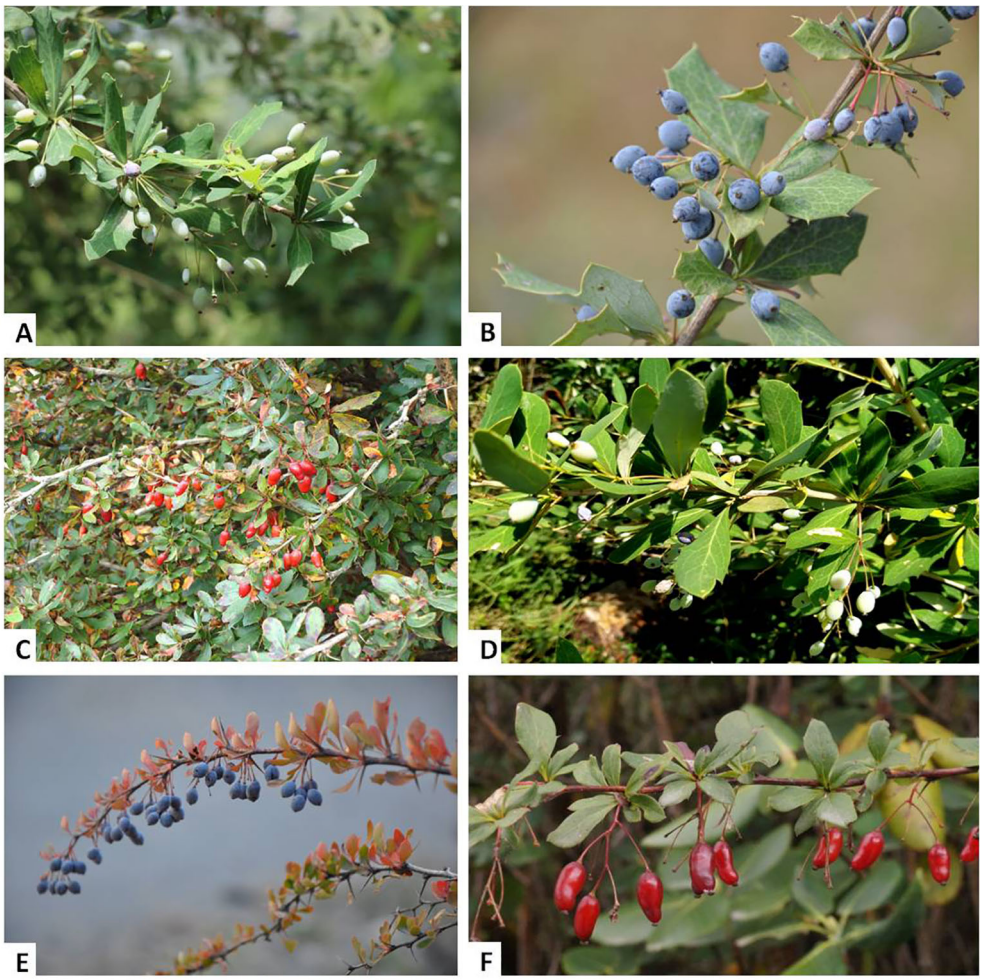

FIGURE 1 | Some *Berberis* species of Indian Himalayan Region (IHR). **(A)** *B. aristata* DC., **(B)** *B. asiatica* Roxb. ex DC., **(C)** *B. jaeschkeana* C.K.Schneid., **(D)** *B. lycium* Royle, **(E)** *B. pseudumbellata* R. Parker, **(F)** *B. thomsoniana* Schneider.

agents locally in Turkey, and the decoction or infusion prepared from dried roots was taken orally one to two times a day for the treatment of diabetes (Durmuskahya and Öztürk, 2013). The anti-diabetic activity has also been reported for *B. brevissima* Jafri and *B. parkeriana* C.K.Schneid. (Alemardan et al., 2013). Bahmani et al. (2016) reported that the inhabitant of Urmia, Iran, use boiled and steamed *B. integerrima* Bunge extract for the treatment of diabetes.

FIGURE 2 | Various plant parts of **(A)** *Berberis asiatica* collected from Indian Himalayan Region (IHR), includes, **(B)** roots, **(C)** stems and **(D)** stem barks. These parts are the major sources to extract Berberine (yellow color) from *Berberis* species.

ALKALOIDS FROM *BERBERIS* SPECIES: POTENTIAL COMPOUNDS AGAINST METABOLIC DISEASES

A large number of studies have been conducted on the isolation and quantification of bioactive compounds from *Berberis* species. The phytochemical investigations of the genus *Berberis* have shown the presence of more than 105 compounds with varying structural confirmations. Most of the studies on *Berberis* species are focused on phytochemical screening; for the presence and estimation of different secondary metabolites, such as alkaloids, flavonoids, steroids, sugars, triterpenoids, tannins, and other preliminary assays such as total ash content, acid soluble ash content, and moisture content (Belwal et al., 2016; Belwal et al., 2017; Andola et al., 2018; Srivastava et al., 2006; Shahid et al., 2009). However, the isolation and characterization of alkaloids from genus *Berberis* is well documented. Alkaloids are one of the major bioactive chemical constituents of the *Berberis* species, and they are responsible for various pharmacological activities of either whole extract or isolated individual compounds. Berberine (BBR) is one of the most commonly reported alkaloids from various *Berberis* species along with palmatine, magnoflorine, and jatrorrhizine, etc. (**Figure 3**) (Bhardwaj and Kaushik, 2012; Feng et al., 2018). Simple isoquinolone alkaloids are mainly reported from these species; however, studies have also reported their dimmers or dimeric benzylisoquinoline alkaloids (Leet et al., 1983). The detailed list of different alkaloids isolated from various *Berberis* species are given in **Table 1**. Among other compounds, BBR and its various natural and synthetic derivatives have also been evaluated and found effective in prevention and treatment of MS (Pérez-Rubio et al., 2013; Li et al., 2015; Zhao et al., 2017).

The effect of different habitat conditions (altitudinal variations and edaphic factors) of *Berberis* species has been investigated. Chandra and Purohit (1980) investigated eight *Berberis* species from different altitudinal range for determining the BBR concentration in different parts. Among these, *B. asiatica* was found to contain higher content of BBR than other species. Lower altitudinal range was found to contain higher BBR content within a species as compared to high altitude habitat. Among plant parts, roots contained a higher concentration of BBR (Chandra and Purohit, 1980). Similarly, variations in the BBR content of five *Berberis* species (i.e., *B. aristata, B. asiatica, B. jaeschkeana, B. lycium,* and *B. pseudumbellata*) depending upon the habitat have also studied. The presence of higher BBR content was recorded from rocky habitats in *B. jaeschkeana* (Andola et al., 2018). Both altitude and edaphic conditions were found to be responsible for the variation in BBR content in root and stem bark. Lower altitude populations showed significantly higher BBR content and positively correlated with moisture and potassium availability in soil species. Among these, *B. asiatica* contain significantly higher BBR content as compared to other species (Andola et al., 2010) Seasonal variations in the BBR content revealed higher percentage in summer and lower in rainy season (Andola et al., 2018). Low moisture and high soil potassium level is reported to be well correlated with high BBR content (Andola et al., 2011).

IN VITRO ACTIVITIES AGAINST DIABETES AND OTHER METABOLIC DISEASES

It has been suggested that physical exercise and a proper diet can act as controllers of the cause of T2DM and metabolic diseases. Currently available pharmacological interventions can control many aspects of diabetes and metabolic diseases, like microvascular and macrovascular complications, hypertension, dyslipidemia, and obesity. However, there is also a need for novel therapeutic agents that work alone or in combination with

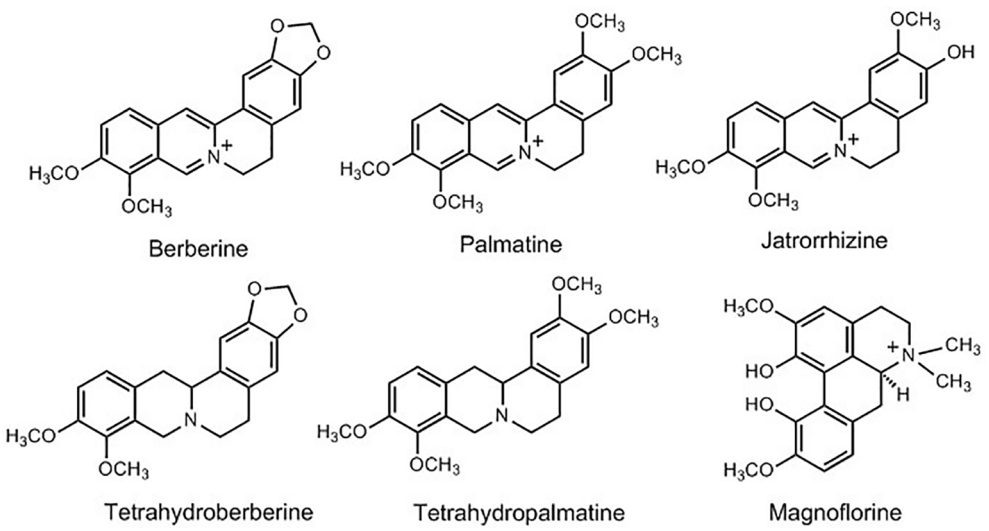

FIGURE 3 | Structures of some of the main bioactive alkaloids from *Berberis* species.

TABLE 1 | List of alkaloids isolated from various *Berberis* species.

Plant source	Plant parts	Alkaloids	References
B. acanthifolium Mart. ex Schult. & Schult.f.	Stem bark	Berberine, tetrahydropalmatine	(Tiwari and Masood, 1977)
B. aetnensis C. Presl.	Root	Berberine	(Alamzeb et al., 2015)
B. amurensis Rupr.	Stem	Berberine, palmatine, berberine	(Wu et al., 2015)
B. amurensis Rupr.	Young shoot	Berberubine, oxyacanthine, pseudopalmatine, amurenine	(Yusupov et al., 1993b)
B. aristata DC.	Stem bark	Berberine phenoxide, ketoberberine benzoate A, ketoberberine benzoate B	(Ahamad et al., 2014)
	Root and stem bark	Berberine, palmatine, berberrubine, jatrorrhizine, ketoberberine, dihydropalmatine, berbamine, pakistanamine	(Bajpai et al., 2015)
B. asiatica Roxb. ex DC.	Root	Berberine, oxyacanthine, berbamine, palmitine, jatrorrhizine, oxyberberine, tetrahydropalmatine, columbamine	(Bhakuni et al., 1968)
B. baluchistanica Ahrendt	Root	Pakistanine, pakistanamine, baluchistanamine, gandharamine	(Shamma et al., 1973; Shamma et al., 1974; Miana et al., 1979; Abu Zarga et al., 1982)
B. buxifolia Lam.	–	Chillanamine, (-)-osornine, (-)-curacutine, (-)-talcamine	(Leet et al., 1983)
B. calliobotrys Bien. ex Koehne	Root	Khyberine, pakistanamine, 1-O-methylpakistanine, pakistanine, chitraline, kalashine	(Fazal Hussain et al., 1980)
B. chitria Buch.-Ham. ex Lindl.	–	Berberine, palmatine, jatrorrhizine, oxyacanthine, *O*-methylcorydine-*N*-oxide	(Hussaini and Shoeb, 1985)
	Root bark	Palmatine,	(Choudhary et al., 2010)
B. coletioides Lechl.	-	Pronuciferine *N*-oxide, pronuciferine	(Fajardo et al., 2009)
B. concinna Hook.f.	Stem bark	Berberine, tetrahydropalmatine	(Tiwari and Masood, 1977)
B. crataegina DC.	Stem and root	Berberine, palmitine	(Petcu, 1968)
	Seed	Berbaine, oxyacanthine	
B. darwinii Hook.	–	magallanesine	(Valencia et al., 1985)
B. densiflora Boiss. & Buhse	Leaf	Berberine, β-allocryptopine, densinine, densiberine, glaucine, oxyacanthine, thalicmidine, isocorydine, *O*-methylcorypalline	(Khamidov et al., 1997c)
B. diaphana Maxim.	Bark	Berberine, palmatine, magnoflorine, jatrorrhizine	(Feng et al., 2018)
B. dictyophylla Franch.	Bark	Berberine, palmatine, magnoflorine, jatrorrhizine	(Feng et al., 2018)
B. glaucocarpa Stapf	Root	Oxyacanthine, tetrandrine	(Alamzeb et al., 2018)
B. heterobotrys E.L.Wolf	–	Berberine, palmatine, yatrorizine, oxyacanthine, berbamine, reticuline, obaberine, isocorydine, talikmidine, berberal.	(Karimov et al., 1993b)
B. heteropoda Schrenk	Young shoot and leaf	*N*-Methyldihydroberberine, 8-oxoberberrubine, berbamunine, aromoline, glaucine, talikmidine, isocorydine, reticuline, Pseudopalmatine, laudanosine, berpodine, isotetrandrine	(Karimov et al., 1992; Karimov et al., 1993a; Yusupov et al., 1993a)
B. hispanica Boiss. & Reut.	Root bark	Berberine tannate	(Aribi et al., 2017)
B. ilicifolia L.f.	–	Ilicifoline	(Fajardo et al., 1996)
B. iliensis Popov	Young shoot	(+)-β-*N*-Methylcorypalmine, berberrubine, berberine, magnoflorine	(Karimov and Shakirov, 1993)
B. integerrima Bunge	–	Berberine, berbamunine, oxyacanthine, magnoflorin, intebrine, intebrinine, intebrimine	(Karimov et al., 1977; Karimov et al., 1993g; Karimov et al., 1993h)
B. jaeschkeana C.K. Schneid	Root and bark	Berberine	(Andola et al., 2018)
B. jaeschkeana Schneid var. *jaeschkeana*	–	Berberine, palmatine, jatrorrhizine, chondrofoline, berberidione	(Alamzeb et al., 2015)
B. julianae C.K. Schneid.	Aerial part	Berberine, magnoflorine, glaucine, tetrahydrojatrorrhizine	(Brazdovicova et al., 1975)
B. kansuensis Schneid.	Bark	Berberine, palmatine, magnoflorine, jatrorrhizine	(Feng et al., 2018)
B. laurina Thunb.	Leaf	Berberine, (-)-tetrahydropalmatine, protopine	(Falco et al., 1968)
	Trunk bark and root	Berberine, obaberine (*O*-methyloxyacanthine), *O*-methylisothalicaberine, lauberine	(Falco et al., 1968)
B. libanotica Ehrenb.	Root, fruit	Oxycanthine, berbamine, jatrorrhizine, palmatine, berberine	(Alamzeb et al., 2015; Hosry et al., 2016)
B. lycium Royle	Fruit	Berberine, magnoflorine	(Sharma et al., 2018)
	–	Berberine, berbericine	(Sehdev et al., 1971)
B. nummularia Bunge	Leaf	Bernumine bernumidine and bernumicine, nummularine	(Karimov et al., 1993d; Faskhutdinov et al., 1997)
B. oblonga Scheid	Leaf	Glaucine, hydroxyacanthin, berbamine, berberin, isocoridin	(Khamidov et al., 2003)
	–	Berberine, berbamunine, oxyacanthine, magnoflorine, palmitine, oblongamine	(Karimov et al., 1977)
	Root	Berberine iodide, magnoflorine iodide, columbamine iodide, oxyacanthine, berbamine, 2'-*N*-methylisotetrandrine iodide	(Karimov and Lutfullin, 1986)

(Continued)

TABLE 1 | Continued

Plant source	Plant parts	Alkaloids	References
	Leaves and shoots	Thalicmidine and in the shoots, berberin. Other alkaloids isolated included glaucine, hydroxyacanthine, berbamine, isocoridine	(Khamidov et al., 2003)
B. pachycantha Koehne	Whole plant	Pachycanthine	(Ahmed et al., 2008)
B. petiolaris Wall. ex G. Don	Fruits, leaf, root and stem	Berberine, palmatine, magnoflorine, jatrorrhizine, tetrahydropalmatine, tetrahydroberberine, thalifendine/berberrubine, demethyleneberberine, reticuline, 8-oxoberberine, *N*-methyltetrahydroberberine,	(Singh et al., 2015)
	Root	Berbamine, berberine chloride, palimitine	(Miana and Ikram, 1970)
B. sibirica Pall.	Aerial part	(-)-Tetrahydropseudocoptisine, pseudoprotopine, (+)-chelidonine, (+)-glaziovine, berberine, palmatine, columbamine, berberubine, oxyacanthine, berbamine, 8-oxoberberine, 8-oxoberberubine, pakistanine, pronuciferine, *N*-acetylhomoveratrylamine	(Karimov et al., 1993e; Istatkova et al., 2007)
B. tabiensis L.A. Camargo	Stem	Tabienine	(Quevedo et al., 2008)
B. thunbergii DC	Stem	Berberine, berbamine, glaucine, isocorydine, oxycanthine, palmatine, thalicmidine	(Khamidov et al., 1997a)
	Leaf	Thalicmidine, oxycanthine, isocorydine, heliamine, berberine	(Khamidov et al., 1997a)
	Fruit	Oxyxanthine, isotetrandrine, thalicmidine	(Khamidov et al., 1997a)
	–	Berberine, columbamine	(Och et al., 2017)
	–	Oxyacanthine, palmatine, thalicmidine, isotetrandrine, berberine, berbamine, glaucine, isocorydine,heliamine	(Khamidov et al., 1997b)
B. turcomanica Kar. ex Ledeb.	Young shoot	Turconidine	(Karimov et al., 1993f)
	–	Turcberine	(Karimov et al., 1993c)
	Young shoot	Berberine, isocorydine, glaucine, thalicmidine, aromoline, oxyacanthine, turcomanine, berberine, papaverine, cyclotriveratrilene	(Khamidov et al., 1996d; Khamidov et al., 1996a)
	Leaf	Turcomanidine, Turcamine,	(Khamidov et al., 1996b; Khamidov et al., 1996c)
B. vernae Schneid.	Bark	Berberine, palmatine, magnoflorine, jatrorrhizine	(Feng et al., 2018)
B. virgetorum C.K. Schneid.	Whole plant	(-)-Berbervirine, berberine, jatrorrhizine, noroxyhydrastinine	(Liu et al., 1995)
B. vulgaris L.	Root bark	Berberine, palmatibne, bersavine, muraricine, berbostrejdine, berbamine, aromoline, obamegine, 8-oxoberberine, berbidine, bargustanine, Berberine, oxyacanthine, talikmidine, yatrorizine, berbamine, berbamunine, isocorydine	(Karimov et al., 1993i; Khamidov et al., 1995; Hošťálková et al., 2013; Hostalkova et al., 2019)
B. vulgaris subsp. *australis* (Boiss.)	Root bark	Berbamine, sotetrandrine, oxyacanthine, obaberine, aromoline, obamegine, thaligrisine, thalifoline, 8-oxyberberine, chilenine, (-)-tejedine	(Suau et al., 1998)

(Continued)

currently available drugs. Within the pharmacological options, phytochemicals have a great potential to act against T2DM, MS, and associated complications (Davì et al., 2010). Extracts of *Berberis* species and their components, especially alkaloids, have been documented for their potential activity against T2DM and MS in various *in vitro* studies (**Table 2**) (Potdar et al., 2012)

Studies in mouse 3T3-L1 cells suggested that BBR has an pivotal role in regulating adipose tissues (Kishimoto et al., 2015). Experiments in mitochondria isolated from the liver of high-fat-fed rats have shown that BBR exhibited protective effects against MS that was associated with the increased mitochondrial sirtuin-3 (SIRT3) activity, normalizing mitochondrial function, and preventing a state of impaired oxidative phosphorylation (OXPHOS) that caused energetic deficit (Teodoro et al., 2013). In the same way, the preventive effects of BBR on diet-induced insulin resistance (InsR) was suggested to be linked to sirtuin-1 (SIRT1) and mitochondrial biogenesis (Gomes et al., 2012). It has been suggested that BBR is a unique natural medicine against insulin resistance in T2DM and MS (Kong et al., 2009). Different investigations have concluded that BBR as a new hypolipidemic drug works by a different mechanism of action to that of statin drugs (Kong et al., 2004). BBR works on multiple molecular targets as an inhibitor of peroxisome proliferator-activated receptor (PPAR) γ and α and is a potential weight reducing, hypolipidemic, and hypoglycemic agent (Huang et al., 2006). Prolonged activation of AMP-activated protein kinase (AMPK) by BBR improved CD36 expression in hepatocytes and was evoked in fatty acid uptake *via* processes associated with hepatocellular lipid accumulation (Choi et al., 2017). Also, BBR improved insulin sensitivity (InsS) by inhibiting fat storage and adjusting the adipokine profile in human preadipocytes (Yang et al., 2012). The hypoglycemic effects of BBR have also been attributed to its acute activation of the transport activity of glucose transporter 1 (GLUT1) (Cok et al., 2011).

Numerous studies of BBR in *in vitro* models have shed light on its positive effect on T2DM. BBR promoted glucose uptake and inhibited gluconeogenesis by inhibiting SIRT3, and regulating the mitochondria-related pathways (Zhang et al., 2018). BBR treatment attenuated a palmitate-induced reduction in glucose uptake and consumption through a

TABLE 2 | *In vitro* activity of extracts and/or isolated compounds from *Berberis* species against diabetes and metabolic diseases.

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
Berberine			
Berberine (BBR)	Mouse 3T3-L1 cells	Downregulated transcription factors (CCAAT/enhancer binding protein β, CCAAT/ enhancer binding protein α) and PPARγ, suppress PPARs, A-FABP and FASN and inhibit 3T3-L1 fibroblast differentiation to adipocytes	(Kishimoto et al., 2015)
Berberine (BBR)	Mitochondria isolated from the liver of high-fat-fed rats	↓capacity to accumulate calcium and OXPHOS capacity (MMP, oxygen consumption, and cellular ATP levels). ↑ mitochondrial SirT3 activity, normalizing mitochondrial function, and preventing a state of energetic deficit caused by impaired OXPHOS	(Teodoro et al., 2013)
Berberine (BBR)	C2C12 cell line	Reverted mitochondrial dysfunction induced by HFD and hyperglycemia in skeletal muscle, in part due to an ↑ in mitochondrial biogenesis. The prevention of mitochondrial dysfunction, ↑ in mitochondrial biogenesis, and BBR-induced AMPK activation, are blocked in cells in which SIRT1 has been knocked down.	(Gomes et al., 2012)
Berberine (BBR)	Cultured human liver and L6 rat skeletal muscle cells	↑ InsR mRNA and ↑ protein expression in dose- and time-dependent results. InsR expression in the L6 rat skeletal muscle cells. BBR-enhanced InsR expression improved cellular glucose consumption only in the presence of insulin. Silencing InsR gene with small interfering RNA or blocking the pi3k ↓ this effect. BBR-induced InsR gene expression through a PKC-dependent activation of its promoter. Inhibition of PKC abolished BBR-caused InsR promoter activation and InsR mRNA transcription.	(Kong et al., 2009)
Berberine (BBR)	3T3-L1 preadipocytes	Inhibitor of PPARγ and α	(Huang et al., 2006)
Berberine (BBR)	Human platelet	Inhibited platelet aggregation, superoxide production *via* modulating AR, NOX, and glutathione reductase activities in HG	(Paul et al., 2019)
Berberine (BBR)	Primary hepatocytes	Promotion of glucose uptake and prevention of gluconeogenesis by inhibition of SIRT3, and by regulation of mitochondria-related pathways.	(Zhang et al., 2018)
Berberine (BBR)	HepG2 and mouse primary hepatocytes	Prolonged activation of AMPK BBR-induced ↑CD36 expression in hepatocytes, evoking in FA uptake *via* processes associated to hepatocellular lipid accumulation and fatty liver.	(Choi et al., 2017)
Berberine (BBR)	H9c2 cardiomyocytes	Attenuation of palmitate-induced reduction in glucose uptake and consumption by ↓cellular DAG levels and accumulation of TAG.	(Chang et al., 2016)
Berberine (BBR)	Rat MCs	Inhibition of mesangial cell proliferation and hypertrophy by modulating cell cycle progress. Suppression of high glucose-induced TGF-β1 and FN expression through blocking NF-κB/AP-1 pathways.	(Lan et al., 2014)
Berberine (BBR)	human hepatoma cells	Upregulated LDLR expression independent of sterol regulatory element-binding proteins, but dependent on ERK activation. Also ↑ LDLR expression through a post-transcriptional mechanism that stabilizes the mRNA.	(Kong et al., 2004)
Berberine (BBR)	Omental adipose tissue biopsies	Inhibition of human preadipocyte differentiation and leptin and adiponectin secretion accompanied by downregulation of PPARγ2, C/EBPα, adiponectin, and leptin mRNA expression	(Yang et al., 2012)
Berberine (BBR)	3T3-L1 adipocytes, L6 myotubes, and L6 cells	↑AMPK in 3T3-L1 adipocytes and L6 myotubes, ↑GLUT4 translocation in L6 cells in a pi3k -independent manner, and ↓ lipid accumulation in 3T3-L1 adipocytes	(Lee et al., 2006)
Berberine (BBR)	CEM, HCT-116, HepG2.2.15, SW1990, HT1080 and 293T cell lines	↑gene expression of the insulin receptor	(Zhang et al., 2010)
Berberine (BBR)	L929 cells	Activation of GLUT 1 transporter	(Cok et al., 2011)
Berberine (BBR	3T3-L1 and L6 cells	Inhibition of PTP1B, and ↑IR and ↑IRS1 phosphorylation	(Chen et al., 2010)

TABLE 2 | Continued

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
Berberine			
Berberine (BBR)	3T3-L1 cells	↓TG accumulation by ↑pIRS1-PI3KpAkt, ↑GLUT4 translocation and ↑insulin tropic action by pCREB-pIRS2-pAkt	(Ko et al., 2005)
Berberine (BBR)	L6 cells	↑AMPK and ↑p38 MAPK phosphorylation	(Cheng et al., 2006)
Berberine (BBR)	3T3-L1 cells	Regulation of PPARs and positive transcription elongation of factor b expression	(Zhou and Zhou, 2010)
Berberine (BBR)	HepG2 and C2C12 cells	↑glucose metabolism by glycolysis stimulation and mitochondrial respiratory chain inhibition	(Xu et al., 2014)
Berberine (BBR)	HL-7702, normal human liver cell lines	LDLR up-regulation by AMPK-dependent Raf-1 activation	(Li et al., 2014)
Combination of berberine and/or derivatives			
Berberine (BBR) and dihydroberberine	L6 and LKB1 –/– cells	AMPK activation, by complex I inhibition of the mitochondrial transport chain	(Turner et al., 2008)
9-*O*-lipophilic group substituted) berberine (9-*O*-BBR)	HepG2 cells	↑ hypoglycemic activity	(Zhang et al., 2016)
13-Methylberberine (13-Me-BBR)	Mouse 3T3-L1 cells	Downregulated the expression of adipocyte differentiation transcription factors (PPARγ and C/EBPα). ↓PPARγ, ↓C/EBPα, and ↓SREBP-1 protein levels. Effect require AMPK signaling pathway	(Chow et al., 2016)
Berberine (BBR) and metformin	HepG2 hepatocytes and C2C12 myotubes	Promotion of glucose metabolism *via* stimulation of glycolysis, not be related to AMPK activity.	(Xiao et al., 2018)
BBR derivatives: thalifendine	Human HepG2 liver cells	↑LDLR or InsR protein expression.	(Wang et al., 2009)
BBR amide derivatives	HL-7702 cells	↑ glucose-lowering efficacies	(Ren et al., 2017)
Mannose modified berberine (m-BBR)	HepG2 cells	↑ antidiabetic activity	(Han et al., 2019)
Pseudoberberine (pBBR)	HepG2 cells	AMPK activation and LDR up-regulation.	(Wang et al., 2012)
Palmatine	Differentiated myocytes, L6 cells	anti-diabetic activity may be mediated through insulin dependent pathway by the activation of IRTK and PI3K	(Sangeetha et al., 2013)
***Berberis* extracts**			
B aristata bark methanolic extract	Dipeptidyl peptidase IV	Inhibition of dipeptidyl peptidase IV activity	(Chakrabarti et al., 2011)
B. mycrophylla roots ethanolic extract	non-resistant and insulin-resistant HepG2 cells	hypoglycemic effects and ↑ glucose uptake by activating AMPK protein.	(Furrianca et al., 2017)
B. vulgaris roots (ethanolic extract) and berberine (BBR)	α-Glucosidase	↑ α-glucosidase activity, extract > BBR	(Abd El-Wahab et al., 2013)
B. vulgaris roots (methanolic extract)	α-Amylase	↑ α-amylase activity	(Boudjelthia et al., 2017)
Jinqi Jiangtang tablet (berberine-contain)	α-Glucosidase, lipase and aldose	↑α-glucosidase, ↑lipase, and ↑aldose reductase activities,	(Chang et al., 2015)

The ↑ and ↓ signs shows significant increase and significant decrease of evaluated factors during mentioned studies.

reduction in cellular diacylglycerol (DAG) levels and the accumulation of triacylglycerol (TAG) in H9c2 cells (Chang et al., 2016). In addition, BBR displayed beneficial effects in the treatment of diabetes and obesity *via* stimulation of AMPK activity (Lee et al., 2006). The mechanisms of action of BBR in treatment of T2DM are suggested to be different than that of metformin and rosiglitazone (Zhang et al., 2010). BBR, as an insulin signal activator, had shown insulin-mimicry effects through the inhibition of protein tyrosine phosphatase 1B (PTP1B) activity on both adipocytes and myocytes (Chen et al., 2010) and acted as an effective insulin sensitizing and insulinotropic agent (Ko et al., 2005). Moreover, BBR and metformin promoted glucose metabolism by stimulating glycolysis through the inhibition of mitochondrial respiratory chain complex I and independent of AMPK activation (Xu et al., 2014). Besides, BBR circumvented the insulin signaling pathways and stimulated the glucose uptake through the AMP-AMPK-p38 MAPK pathway (Cheng et al., 2006). BBR modulated metabolism-related PPARs expression and differentiation-related positive transcription elongation factor b (P-TEFb) expression in adipocytes, which are associated with its hypoglycemic and hypolipidemic effects (Zhou and Zhou, 2010). In addition, BBR upregulated LDL receptor expression through Ras-independent (but AMPK-dependent) Raf-1 activation in liver cells (Li et al., 2014). BBR and metformin induced glycolysis and glucose consumption but are not related to the AMPK status (Xiao et al., 2018).

Different natural and synthetic derivatives of berberine are also evaluated for their *in vitro* activities. A BBR derivative, thalifendine, showed upregulatory activities for both LDLR and InsR, proving to be a potential treatment of both hyperlipidemia and hyperglycemia (Wang et al., 2009). Similarly, BBR amide derivatives improved the glucose-lowering effects (Ren et al., 2017). Mannose-modified BBR derivative exhibited high anti-diabetic activity at both high and low drug concentrations (Han et al., 2019). Palmatine showed anti-diabetic activity

mediated through an insulin-dependent pathway by the activation of IRTK and PI3K (Sangeetha et al., 2013). Pseudoberberine (pBBR) has exhibited a potential effect on AMPK activation and LDLR upregulation as compared with BBR (Wang et al., 2012).

In the same way, the effects of extracts of species of the genus *Berberis* have been studied in several *in vitro* models and found effective. For instance, *B. mycrophylla* root extracts showed hypoglycemic effects and stimulated glucose uptake in HepG2 cells with and without resistance by activating AMPK protein (Furrianca et al., 2017). *B. aristata* bark methanolic extracts also inhibited the dipeptidyl peptidase–IV (DPP-IV) enzyme activity (Chakrabarti et al., 2011). *B. vulgaris* roots (ethanolic extract) and BBR showed α-glucosidase inhibition, where the inhibition caused by the extract was found to be higher than that of the BBR alone (Abd El-Wahab et al., 2013), and the extract also showed α-amylase inhibition activity (Boudjelthia et al., 2017).

Some of the mechanisms of *Berberis* species and BBR against diabetes and metabolic diseases are depicted in **Figure 4**.

IN VIVO ACTIVITIES AGAINST DIABETES AND METABOLIC DISEASES

Extracts of *Berberis* species and their components, especially alkaloids, have been documented for their potential activity against T2DM and MS in *in vivo* models (**Table 3**). In the MS condition, BBR improved vascular inflammation and remodeling that was found to be correlated with the ability to inhibit p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression (Li et al., 2015). Long-term treatment with BBR diminished the adipose tissue weight and decreased the renal injury (MS related diseases) in spontaneously hypertensive rats (Kishimoto et al., 2015). In normal diet-fed mice treated with BBR, hepatic CD36 expression and TG levels were increased; however, these effects were prevented when hepatic CD36 was silenced with an adenovirus containing CD36-specific short hairpin RNAs (shRNA) (Choi et al., 2017). BBR also improved the insulin-mediated vasodilatation of mesenteric arteries in diabetic rats through upregulation of insulin receptor-mediated signaling and increasing vascular InsS (Geng et al., 2016). Similarly, BBR increased both InsR and the low-density lipoprotein receptor (LDLR) expression, which resulted in a cellular response against InsR (Kong et al., 2009). In hyperlipidemic hamsters, the cholesterol-lowering effect of BBR was found to be due to its activity on upregulation of hepatic LDLR (Kong et al., 2004). Administration of BBR in hyperlipidemic and InsR rats decreased blood free fatty acid levels and increased the activity of lipoprotein lipase, leading to the amelioration of blood lipid and glucose metabolism (He et al., 2004). BBR administration resulted in the decrease of fasting blood glucose (FBL) level and ameliorated glycogen structural fragility (Li et al., 2019). Furthermore, BBR displayed beneficial effects in the treatment of obesity, and this was in part *via* improvement of adipose tissue fibrosis (Wang L. et al., 2018). BBR was reported to act in the liver to regulate lipid utilization and to maintain whole-body energy metabolism by mediating autophagy and FGF21 activation (Sun Y. et al., 2018). Additionally, BBR is also reported to reduce the systemic low-grade inflammation of T2DM mice to alleviate disease, and this effect may be achieved through regulating the gut microbes or inhibiting the TLR4 signaling pathway (Cao et al., 2017). Other *in vivo* investigations also showed the hypoglycemic effects of BBR through the improvement in gut-derived hormones and the attenuation of both intestinal mucosal mechanic and immune

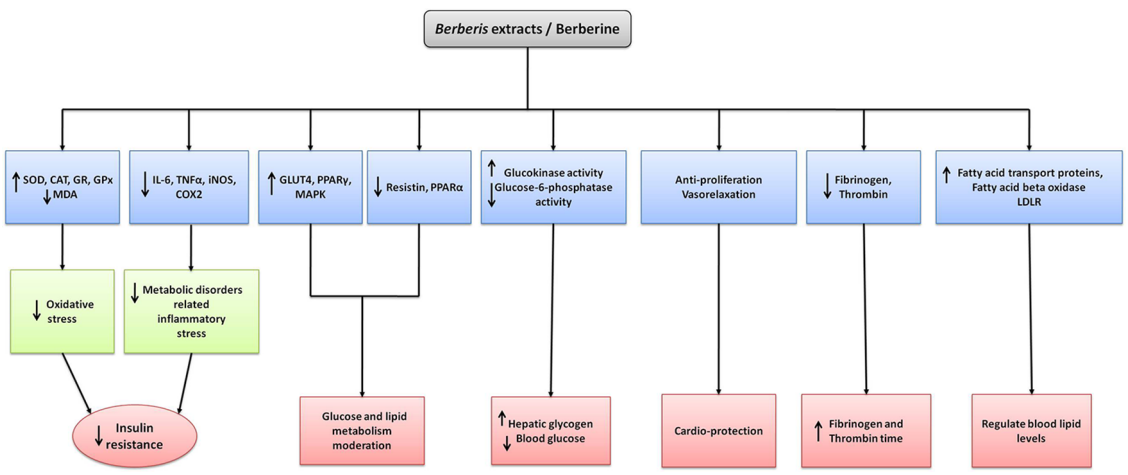

FIGURE 4 | The mechanism of action of extracts and its major isolated alkaloid of *Berberis* species in the treatment of diabetes and metabolic syndrome. *Berberis* spp. and berberine upregulate the anti-oxidant enzymes while decreasing reactive oxygen species and inflammatory mediators which in turn decreases oxidative and inflammatory stresses and thus decreasing insulin resistance. Upstream regulating expression of GLUT4, PPARγ, MAPK and downstream regulation of resistin, PPARα results glucose and lipid metabolism moderation. Increase in AMPK and glucokinase activities while decrease in glucose-6-phosphate activity results in decreasing gluconeogenesis, restoring hepatic glycogen and blood glucose. Upregulating AMPK and p38 MAPK activities also cause increasing insulin action and decreasing lipid synthesis. Antiproliferative action and vasorelaxation results in cardioprotection whereas decrease in fibrinogen and thrombin results in increasing fibrinogen and thrombin time respectively. Increasing expression of fatty acid transport proteins, fatty acid beta oxidase and LDLR aids in regulating blood lipid levels.

TABLE 3 | *In vivo* activity extracts and/or isolated compounds from *Berberis* species against diabetes and metabolic diseases.

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
Berberine			
Berbamine (BBA)	STZ-induced diabetic Sprague-Dawley rats	↑metabolic enzymes activities and preserved the glucose homeostasis	(Chandrasekaran et al., 2018)
Berberine (BBR)	Specific-pathogen-free male C57BL/6 mice	prolonged activation of AMPK BBR-induced ↑CD36 expression and fatty acid uptake	(Choi et al., 2017)
Berberine (BBR)	male Sprague–Dawley diabetic rats	↑DVIS and ↑mesenteric vasodilatation by insulin receptor-mediated signaling upregulation.	(Geng et al., 2016)
Berberine (BBR)	male Wistar rats	↓secretion of inflammatory factors and ↑vascular remodeling. Inhibition of p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression.	(Li et al., 2015)
Berberine (BBR)	Male spontaneously hypertensive rats	↓BWG, ↓retroperitoneal adipose tissues, ↓mesenteric adipose tissues, and ↓urinary albumin excretion.	(Kishimoto et al., 2015)
Berberine (BBR)	T2DM STZ-induced Wistar rats	↓FBGL, ↓FSIL, ↑InsS, ↑InsR-mRNA, and ↑PKC activity in the liver.	(Kong et al., 2009)
Berberine (BBR)	hyperlipidemic hamsters	↓TC, ↓LDL-C, ↑hepatic LDLR mRNA, and ↑hepatic LDLR protein	(Kong et al., 2004)
Berberine (BBR)	Hyperlipidemic and IR rats	↓TC, ↓TG, ↓ApoB, ↓LDL-C, ↓FFA, ↑HDL-C, ↑ISI, ↑ApoAI, and ↑lipoprotein lipase activity	(He et al., 2004)
Berberine (BBR)	T2DM db/db mice	↓FBGL and ameliorated glycogen structural fragility	(Li et al., 2019)
Berberine (BBR)	HFD Obese rats	↓BWG, ↑glucose tolerance, ↓collagen deposition and reversed the upregulation of fibrosis related genes in the adipose tissue of HFD.	(Wang L, et al., 2018)
Berberine (BBR)	Liver-specific SIRT1 knockout mice	Regulation of lipid usage and preserved whole-body energy metabolism *via* autophagy and FGF21 activation.	(Sun Y, et al., 2018)
Berberine (BBR)	Rat islets	Inhibition of glucose-stimulated insulin secretion with AMPK activation, ↓OCR and ↓ATP production induced by high glucose, and attenuation of glucose-stimulated expression of fatty acid synthase	(Bai et al., 2018)
Berberine (BBR)	T2DM mice	↓systemic low-grade inflammation to alleviate disease, by regulating the gut microbes and/or inhibiting TLR4 signaling pathways.	(Cao et al., 2017)
Berberine (BBR)	Diabetic rats	hypoglycemic effects associated to ↑ gut-derived hormones.	(Gong et al., 2017)
Berberine (BBR)	T2DM rats	↓MALA, ↑InsR and ↑liver enzymes by	(Almani et al., 2017)
Berberine (BBR)	Diabetic rats	Attenuation of hyperglycemia, oxidative stress and inflammation by potentiation of the antioxidant defenses and up-regulation of PPARγ expression	(Mahmoud et al., 2017)
Berberine (BBR)	SD rats	↓2h-PPG level by local inhibition of intestinal DPP-IV.	(Wang J, et al., 2016)
Berberine (BBR)	Diabetic rat model	↓ expressions of Nrf2 and HO-1	(Tao et al., 2017)
Berberine (BBR)	Diabetic rats	Inhibition of hepatic gluconeogenesis *via* the regulation of the LKB1-AMPK-TORC2 signaling pathway.	(Jiang et al., 2015)
Berberine (BBR)	Diabetic hamsters	↓BGL, ↓TC, ↓TG, ↓FFA, ↓LDL-C, ↓Glucose, ↓insulin levels, ↓malondialdehyde, ↓thiobarbituric acid-reactive substance, and ↓8-isoprostane levels, ↑expression of skeletal muscle glucose transporter 4 mRNA and ↓liver LDL receptor mRNA expression.	(Liu et al., 2015)
Berberine (BBR)	Zucker Diabetic Fatty Rats	↓HbA1c, ↓TC, ↓TG, ↑insulin secretion, regulation of glucose and lipid metabolism and activation of pAMPK.	(Dong et al., 2016)

(Continued)

TABLE 3 | Continued

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
Berberine			
Berberine (BBR)	db/db mice and high-fat–fed Wistar rats	↓BWG, ↑glucose tolerance, ↓TG, and ↑ insulin action	(Lee et al., 2006)
Berberine (BBR)	Diabetic rats	Direct inhibition of liver gluconeogenesis	(Xia et al., 2011)
Berberine (BBR)	Diabetic rats	Intestinal microbiome modulation	(Han et al., 2011)
Berberine (BBR)	Diabetic rats	Lipid metabolism regulation and ↑ elimination of free radicals	(Tang et al., 2006)
Berberine (BBR)	Diabetic rats	PPAR α/δ up-regulation and PPARδ repression in liver	(Zhou et al., 2008)
Berberine (BBR)	Non-obese Diabetic rats	Regulation of MAPK activity to control the differentiation of Th17 and Th1	(Cui et al., 2009)
Berberine (BBR)	Diabetic rats	Promotes secretion of glucagon-like peptide type I	(Lu et al., 2009)
Berberine (BBR)	Diabetic rats	Tyrosine phosphatase 1B activity inhibition and insulin-like effect	(Chen et al., 2010)
Berberine (BBR)	Diabetic hamster	Up-regulation of LXRα, PPARα, and down-regulation of SREBPs	(Liu et al., 2010)
Berberine (BBR)	Diabetic rats	↓ intestinal disaccharidases and β-glucuronidases activities	(Liu et al., 2008)
Berberine (BBR)	Diabetic rats	Glucose metabolism modulation by GnRH-GLP-1 and MAPK pathway in the gut	(Zhang et al., 2014)
Berberine chloride (BC)	Diabetic rats	↓FBG, ↓WBC, ↓HbAlc ↑plasma insulin, ↑hemoglobin, ↑RBC, ↑Ht, ↑MCH and ↑MCHC.	(Chandirasegaran et al., 2017)
Berberine chloride (BC)	Diabetic rats	↓TC, ↓TG, ↓phospholipids, ↓LDL-C, ↓VLDL, ↓LOOH, ↓TBARS. ↑SOD, ↑CAT, ↑GPx, non-enzymatic antioxidant (↑GSH, ↑vitamin C, ↑vitamin E) and ↑IRS-1, ↑PKB, ↑Akt and ↑GLUT-4)	(Chandirasegaran et al., 2019)
Berberine fumarate (BF)	T2DM rats	↑metabolic disorder and ↓ inflammation by ↓over-expression of TLR4 and p-JNK and ↑PI3K and VGLUT2 expression.	(Cui et al., 2018)

(Continued)

TABLE 3 | Continued

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
Combination of berberine and other compounds/extracts			
Berberine chloride (BC), oryzanol and vitamin B2	Male Wistar hyperlipidemic rats	↓lipid effect without apparent adverse side effects.	(Li et al., 2016)
Berberine (BBR), *Ortosiphon staminensis,* policosanol, red yeast rice extract, folic acid and coenzyme Q10	Rats	↓TC, ↓LDL-C, ↓DBP, ↓TG, and ↑HDL-C. antihypertensive effect, which allows an effective control of blood pressure	(Rozza et al., 2009)
Berberine - Metformin Hybrid (BMH473)	T2DM obese rats	↑maintaining glucose and ↑ lipid homeostasis, ↑antihyperlipidemic activity.	(Jia et al., 2019)
berberine (BBR) and Timosaponin B2 (TB-2)	Goto-Kakizaki rats	↑anti-diabetic efficacy.	(Huang et al., 2019)
berberine (BBR) and Glycyrrhizic acid	Rats	↓FBG, and ↑Insulin level	(Qiao et al., 2018)
Berberine (BBR) with resveratrol	High fat diet-induced mice	↓TC, ↓TG, and ↓LDL-C	(Zhu et al., 2018)
Berberine (BBR) and Gelucire44/14	diabetic mice	Gelucire44/14 showed potential ↑oral absorption of BBR thus ↑ anti-diabetic efficacy.	(Sun J, et al., 2018)
Berberine organic acid salts (BOAs), including berberine citrate, berberine fumarate, berberine malate, and berberine succinate	T2DM rats	↑ hypoglycemic effects	(Li et al., 2017)
Berberine (BBR) and *Coptis chinensis* extract (CCE)	T2DM rats	↑pancreatic insulin secretion *via* ↑ islet β-cell proliferation and ↑ protein expression of PARP-1.	(Jiang et al., 2017)
Berberine (BBR) combined with Canagliflozin	Diabetic mice	↓FBG and ↓insulin. Antidiabetic effect associated with ↑ pAMPK and ↓ TNFα in kidneys.	(Cai-Ming et al., 2016)
Berberine (BBR) and Ginsenoside Rb1 (Rb1)	Diabetic mice	Improved abnormal metabolism of glucose and lipid.	(Shang et al., 2015)
Berberin glycyrrhizinate complex salt (BGC)	GK rats	↓PBG, ↓insulin level, ↓GSP, ↓LDL-C and ↓MDA, and ↑ histopathological changes in kidney and pancreas.	(Wang et al., 2014)

(Continued)

TABLE 3 | Continued

Extracts from *Berberis* spp./ isolated compounds	Model	Outcomes	References
***Berberis* extracts**			
B. aristata roots (ethanolic extract)	Diabetic rats	↓dose-dependent in hyperglycemia, ↓TC, ↓TG, ↓AST, and ↓ALT levels of serum, ↓serum creatinine and ↓blood urea.	(Mittal et al., 2012)
B. aristata stem (ethanolic extract)	T1DM and T2DM albino rats	↑Liver glycogen and ↓FBS	(Rameshwar et al., 2009)
B. aristata roots (ethanolic extract)	STZ-induced diabetic rats	↓PBG	(Pareek and Suthar, 2010)
B. aristata stem bark (aqueous extract)	STZ-induced diabetic rats	↓TC and ↑HDL-C	(Ahamad et al., 2012)
B. aristata bark (ethanolic extract)	alloxan-induced diabetic rats	↓PBG	(Semwal et al., 2008)
B. aristata stem bark (methanolic extract)	Alloxan-Induced DiabeticRats	↓PBG	(Gupta et al., 2010)
B. aristata roots (methanolic-water extract	Diabetic rabbits	↓PBG	(Akhtar et al., 2008)
B. aristata roots (water-ethanolic extract)	Diabetic rats	Regulated glucose homeostasis *via* ↓ gluconeogenesis and ↓oxidative stress.	(Singh and Kakkar, 2009)
B asiatica roots (water-ethanolic extract)	Diabetic rats	↓BW	(Singh and Jain, 2010)
B. dictyophylla roots (extract)	Diabetic mice and normal mice	↓FBG, ↓ICAM-1, ↓ANGII, and ↓SOD in serum expression	(Yue et al., 2013)
B. holstii roots (aqueous extract)	Alloxan-induced diabetic male mice	↓FBGL	(Kimani et al., 2017)
B. integerrima roots (aqueous extract)	Diabetic male Wistar rats	↑renal by control of blood glucose and renal protective effects.	(Ashraf et al., 2013)
B. integerrima fruits (anthocyanin fraction)	Diabetic Male Sprague Dawley rats	↓FBG, ↑ liver glycogen level, and ↑ body weight.	(Sabahi et al., 2016)
B. julianae roots (methanolic extract)	T2DM mice	↑ GLUT4 translocation, ↑ oral glucose tolerance, ↑LDL-C, ↓BWG, ↓blood glucose and ↓other related blood-lipid contents.	(Yang et al., 2014)
B. lycium roots (aqueous extract)	Diabetic rabbits	↓ FBG.	(Ahmad and Alamgeer, 2009)
B. lycium extract (BLE)	Diabetic rabbits	↓TG, ↓TC, ↓LDL-C, and ↑HDL-C	(Ahmad et al., 2008)
B. lycium leaves (methanolic extract)	Female diabetic rabbits	↓FBG	(Hussain et al., 2017)
B. lycium roots (ethanolic extract)	Alloxan treated rats	↓FBG	(Gulfraz et al., 2007)
B. lycium roots (powder)	Broilers chickens	↓TG, ↓TC, ↓LDL-C, and ↑HDL-C	(Chand et al., 2007)
B. lycium roots (aqueous extract)	Diabetic rats	↓FBG, ↓TC, ↓TG, ↓LDL-C, ↓VLDL, ↓SGOT, ↓SGPT, and ↓ALP	(Mustafa et al., 2011)
B. lycium fruits (aqueous extract)	Diabetic rats	↓TC, ↓TG, ↓LDL-C, ↓VLDL, and ↓MDA	(Rahimi Madiseh et al., 2014)
B. lycium root (methanolic extract) and berberine (BBR)	Diabetic rats	↓FBG, ↑glucose tolerance, positive serum lipid profiles, glycosylated hemoglobin and body weight.	(Gulfraz et al., 2008)
B. vulgaris roots (aqueous extract)	Diabetic rats	↓TC and ↓TG.	(Meliani et al., 2011)
B. vulgaris fruits (aqueous and hydro-ethanolic extract)	T1DM Rats	↑ serum glucose levels, ↑ serum alanine aminotransferase activities, and ↓ HbA1c.	(Karami et al., 2016)
B. vulgaris fruits (ethanolic extract)	Diabetic rats	↑total antioxidant levels, ↓MDA and ↓FBG, and ↑mRNA level of GK	(Hemmati et al., 2016)
B. vulgaris fruits (Hydro-ethanolic extract)	Diabetic rats	↓ liver damage by influencing hepatic histopathological and biochemical markers	(Rahimi-Madiseh et al., 2017)
Jatrorrihizine	Hyperglycemic mice	↓FBG and ↑aerobic glycolysis	(Yan et al., 2005)
Jatrorrihizine and berberine	Diabetic rats	↓FBG. Berberine > Jatrorrihizine	(Fu et al., 2005)
Palmatine	Normal rats	↓FBG.	(Patel and Mishra, 2011)

The ↑ and ↓ signs show significant increase and significant decrease, respectively, of evaluated factors during mentioned studies.

barrier damages (Gong et al., 2017). In the same way, the gut microbiota modulation was also suggested to be an effective mechanism of the antidiabetic effect of BBR (Han et al., 2011). The lipid-lowering effect of BBR chloride treatment in hyperlipidemic rats was found to be associated with a global change in the metabolism of lipids, carbohydrates, and amino acids as well as the structure of microbiota (Li et al., 2016).

On the other hand, BBR protects against metformin-associated lactic acidosis (MALA) in streptozotocin (STZ)-induced T2DM (Almani et al., 2017). BBR attenuated hyperglycemia and its associated oxidative stress and inflammation through, possibly, the potentiation of the antioxidant defenses and upregulation of PPARγ expression (Mahmoud et al., 2017). BBR decreased 2-hour postprandial plasma glucose (2h-PPG) level in STZ-induced diabetic rats by locally inhibiting intestinal DPP-IV (Wang J. et al., 2016). Moreover, BBR also reduced the blood glucose level in diabetic

rats, improving the blood lipid and decreasing the retinal vascular injury, suggesting its association with the reduced expressions of Nrf2/HO-1 (Tao et al., 2017). BBR also upregulated protein expressions of LKB1, AMPK, p-AMPK, and p-TORC2 and also inhibited the translocation of TOCR2 into the cell nucleus (Jiang et al., 2015). Moreover, BBR was also found to be effective in lowering blood glucose and lipid levels, reducing the body weight, and alleviating the oxidative stress in diabetic hamsters (Liu et al., 2015).

The anti-diabetic effect of BBR was suggested to be mainly due to its activity in the regulation of glycometabolism and lipometabolism and the activation of AMPK (Lee et al., 2006; Dong et al., 2016). BBR improved glucose metabolism through an insulin-independent pathway (Xia et al., 2011). BBR also significantly inhibited the progression of diabetes induced by alloxan, and the effect of BBR on diabetes was suggested to be associated with its hypoglycemic effect, modulating lipids metabolic effects and its ability to scavenge free radicals (Tang et al., 2006). BBR improved glucolipid metabolism in diabetic rats both in the blood and liver, possibly through modulating the metabolic related PPARα/δ/γ protein expression in liver (Zhou et al., 2008). BBR targeted MAPK to suppress Th17 and Th1 differentiation in T1DM NOD mice and showed a novel role of ERK in Th17 differentiation through downregulation of STAT3 phosphorylation and RORt expression (Cui et al., 2009). Altered hepatic SREBPs, LXRα, and PPARα transcriptional programs were suggested to be involved in the therapeutic mechanisms of BBR on fat-induced hepatic insulin resistance (FIHIR) in T2DM hamsters (Liu et al., 2010). The inhibitory effect on intestinal disaccharidases and β-glucuronidase of BBR might be one of the mechanisms for BBR as an antihyperglycaemic agent (Liu et al., 2008). BBR caused the glucose metabolism modulation by the GnRH-GLP-1 and MAPK pathway in the gut (Zhang et al., 2014). The treatment of BBR chloride notably protected the blood components (Chandirasegaran et al., 2017) and significantly reversed the abnormal levels of lipids, oxidant status, and insulin signaling molecules in the diabetic rat model (Chandirasegaran et al., 2019). BBR also reduced the release of lipopolysaccharides and ameliorated inflammation by reducing the level of lipolysaccharide binding protein (LBP), thus alleviating intestinal injury and improving InsR (Cui et al., 2018).

The combination of *Ortosiphon staminensis*, policosanol, red yeast rice extract, BBR, folic acid, and coenzyme Q_{10} provided an antihypertensive effect, which allowed for an effective control of blood pressure in patients with MS (Rozza et al., 2009). The berberine-metformin hybrid compound BMH473 was found to be beneficial for maintaining glucose and lipid homeostasis in T2DM rats, and it exhibited better anthyperlipidaemic effects compared to metformin and BBR alone (Jia et al., 2019).

Combining timosaponin B2 (TB-2) and BBR in a single formulation enhanced the anti-diabetic efficacy by improving the intestinal absorption (Huang et al., 2019). Glycyrrhizic acid was also reported to improve the oral absorption of BBR by inhibiting P-gp, and it thus increased the anti-diabetic effects of BBR in db/db mice (Qiao et al., 2018). Lipid-lowering effects of BBR were also reported to be increased with resveratrol, which may be associated with upregulation of a low-density-lipoprotein (LDL) receptor (Zhu et al., 2018). Similarly, gelucire44/14 was found to enhance the oral absorption of BBR and thus improve the antidiabetic efficacy of BBR (Sun J. et al., 2018). Berberine organic acids (BOAs) were found to be comparable to berberine hydrochloride (BH) in terms of hypoglycaemic effects, they were but superior with regard to safety from hyperchloraemia in T2DM rats (Li et al., 2017). *Coptis chinensis* (containing berberine) and BBR exerted similar effects when used for the treatment of T2MD rats, mainly *via* the stimulation of the pancreatic secretion of insulin (Jiang et al., 2017). Berberine chloride was a stronger antidiabetic agent than BBR or canagliflozin alone with fewer side effects on kidneys in the diabetic mice (Cai-Ming et al., 2016). BBR and ginsenoside Rb1 (Rb1) improve abnormal metabolism of glucose and lipid (Shang et al., 2015).

Extracts of *Berberis* plants have shown interesting results in *in vivo* models. The ethanolic extract of *B. aristata* showed antidiabetic activity due to its significant dose-dependent reduction effect on the blood glucose levels (Semwal et al., 2008; Mittal et al., 2012), which were also reported to be better than glibenclamide (Rameshwar et al., 2009) and comparable to metformin in diabetic rats (Pareek and Suthar, 2010). In addition, the aqueous extract of *B. aristata* showed significant antidiabetic activity, decreased total cholesterol, increased HDL-C levels, and prevented the body weight loss in diabetic rats (Ahamad et al., 2012).

The aqueous extract of *B. lycium* roots showed an antihyperlipidemic effect (Ahmad et al., 2008). *B. lycium* leaf extracts alleviated lipid profile levels and might be used efficiently in hyperglycemic and diabetic patients (Hussain et al., 2017). Also, the root extract of *B. lycium* reduced the serum glucose levels in normal and diabetic rats (Gulfraz et al., 2007). In chicken Broilers, the powder of *B. lycium* reduced the serum cholesterol (Chand et al., 2007). The oral administration of extracts of *B. lycium* showed hypoglycemic activity (Mustafa et al., 2011) and alleviated lipid profile levels (Rahimi Madiseh et al., 2014). Similarly, the methanolic extract of the *B. lycium* root and its main alkaloid BBR showed hypoglycemic activity (Gulfraz et al., 2008) and showed antiglycation activity (Khan et al., 2014).

On the other hand, in diabetic rats, the beneficial effects of *B. vulgaris* extracts showed positive effects in attenuating the side effects of T2DM (Karami et al., 2016), ameliorating oxidative stress (Hemmati et al., 2016), decreasing the liver damage by influencing hepatic histopathological and biochemical markers (Rahimi-Madiseh et al., 2017), and showed that the serum cholesterol and serum triglycerides levels were decreased (Meliani et al., 2011).

Other species of *Berberis* have also been studied. For instance, *B. asiatica* hydro-ethanolic root extracts have shown to be a potent orally effective antidiabetic extract (Singh and Jain, 2010). Likewise, the *B. dictyophylla* cortex could significantly reduce the level of fasting blood glucose, ICAM-1, and ANG II expression (Yue et al., 2013). The *B. holstii* extract showed the reduction of blood glucose levels (Kimani et al., 2017). Furthermore, the aqueous extract of *B. integerrima* roots improved renal dysfunction in STZ-induced diabetic rats through controlling blood glucose, and it also showed renal protective effects (Ashraf

et al., 2013). The anthocyanin fraction of the fruits of *B. integerrima* also showed hypoglycemic effects (Sabahi et al., 2016). Moreover, the methanolic extract of *B. julianae* roots was also reported to possess promising beneficial effects for the treatment of T2DM with the possible mechanism *via* stimulating AMPK activity (Yang et al., 2014).

Other alkaloids isolated from *Berberis* species have also shown promising activities against T2DM and MS. For example, berbamine increased the activity of metabolic enzymes and preserved the glucose homeostasis in HFD/STZ induced diabetic rats (Chandrasekaran et al., 2018). Jatrorrihizine (JAT) induced an important decrease in FBG in normal and hyperglycemic mice, attributed to improve in aerobic glycolysis (Yan et al., 2005). JAT, BBR, and a combination of BBR and JAT decreased the FBG of diabetic and normal mice at different degrees. JAT also possessed the function of decreasing FBG, which was found less than that of BBR at the same dose level (Fu et al., 2005). Palmatine was also found to decrease FBG and suppressed the increase of blood glucose level in normal rats (Patel and Mishra, 2011).

STUDIES IN HUMANS

Several pilot studies as well as pre-clinical studies and clinical trials have evaluated the beneficial effects of *Berberis* extracts and isolated compounds on diabetes, metabolic syndrome, and other metabolic diseases (**Table 4**).

The administration of BBR in patients with MS was found to be effective in regulating the blood glucose and blood lipid levels, improving the InsR, and reducing the level of inflammatory responses in the body (Cao and Su, 2019). BBR also decreased the waist circumference, systolic blood pressure (SBP), triglycerides, and total insulin secretion along with an increase in InsS (Pérez-Rubio et al., 2013). BBR was suggested as a promising new hypolipidemic drug that acts through signaling pathways distinct from those of statins in the treatment of hyper mild mixed hyperlipidemia patients (Kong et al., 2004). Besides, BBR has been shown to have a good potential as a drug to control lipid metabolism alone or in combination with other drugs for hyperlipidemic hepatitis or liver cirrhosis patients (Zhao et al., 2008). Moreover, BBR improved the InsS by limiting fat storage and adjusting adipokine profile in human preadipocytes and MS patients (Yang et al., 2012), and attenuated some of the metabolic and hormonal derangements in women with polycystic ovary syndrome (PCOS (Wei et al., 2012). The administration of BBR was found to be effective in the regulation of blood glucose and blood lipid in T2DM patients (Ming et al., 2018) and in improving diabetic kidney disease by reducing UACR and serum Cys C (Li et al., 2018). On the other hand, BBR had also shown glucose-lowering activity with a mechanism different from metformin and rosiglitazone (Zhang et al., 2010). In pilot study, BBR demonstrated a potent oral hypoglycemic activity with positive effects on lipid metabolism (Yin et al., 2008). Also, the benefits of BBR in lowering blood glucose, lipids, body

TABLE 4 | Studies in diabetic and/or metabolic syndrome patients using treatment with extract and/or isolated compounds of *Berberis* species.

Berberis spp./isolated compound	Study design/Model	Results	References
Berberine			
Berberine (BBR, 0.05g, 4 tablets/time, 3 times/day)	MS patients (*n*=80) RCT, 1 month	↓FBG, ↓PBG, ↓InsR, ↓TG, ↓TC, ↓hs-CRP, and ↓IL-6 and ↓TNF-α	(Cao and Su, 2019)
Berberine (BBR, 0.5 g, 2 times/day)	T2DM patients (*n* = 300), double-blind, RCT, 16 weeks	↓FPG	(Ming et al., 2018)
Berberine (BBR, 0.5 g, 3 times/day)	MS patients (*n*=24) double-blind, placebo-controlled, RCT, 3 months	↓WC, ↓SBP, ↓TG, ↓AUC of glucose, ↓AUC of insulin, ↓insulinogenic index, and ↑Matsuda index	(Pérez-Rubio et al., 2013)
Berberine (BBR, 0.4 g, 3 times/day)	T2DM patients (*n*=114), RCT, 6 months	↓HbA1c, ↓BUN, ↓SP, ↓hs-CRP, ↓ESR, and ↓eGFR	(Li et al., 2018)
Berberine (BBR, 0.5 g, 2 times/day)	Mild mixed hyperlipidemia (*n*=32), double-blind, RCT, 12 weeks	↓TC, ↓LDL-C and ↓TG.	(Kong et al., 2004)
Berberine (BBR, 1 g, 1 time/day)	T2DM and mixed hyperlipidemia patients (*n*=116), double-blind, RCT, 3 months	↓FPG, ↓PPG, ↓HbA1c, ↓TG, ↓TC, ↓LDL-C, and ↑GDR	(Zhang et al., 2008)
Berberine (BBR, 0.5 g, 3 times/day)	Newly diagnosed T2DM patients (*n*=36) double-blind, RCT, 3 months	↓HbA1c, ↓FBG, ↓PBG, ↓TG, ↓TC ↓FPI, ↓IR, and ↓LDL-C.	(Yin et al., 2008)
Berberine (BBR, 0.5 g, 2 times/day)	Hyperlipidemic patients (*n* =86), Open study, 3 months	↓LDL-C, ↓TC and ↓TG.	(Zhao et al., 2008)
Berberine (BBR, 0.3g, 3 times/day)	MS patients (*n*=41) Double-blind, RCT, 3 months	↓BMI, and ↓leptin levels, ↓leptin/adiponectin ratio, ↓HOMA-IR, and ↑IS	(Yang et al., 2012)
Berberine (BBR, 0.5 g, 3 times/day)	PCOS and IR patients (*n*=89) randomized, single center, placebo-controlled, 3 months	↓WHR, ↓TC, ↓TG, ↓LDLC, ↓FPG, ↓HOMA-IR, ↓AUC of insulin, ↑HDLC, and ↑SHBG	(Wei et al., 2012)
Berberine (BBR, 1.0 g, 1 time/day)	T2DM and dyslipidemic patients (*n* = 116) double-blind, placebo-controlled and multiple-center trial consisting of a screening visit, RCT, 2-week	↓FFA	(Gu et al., 2010)
Berberine (BBR, 1.0 g, 1 time/day)	T2DM patients with fasting blood glucose (*n* = 96), 2 months	↓FBG, ↓HA1c, ↓TG, and ↓insulin levels	(Zhang et al., 2010)
Berberine (BBR, 0.5 g, 2 times/day)	T2DM patients (*n*=228) double-blind	↓FPG, ↓PMBG, and ↓FA.	(Rashidi et al., 2018)

(Continued)

TABLE 4 | Continued

Berberis spp./isolated compound	Study design/Model	Results	References
Berberine			
	randomized controlled placebo, 4 weeks		
Berberine (BBR, 0.5 g, 2 times/day)	T2DM patients (*n*=30), open labelled, observational and single centre study, 12 weeks	↓FBG, ↓PPBG, and ↓GHb	(Dange et al., 2016)
Berberine (BBR, 0.3 g, 3 times/day)	T2DM patients (*n*=30), 8 weeks	↓BMI, ↓FBG, ↓HbAlc, ↓fasting insulin, ↓TG, ↓TC, ↓HDL-C, ↓LDL-C, ↓CPR, ↓TNF-α, and ↓LPS	(Chen et al., 2016)
Berberine (BBR, N.I., 2 times/day)	T2DM patients (*n*=41), open-label interventional RCT, 3 months	↓HbA1C, ↓FBG, and ↓PPG	(Rao, 2017)
Berberine (BBR, 0.3 g, 3 times/day)	Mild hyperlipemic patients (*n*=97) Double-blind, RCT, 3 months	↓TG, ↓TC, and ↓LDL-C	(Wang L et al., 2016)
Berberine (BBR, 0.4 g, 1 time/day)	Hypercholesterolemia in tolerance to more than one statin (*n*=91), 3 months	↓ LDL-C and ↓TG.	(Cicero and Ertek, 2008)
Berberine combined with others compounds and extracts			
Berberine (BBR, 1.0 g, 1 time/day.) and simvastatin (SIMVA)	Hypercholesterolemic patients (*n*=63), double-blind, RCT, 2 months	↓LDL-C, ↓TC, and ↓TG	(Kong et al., 2008)
(Berberine, BBR, 0.5 g; red yeast, 200 mg; and policosanol, 10 mg; 1 time/day)	Hypercholesterolemic patients (*n*=50), double-blind, single-centered, placebo-controlled, RCT, 6 weeks	↓TC, ↓LDL-C, ↓TG, ↑FMD, and ↑InsS	(Affuso et al., 2010)
(Berberine, BBR, 0.5 g; policosanols, 10 mg; and red yeast rice, 200 mg; 1 time/day)	Hypercholesterolemic patients (*n*=135) randomized, double-blind, EZE-controlled, 6 months	↓LDL-C, and ↓TG	(Pisciotta et al., 2012)
Armolipid Plus ™ composed by (Berberine, BBR, 0.5 g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Hypercholesterolemic patients (*n*=106), single-blind, single centered, placebo-controlled, RCT, 12 months	↓TC, ↓LDL-C, and ↓InsR	(Marazzi et al., 2011)
Armolipid Plus ™ composed by (Berberine, BBR, 0.50g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Hyperlipidemic patients (*n*=102), double-blind, parallel, controlled, Multiple centered, placebo-controlled, RCT, 12 weeks	↓LDL-C, ↓apo B-100, ↓TC/HDL-C, ↓ApoB/ApoA1 ratio, and ↑ApoA1	(Sola et al., 2014)

(Continued)

TABLE 4 | Continued

Berberis spp./isolated compound	Study design/Model	Results	References
Berberine combined with others compounds and extracts			
Armolipid Plus ™ composed by (Berberine, BBR, 0.5g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Dyslipidemic patients (*n* = 1751) Double-blind, RCT, 16 weeks	↓TC and ↓LDL-C	(Trimarco et al., 2011)
Armolipid Plus ™ composed by (Berberine, BBR, 0.5g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Hypercholesterolemic patients (*n*=66), single-blind, placebo-controlled, RCT, 3 weeks	↓TC, ↓LDL-C, and ↓TG	(Gonnelli et al., 2015)
Armolipid Plus ™ composed by (Berberine, BBR, 0.5 g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Moderate dyslipidemic and MS patients (*n*=30), double-blind, centered, placebo-controlled, RCT,	↓TC, ↓LDL-C, ↓leptin-to-adiponectin ratio, and ↑HDL-C	(Ruscica et al., 2014)
Armolipid Plus ™ composed by (Berberine, BBR, 0.5 g; red yeast rice, 200 mg; policosanol, 10 mg; folic acid, 0.2 mg; coenzyme Q_{10}, 2.0 mg; and astaxanthin, 0.5 mg; 1 time/day)	Dyslipidemic with ischemic heart disease treated patients (*n*=100), single-blind, EZE-controlled, RCT, 12 months	↓LDL-C, ↓TC, ↓TG, and ↑HDL-C	(Marazzi et al., 2015)
Berberine (BBR, 500mg) and Armolipid Plus ™ Composed by (Berberine, BBR, 0.5 g; red yeast extract, 200 mg; policosanol, 10 mg; folic acid, 200 mg; coenzyme Q_{10}, 2 mg; and astaxanthin, 0.5 mg; 1 time/day)	Hyperlipidemic patients (*n*=40) single-blind, no placebo-controlled, 4 weeks	↓TC, ↓LDL-C, ↓ApoB, ↓TG, and ↑HDL-C	(Cicero et al., 2007)
Body Lipid ™ composed by (Berberine, BBR,	Hypercholesterolemic patients (*n* = 158)	↓TC and ↓LDL-C	(D'Addato et al., 2017)

(Continued)

TABLE 4 | Continued

Berberis spp./isolated compound	Study design/Model	Results	References
Berberine combined with others compounds and extracts			
0.5 g; red yeast rice, 10 mg; coenzyme Q_{10}, 2 mg; and hydroxytyrosol, 5 mg; 1 time/day)	Double-blind, RCT, 4 weeks		
Berberine (BBR, 0.2g; monacolin K, 3 mg; chitosan, 10 mg; and coenzyme Q_{10}, 10 mg; 1 time/day)	Hypercholesterolemic patients (n =36) Double-blind phase II placebo-controlled study, 12 weeks	↓nHDL-C, ↓LDL-C and ↓apoB	(Spigoni et al., 2017)
Estromineral lipid ™ composed by (Berberine, BBR, 0.5 g; soy isoflavones, 60 mg; *Lactobacillus sporogenes*, $1x10^9$ spores; calcium phosphate dehydrate, 137 mg; vitamin D_3, 5 µg; and folic acid, 0.2 mg; 1 time/day)	Menopausal women (n=120) RCT, 12 weeks	↓TC, ↓LDL-C, and ↓TG	(Cianci et al., 2012)
Berberine (BBR, 1.0 g; phytosterols, 4 g; antioxidants, 2 capsules; probiotics, 12 billion colony forming units; fish oil, 2g; and soy, pea, and whey proteins, 40 g, 2-3 times/day)	CMS patients (n=44) open-label, 2-arm, RCT, 13 weeks	↓body mass, ↓fat mass, ↓TC, ↓LDL-C, ↓TG, ↓TC/HDL-C, ↓TG/HDL-C, ↓apoB/apoA1, and ↓hs-CRP.	(Dahlberg et al., 2017)
Berberine sulfate trihydrate (0.1 g, equiv. 69 mg berberine, BBR); Hop rho iso-alpha acids, 200 mg; vitamin D_3, 500 IU; and vitamin K_1 500 µg; 2 times/day)	MS postmenopausal women patients (n=51), randomized, single-blind, 2-arm placebo-controlled, RCT, 14 weeks	↓serum OC, serum ↑25(OH) D, and ↑IGF-I	(Lamb et al., 2011)
Berberine (BBR, 0.5 g, 3 times/day) and methylglyoxal (0.5 g ×3 times/day)	T2DM patient (n=200), case–control study, 3 months	↓HOMA-IR, and ↓MGO	(Memon et al., 2018)
Berberine (BBR, 0.5 g; orthosiphon, 300 mg; red yeast rice, 60 mg; monacolin, 3 mg; policosanol, 10 mg; folic acid, 0.2 mg; and coenzyme Q_{10}, 15mg; 1 time/day)	MS patients (n=1161), Double-blind, Randomized, controlled, 1 year	↓TC, ↓LDL-C, ↓HDL-C, ↓TG, ↓SBP, and ↓DBP	(Manzato and Benvenuti, 2014)

(Continued)

TABLE 4 | Continued

Berberis spp./isolated compound	Study design/Model	Results	References
***Berberis* extracts**			
B. aristata stem powder (1.5 and 3 g in two divided doses daily)	T2DM with dyslipidemic patients (n=90) open parallel, RCT, 9 months	↓FBS, ↑HDL, ↓TC, ↓TG, and ↓LDL.	(Sharma et al., 2017)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) and only *B. aristata* extract (Berberine, BBR, 1.0 g) 2 time/day	T2DM patients (n=69), single-blind, RCT, 120 days	↓IFG, ↓HbA1c, ↓TC, ↓TG, ↓LDL (only Berberol ®), ↓AST, and ↓ALT	(Di Pierro et al., 2013)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	T1DM patients (n=85) double-blind, randomized, placebo-controlled, 6 months	↓TIC, ↓HgbA1c, ↓FPG, ↓PPG, ↓TC, ↓TG, ↓LDL-C, and ↑HDL-C	(Derosa et al., 2016)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	Dyslipidemic patients (n=105), Double-blind, RCT, 3 months	↓TC, ↓LDL-C, ↓TG, ↑HDL-C, ↓FPI, and ↓HOMA-IR	(Derosa et al., 2013)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	T2DM and MS patients (n=50) double-blind placebo-controlled, 6 months	↓BMI, ↓HOMA-R, ↓TC, ↓WC, ↓HbA1c, and ↓TF%	(Guarino et al., 2015)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	T2DM and MS patients (n=136), placebo RCT, 52 weeks	↓TC, ↑HDL-C, ↓TG, ↓LDL-C, ↓HOMA-R, ↓WC, ↓TF(%), ↓VF(%), ↓UA, ↓HbA1c, ↓SBP, and ↓DBP	(Guarino et al., 2017)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	T2DM patients (n = 26), 6 months	↓HbA1c, ↓basal insulin, ↓TC, ↓LDL-C, ↓TG, ↓HOMA-R, ↓ ALT, and ↓AST	(Di Pierro et al., 2012)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) 2 times/day	Dyslipidemic patients (n =175), double blind, placebo-controlled, RCT, 6 months	↓FPG, ↓IC, ↓HOMA, and ↓dosage of statin	(Derosa et al., 2015a)
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S.*	Euglycemic, dyslipidemic subjects (n=137) double-blind,	↓FPG, ↓IC, and ↓HOMA-index	(Derosa et al., 2015b)

(Continued)

TABLE 4 | Continued

Berberis spp./isolated compound	Study design/Model	Results	References
***Berberis* extracts**			
marianum (silymarin, 210 mg) 2 times/day	RCT, placebo-controlled, 6-months		
Berberol ® compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg), Berberol ® + statin, and Berberol ® +ezetimibe; 2 times/day	T2DM and hypercholesterolemic patients (*n*=45), 6-months	↓TC, ↓LDL-C, ↓HDL-C (only Berberol ®), ↓FPG, and ↓HbA1c.	(Di Pierro et al., 2015)
Berberol ® K compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg) and Monakopure™-K20, 50 mg; 1 time/day	Dyslipidemic patients (*n*=226), non-blind non-randomized, 6 months	↓TC, ↓LDL-C, ↓TG, and ↓CPK.	(Di Pierro et al., 2018)
Berberol ® K compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg), and Monakopure™-K20, 50 mg; 1 time/day	Low cardiovascular risk patients (*n*=73), double-blind, placebo-controlled, RCT, 3 months	↑FPI, ↓HOMA, ↓TC, ↓TG, ↓LDL-C, and ↓hs-CRP	(Derosa et al., 2017)
Berberol ® K compose by *B. aristata* (Berberine, BBR, 1.0 g) and *S. marianum* (silymarin, 210 mg), and Monakopure™-K20, 50 mg; 1 time/day	Diabetic and dyslipidemic patients (*n* = 59), 6 months	↓HbA1c, ↓TC, ↓LDL-C), and ↓TG	(Di Pierro et al., 2017)
B. aristata (83.3 mg), *Cyperus rotundus* (83.3 mg), *Cedrus deodara* (83.3 mg), *Emblica officinalis* (83.3 mg), *Terminalia chebula* (83.3 mg) and *T. bellirica* (83.3 mg) 1-6 timea/day	T2DM patients (*n*=93) Pilot RCT, 24 weeks	↓PBG, ↓FBG, ↓TC, and ↓HbA1c.	(Awasthi et al., 2015)
B. vulgaris fruit (aqueous extract, 3 g/day)	T2DM patients (*n*=31) Double-blind, RCT, 3 months	↓TG, ↓TC, ↓LDL-C, ↓apoB, ↓glucose, ↓insulin, and ↑TAC.	(Shidfar et al., 2012)
B. vulgaris fruit (600 mg/day)	MS patients (*n*=106) Double-blind, RCT, 6 weeks	↓PAB	(Mohammadi et al., 2014)
B. vulgaris juice (10 c.c. of processed extract/day)	MS patients (*n*=57) Double-blind, RCT, 8 weeks	↓LDL-C, ↓TC/HDL-C ratio, ↑HDL, ↑IC, and ↑IR.	(Ebrahimi-Mamaghani et al., 2009)
B. vulgaris fruit (ethanolic extract 1 mg, 3 times/day)	T2DM patients (*n*=30) Double-blind, RCT, 8 weeks	↓SGL, ↓FG, and ↓HbA1c	(Moazezi and Qujeq, 2014)
B. vulgaris juice (480 mL/day)	women diagnosed with BBD (*n* =85), 8 weeks	↓IC, ↓C-peptide, ↓HOMA-IR, ↓glucose/insulin ratio, and ↑HOMA-B.	(Asemani et al., 2018)
B. vulgaris fruit (600 mg/day)	(*n* = 106) Double-blind, RCT, 6 weeks	↓LDL-C, ↓TC, ↑HDL-C, ↓anti-HSPs 27, ↓anti-HSPs 60, and ↓hs-CRP	(Zilaee et al., 2014)

The ↑ and ↓ signs show significant increase and significant decrease, respectively, of evaluated factors during mentioned studies. N.I., not informed.

weight, and blood pressure have been confirmed in T2DM and MS patients (Zhang et al., 2008). BBR played an important role in the treatment T2DM through downregulating the higher levels of free fatty acids (Gu et al., 2010). In another study, BBR reduced the fasting plasma glucose, post-meal blood glucose, and fructosamine; however, no signification changes were found in lipid profiles, fasting insulin, HOMA-IR, and HOMA-β% in T2DM patients (Rashidi et al., 2018).

In addition, BBR improved the glycemic parameters comparable to metformin in T2DM patients (Dange et al., 2016). BBR significantly ameliorated T2DM *via* modulation of *Bifidobacterium* species, TNF-α, and LPS (Chen et al., 2016). BBR improved the blood lipid level in mild hyperlipidemia patients (Wang L, et al., 2016). Likewise, it reduced the plasma LDL-C and TG in mixed hyperlipidaemic subjects (Cicero and Ertek, 2008).

The combination of BBR and simvastatin (SIMVA) in hypercholesterolemic patients significantly improved LDL-receptor upregulation and LDL-cholesterol downregulation compared to monotherapies, and the combined effect also reduce the statins dosage (Kong et al., 2008). The administration of BBR along with red yeast and policosanol on a daily basis was found to be effective in reducing cholesterol levels and was associated with the enhancement of endothelial function and InsS (Affuso et al., 2010). The administration of this supplementation in patients with familial hypercholesterolemia heterozygotes on stable treatment with LDL-C-lowering validated that the supplement reduced the LDL-C superior to that obtained by doubling the dose of statins (Pisciotta et al., 2012).

Also, the dietary supplement Armolipid Plus™ composed of BBR, red yeast rice, policosanol, folic acid, coenzyme Q_{10}, and

astaxanthin showed significant reduction of cholesterolemia and positive plasma LDL-C levels in elderly (statin-intolerant) hypercholesterolemic patients (Marazzi et al., 2011). Moreover, it reduced LDL-C levels as well as total cholesterol/HDLc and ApoB/ApoA1 ratios, and it increased the Apo A1; tjos demonstrated the improvements in CVD risk indicators in patients with hypercholesterolemia (Sola et al., 2014) and amelioration of blood lipids and significant reduction of global CVD risk in dyslipidemic patients (Trimarco et al., 2011). In patients with low- to moderate-risk hypercholesterolemia, Armolipid Plus ™ in association with a hypolipidic diet significantly reduced the total cholesterol and LDL-C levels (Gonnelli et al., 2015). In addition, Armolipid Plus ™ improved the lipid profile similar to a low dose of a standard statin and also increased the HDL-C levels and improved the leptin-to-adiponectin ratio in patients with moderate dyslipidemia and MS (Ruscica et al., 2014). Armolipid Plus™ alone or in combination with ezetimibe enhanced the lipid profile in statin-intolerant patients with coronary heart disease (Marazzi et al., 2015). BBR and Armolipid Plus™ could be a useful alternative to correct dyslipidemias and to reduce CVD risk in subjects with moderate mixed dyslipidemias (Cicero et al., 2007).

Other food supplements containing BBR, including Body Lipid™, were suggested as an alternative to pharmacological treatment for patients with mild-to-moderate hypercholesterolemia (D'Addato et al., 2017). A new nutraceutical formulation containing BBR, monacolin K, chitosan, and coenzyme Q_{10} has proven effective in reducing non-HDL/LDL-C levels, representing an emergent therapeutic strategy in dyslipidemic patients (Spigoni et al., 2017). On the other hand, the combination of BBR and isoflavones was found to be effective in lowering CVD risk factors in menopausal women with moderate dyslipidaemia (Cianci et al., 2012).

Treatment with BBR and rho iso-alpha acids, vitamin D3, and vitamin K1 produced a more favorable bone biomarker profile, indicative of healthy bone metabolism in postmenopausal women with MS (Lamb et al., 2011). In a case–control study, BBR is more effective in decreasing the serum MGO levels and InsR through increasing the glycemic control in newly diagnosed T2DM patients (Memon et al., 2018). The intake of the natural formulation (containing BBR, orthosiphon, red yeast rice equivalent to monacolin, policosanol, folic acid, and coenzyme Q_{10}) has evidenced the effective control of plasma lipids and keeps borderline high blood pressure within normal values compared with diet alone (Manzato and Benvenuti, 2014).

Stem powder of *B. aristata* was found to be effective in improving glycemic control and lipid profiles with no major adverse effects on T2DM patients (Sharma et al., 2017). The effect of *B. vulgaris* extract on T2DM and MS patients has been widely studied in humans. The intake of 3 g/d of *B. vulgaris* fruits aqueous extract for 3 months may have beneficial effects on lipoproteins, apoproteins, glycemic control, and TAC in T2DM patients (Shidfar et al., 2012). *B. vulgaris* juice reduced oxidative burden in patients with MS (Mohammadi et al., 2014). Other study showed the beneficial effects of processed *B. vulgaris* on certain atherosclerosis risk factors in T2DM patients (Ebrahimi-Mamaghani et al., 2009). *B. vulgaris* fruit extract showed beneficial metabolic effects in T2DM patients, improving the glucose catabolism *via* the glycolysis pathway, stimulating the insulin secretion or improving the insulin function, and later decreasing the glucose uptake (Moazezi and Qujeq, 2014). Another study demonstrated that the *B. vulgaris* juice evoked regulatory roles on HOMA-IR and improved HOMA-B with the metabolic controlling insulin-related indices in benign breast disease (Asemani et al., 2018). Also, *B. vulgaris* supplementation in patients with MS significantly diminished anti-HSPs 27 and 60 and hs-CRP levels and improved lipid profiles (Zilaee et al., 2014). It is reported that the Hsp60 protein is able to induce the production of anti-Hsp60 antibodies, which leads to the destruction of β-islet cells. In the same way, Hsp60 acts as a proinflammatory signaling molecule, which plays a role in the non-resolved vascular inflammation, and this is recognized as one of the characteristic of T2DM (Juwono and Martinus, 2016). Others natural formulations containing *Berberis* have also been tested in humans. A clinical trial demonstrated that daily intake of polyherbal capsule composed by *B. aristata* and *Cyperus rotundus*, *Cedrus deodara*, *Emblica officinalis*, *Terminalia chebula*, and *T. bellirica* decreased the glucose level, enhanced lipid homeostasis, and maintained other serum biochemical levels to the normal in patients with T2DM (Awasthi et al., 2015).

The nutraceutical product Berberol®, containing a *B. aristata* extract (titrated in 85% BBR) plus a *Silybum marianum* extract (titrated in 60% silymarin), has been evaluated for its antidiabetic potential in humans. Berberol® was demonstrated to be more effective than BBR alone (administered at the same dose), reducing HbA1c in T2DM patients (Di Pierro et al., 2013). The incorporation of Berberol® into insulin therapy in patients with T1DM has the effect of a diminution of the insulin dose necessary for adequate glycemic control (Derosa et al., 2016). In dyslipidemic patients, Berberol® has proven to be safe and effective in improving lipid profile, InsR, and adipocytokines levels (Derosa et al., 2013). Berberol® also improved the cholesterol-lowering properties of statins and showed the positive effects on liver enzymes and glycemic control in patients with T2DM (Guarino et al., 2015). In addition, Berberol® significantly lowered abdominal adiposity and decreased the circulating uric acid level in overweight/obese patients with T2DM (Guarino et al., 2017). Berberol® was suggested as a good candidate for an adjunctive treatment option in diabetes, especially in patients with suboptimal glycemic control (Di Pierro et al., 2012). Berberol® administered as a single or add-on therapy in statin-intolerant subjects is an effective treatment to improve the lipidic and glycemic profiles in T2DM and hypercholesterolemia patients (Di Pierro et al., 2015). The combination of Berberol® and a reduced dosage of statin is found effective for the treatment of hyperlipidemia in patients intolerant to statins at high dosage (Derosa et al., 2015a) and in dyslipidemic euglycemic patients (Derosa et al., 2015b)

Berberol K®, was found to be a potentially good alternative in primary intervention in low cardiovascular-risk subjects with dyslipidemia, as an add-on therapy in mildly statin-intolerant patients, and as an alternative for dyslipidemic patients with a negative perception of statins (Di Pierro et al., 2017). Berberol K® reduced lipid profile effectively and improved the inflammatory

parameters under a safe dose (Derosa et al., 2017). It was also found to be effective in diabetic subjects with dyslipidemia statin intolerant or with diarrhea caused by IBS or metformin (Di Pierro et al., 2018).

Few studies have also reported the effectiveness of BBR in non-alcoholic fatty liver disease (NAFLD). NAFLD is a result of abnormal fat accumulation in the liver due to the reasons other than alcohol, and it is considered to be a hepatic manifestation of MS. NAFLD results in the overproduction of sugars and triglycerides and plays a central role in the development of InsR and various other glucose- and lipid metabolism-related diseases (Yki-Järvinen, 2014). Recently, Yan et al. (2015) conducted a randomized, parallel controlled, open-label clinical trial in 188 NAFLD patients. Patients received lifestyle intervention (LSI) or LSI and 15 mg of pioglitazone qd or LSI and of BBR for 16 weeks. Parameters, including hepatic fat content, serum glucose level, serum lipid profiles, liver enzymes, and serum and urine BBR concentrations, were measured before and after treatment. LSI and BBR showed a reduction in hepatic fat content as compared to LSI and were better than pioglitazone in reducing body weight and resulted in better lipid profiles (Yan et al., 2015). Furthermore, a mechanism-based study revealed that BBR reduced hepatic TG accumulation and decreased the expressions of hepatic stearyl-coenzyme A desaturase 1 (SCD1) and other TG synthesis-related genes (Zhu et al., 2019). Berberine administration was also reported to recruit and activate BAT in both humans and mice (Wu et al., 2019).

CONCLUSION

Although there are many effective therapeutic drugs for the treatment of metabolic diseases, the current treatment did not control the rapid increasing trend in diabetes mortality and morbidity. Various therapeutic agents from both natural and synthetic sources are being investigated in patients with clinical signs of diabetic and other metabolic diseases. Formulations prepared from the various plant parts of *Berberis* species were found to be used traditionally in the treatment of diabetes and other metabolic diseases and related complications. A review of the scientific literature revealed that the extracts, isolated alkaloids from *Berberis* species including BBR and their derivatives, have shown promising effects in the studies related to diabetes and other metabolic diseases. The relatively low cost of BBR or supplements or extracts containing BBR, compared to other synthetic medications, will be of an advantage to the patients living in developing countries with poor socioeconomic circumstances. However, currently available scientific evidence is still not fully sufficient to prove their efficacy clinically. Further randomized double-blind clinical trials with a large number of patients and standardized clinical assessments are required to prove the effectiveness of the *Berberis* extracts and isolated compounds on metabolic diseases alone or in combinations. Novel pharmacological assessment techniques and analytical techniques will further provide additional opportunities for these agents. Moreover, the development of novel formulations of berberine could be an effective strategy for increasing its effectiveness against diabetes and other metabolic diseases.

AUTHOR CONTRIBUTIONS

TB, IB and JE conceptualized the manuscript. TB, AB, HD, HU, HK, IB and JE wrote the initial manuscript. TB, HD, HU, AP, IB and JE revised the manuscript. All authors agreed on the final version of the manuscript.

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Hepatic Glucose Output Inhibition by Mexican Plants Used in the Treatment of Type 2 Diabetes

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De novo hepatic glucose production or hepatic gluconeogenesis is the main contributor to hyperglycemia in the fasting state in patients with type 2 diabetes (T2D) owing to insulin resistance, which leads to at least twice as much glucose synthesis compared to healthy subjects. Therefore, control of this pathway is a promising target to avoid the chronic complications associated with elevated glucose levels. Patients with T2D in the rural communities of Mexico use medicinal plants prepared as infusions that are consumed over the day between meals, thus following this rationale (consumption of the infusions in the fasting state), one approach to understanding the possible mechanism of action of medicinal plants is to assess their capacity to inhibit hepatic glucose production. Furthermore, in several of these plants, the presence of phenolic acids able to block the enzyme glucose-6-phosphatase (G6Pase) is reported. In the present work, extracts of *Ageratina petiolaris*, *Bromelia karatas*, *Equisetum myriochaetum*, *Rhizophora mangle*, and *Smilax moranensis*, which are Mexican plants that have been traditionally used to treat T2D, were assayed to evaluate their possible hepatic glucose output (HGO) inhibitory activity with a pyruvate tolerance test in 18-h fasted STZ-NA Wistar rats after oral administration of the extracts. In addition, the *in vitro* effects of the extracts on the last HGO rate-limiting enzyme G6Pase was analyzed. Our results showed that four of these plants had an effect on hepatic glucose production in the *in vivo* or *in vitro* assays. *A. petiolaris* and *R. mangle* extracts decreased glucose output, preventing an increase in the blood glucose levels and sustaining this prevented increase after pyruvate administration. Moreover, both extracts inhibited the catalytic activity of the G6Pase complex. On the other hand, even though *S. moranensis* and *B. karatas* did not exhibit a significant *in vivo* effect, *S. moranensis* had the most potent inhibitory effect on this enzymatic system, while the *E. myriochaetum* extract only inhibited hepatic glucose production in the pyruvate tolerance test. Because of the traditional method in which diabetic patients use plants, hepatic glucose production inhibition seems to be a mechanism that partially explains the common hypoglycemic effect. However, further studies must be carried out to characterize other mechanisms whereby these plants can decrease HGO.

Keywords: medicinal plant, hepatic glucose output, type 2 diabetes, glucose 6 phosphatase, traditional medicine

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INTRODUCTION

Diabetes is a chronic condition that occurs when the body cannot produce enough insulin or respond properly to the insulin it does produce. In type 2 diabetes (T2D), the inability of the cells to respond to low levels of insulin, defined as insulin resistance, leads to elevated glucose levels in the bloodstream, or hyperglycemia (International Diabetes Federation, 2017). In the long term, uncontrolled hyperglycemia induces the development of complications (neuropathy, nephropathy, and retinopathy, among others) that impair the quality of life of individuals with T2D.

Hyperglycemia in T2D, among other factors, is caused by an increased hepatic glucose output (HGO), which is triggered by insulin resistance in the fasting state. Poor insulin signaling in the liver cannot efficiently suppress HGO. Overall, hyperglycemia is the sum of two glucose inputs, one from the gastrointestinal tract at the postprandial stage, and another from endogenous production. The liver produces approximately 85% of endogenous glucose, and half of this comes from gluconeogenesis. In a healthy subject, the production rate is approximately 1.8–2.0 mg/kg/min after overnight fasting. On the other hand, this rate increases at least two-fold in patients with T2D due to impaired gluconeogenesis (Cersosimo et al., 2018). Furthermore, when the balance between glucose production and glucose storage is disrupted, as observed in patients with T2D, glucose homeostasis is altered and significantly contributes to hyperglycemia (Sharabi et al., 2015). Some of the identified mechanisms responsible for the increased rate of gluconeogenesis include high circulating levels of gluconeogenic precursors such as lactate, pyruvate, alanine, and glycerol; increased free fatty acid oxidation; enhanced sensitivity to glucagon; and decreased sensitivity to insulin (Cersosimo et al., 2018).

Gluconeogenesis is an anabolic pathway that plays a major role in glucose metabolism by maintaining the glucose demand of the organs during starvation and after a meal high in fat and protein without carbohydrates. Gluconeogenesis can be regulated depending on the energy demand of the organism by activation or inhibition of the rate-limiting enzymes at different levels, such as substrate delivery, mass action regulation, allosteric activation, covalent modification, and alteration of gene expression. Among these enzymes, glucose-6-phosphatase (G6Pase) can control hyperglycemia because it determines the production of glucose released from gluconeogenesis and glycogenolysis (HGO) (Jawad et al., 2016). This enzyme is a multifunctional system attached to the membrane of the endoplasmic reticulum (ER) formed by three specific translocases (T1, T2, and T3) whose functions are: T1, glucose-6-phosphate (G6P) influx transport into the ER; T2, phosphate efflux transport; and T3, free glucose efflux transport; and a phosphatase subunit, in accordance with the widely accepted "substrate-transport" model (van Schaftingen and Gerin, 2002).

Inhibition of the rate-limiting enzymes in gluconeogenesis by phytochemicals as a target to treat T2D has attracted attention in recent years. Medicinal plants could provide new therapeutic compounds that allow regulation of gluconeogenic enzymes at different levels (Andrade-Cetto, 2012). For example, metformin, a first-line drug prescribed for T2D management derived from the medicinal plant *Galega officinalis* L. (Fabaceae) (French lilac or Goat's rue) (Bailey, 2017), can decrease hepatic gluconeogenesis flux by reducing the gene expression of G6Pase and phosphoenolpyruvate carboxykinase (PEPCK), and promoting allosteric inhibition of fructose-1,6-bisphosphatase (F-1,6-Pase) (Hardie, 2013; Viollet and Foretz, 2013; Foretz et al., 2014; Tan et al., 2016; Hunter et al., 2018). Moreover, chlorogenic acid (CA), which is the most abundant isomer of caffeoylquinic acid present in foods, such as coffee and green tea, has been identified as a reversible competitive inhibitor of G6Pase T1 translocase (Hemmerle et al., 1997; Charkoudian et al., 2012; Naveed et al., 2018). This phenolic acid is the most studied natural compound found in a wide variety of plant species that has been associated with improved both glucose tolerance and insulin resistance in animal models (Meng et al., 2013).

To assess the potential inhibitory effects of phytochemicals on HGO, fasting hyperglycemic animal models are needed. The STZ-NA hyperglycemic model developed by Masiello et al. (1998), which consists of generating a "type-2-diabetogenic" syndrome with the ability to respond to glucose-stimulated insulin secretion, is characterized by stable hyperglycemia due to the partial protection of nicotinamide (NA) against the specific β-cytotoxic effect of streptozotocin (STZ). Although this model lacks insulin resistance (Szkudelski, 2012), the decreased insulin secretion due to the residual β-cells is responsible for the reduced glucose tolerance in these induced organisms (Szkudelski et al., 2013). Moreover, since β-cells are able to respond to drugs, this model has been used to assess the potential glucose-lowering effects of natural products, which are always evaluated after a fasting period (Eddouks et al., 2012).

In Mexico, type 2 diabetic patients use medicinal plants together with the prescribed medication to control glucose levels (Andrade-Cetto and Heinrich, 2005). As a result of our fieldwork performed in some regions of Mexico, we select five relevant medicinal plants traditionally used for the treatment of the illness. In brief, in the town of "Nopala" in Oaxaca state, diabetic patients use an infusion of the roots from *Smilax moranensis* M. Martens & Galeotti (Smilacaceae, known as "Cocolmecatl") as well as the infusion of the bark from *Rhizophora mangle* L. (Rhizophoraceae, known as "Mangle Rojo") to control the disease. In the state of Hidalgo, in the towns of "Tlanchinol" and "Tamala," the patients use both the infusions of the aerial parts of *Bromelia karatas* L. (Bromeliaceae, known as "piñuela") and *Equisetum myriochaetum* Schltdl. & Cham. (Equisetaceae, known as "cola de caballo") which were highly recommended by the traditional healer "Isabel Escalante, RIP" to treat T2D. In Mexico City, in the "Sonora" market of medicinal plants, the sellers suggest the disease control by using an infusion of the aerial parts of *Ageratina petiolaris* (Moc. ex DC.) R.M. King & H. Rob. (Asteraceae, traditionally known by its Spanish name "hierba del ángel," or its Nahuatl name "Yolochichotl") which is in part brought to market from "Tenancingo," Mexico State.

In previous works, we analyzed some aspects of their hypoglycemic effect as well as the phytochemical composition (Andrade-Cetto et al., 2000; Wiedenfeld et al., 2000; Andrade-Cetto, 2011a; Andrade-Cetto and Rubalcaba-Mares, 2012;

Andrade-Cetto and Medina-Hernández, 2013; Bustos-Brito et al., 2016) for some of these plants. We also assessed the chronic hypoglycemic effect, the α-glucosidase inhibition or the insulin secretory effect (Revilla et al., 2002; Andrade-Cetto et al., 2008, 2015, 2017; Escandón-Rivera et al., 2019; Romo-Pérez et al., 2019). In these studies, we noticed that the hypoglycemic effect of a plant cannot be attributed to a single factor but to the combination of different mechanisms.

In the field, we documented that these plants are used as an infusion that diabetic patients usually drink over the course of a day in the so-called preparation "agua de uso." Since the infusion is consumed during the fasting state, it is a rational that a possible mechanism contributing to the hypoglycemic effect of the plants can be related to HGO inhibition. To test this hypothesis, we performed pyruvate tolerance tests in fasting STZ-NA hyperglycemic rats *in vivo* and evaluated the ability of these plants to inhibit G6Pase *in vitro*.

MATERIALS AND METHODS

Plant Extracts

In previous works, two kinds of plant extracts, aqueous (like the traditional infusion) and ethanol-water, were tested for their hypoglycemic effects. In the present study, we selected the extracts and doses that previously presented better biological activity (phytochemical profiles and voucher numbers are provided as **Supplementary Material**): ethanol-water extracts from *R. mangle* (bark, collected in "Manialtepec, Oaxaca, Mexico") and *S. moranensis* (roots, collected in "Nopala, Oaxaca, Mexico"), aqueous extracts from *A. petiolaris* (aerial parts, collected in "Tenancingo, Estado de Mexico, Mexico"), *B. karatas*, and *E. myriochaetum* (both aerial parts, collected in "Tamala, Hidalgo, Mexico"). The ethanol-water extracts were prepared with 20 g of the plant in 500 ml of a mixture of ethanol:water (1:1) at 40°C for 4 h with agitation. The ethanol was removed on a Büchi rotatory evaporator, and the aqueous portion was frozen at −40°C. The water was eliminated by sublimation with a Labconco freeze dryer at reduced pressure. Aqueous extracts were made by boiling 20 g of the plant in 500 ml of water for 15 min with agitation. Afterward, the extracts were frozen at −40°C, and the water was finally removed by sublimation.

Hyperglycemic Animals

Eight-week-old Wistar rats were obtained from the bioterium of the School of Sciences, National Autonomous University of Mexico (UNAM). Animals were maintained with free access to food and water in a room at 25°C and 55% humidity under 12:12 h light:dark periods.

Hyperglycemia was induced as described previously (Masiello et al., 1998). Briefly, overnight fasting rats were administered 65 mg/kg i.v. injection of STZ (Sigma-Aldrich S0130) in 0.1 M acetate buffer, pH 4.5, and 15 min later they received a 150 mg/kg i.p. injection of NA (Sigma-Aldrich). One week later, the animals with fasting glucose values over 180 mg/dl were selected to perform pyruvate tolerance tests.

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Committee for the Update of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The protocol was approved by the Committee of Academic Ethics and Scientific Responsibility (CEARC) of the Faculty of Science, UNAM (PI_2020_01_001).

Pyruvate Tolerance Test

Wistar rats fasted for 18 h were administered with vehicle (saline), metformin (Roche®) or extracts by gavage. Fifteen minutes later, vehicle or sodium pyruvate (2 g/kg bw, Sigma-Aldrich) were injected intraperitoneally. Blood samples were taken from the tail vein, and glucose levels were measured just before pyruvate administration (time 0) and 30, 60, 90, and 120 min later by using an Accutrend Plus (Roche®) glucometer. The animals were assigned into ten experimental groups (n = 6 per group): normoglycemic (N); normoglycemic + pyruvate (NP); hyperglycemic (H); hyperglycemic + pyruvate (HP); hyperglycemic + pyruvate + metformin 500 mg/kg (HPM); hyperglycemic + pyruvate + *A. petiolaris* 160 mg/kg (HPAp); hyperglycemic + pyruvate + *B. karatas* 218 mg/kg (HPBk); hyperglycemic + pyruvate + *E. myriochaetum* 330 mg/kg (HPEm); hyperglycemic + pyruvate + *R. mangle* 90 mg/kg (HPRm) and hyperglycemic + pyruvate + *S. moranensis* 80 mg/kg (HPSm).

Liver Microsome Isolation

Four overnight-fasted Wistar rats were anesthetized with pentobarbital (6 mg/100 g b.w., i.p.). Livers were dissected and homogenized in a 7 ml Dounce tissue grinder to obtain a 20% homogenate in buffer (0.25 M sucrose, 1 mM EDTA, 5 mM HEPES, pH 7.4). The homogenate was filtered through a nylon mesh and submitted to differential centrifugation as described previously (Andrade-Cetto and Cárdenas-Vázquez, 2010). The 100,000 $g \times 1$ h pellets were stored at −40°C until use.

Glucose-6-Phosphatase Assay

A colorimetric assay was performed to assess microsomal G6Pase inhibition by the extracts as described previously (Andrade-Cetto and Cárdenas-Vázquez, 2010; Andrade-Cetto, 2011b). The test consists in the addition, from least to greatest, several concentrations of the potential inhibitor in the assayed medium which contains intact rat hepatic microsomes. The reaction starts by supplementing the substrate, while the stop solution, which incorporates sodium molybdate and ascorbic acid, is added at the end. Therefore, a reduced blue phosphomolybdate complex is formed due to the presence of the released inorganic phosphate which is proportional to the enzymatic activity. In brief, in 100 μl of total assay volume, buffer (40 mM imidazole, 0.25 M sucrose, pH 7), 20 mM G6P, microsomes and CA or plant extracts at different concentrations were added. The reaction was started by the addition of G6P, incubated at 22°C for 20 min and stopped with the addition of 900 μl of a solution containing 0.42% ammonium molybdate in 1 N H_2SO_4, 10% SDS and 10% ascorbic acid. After incubation of the media at 45°C for 20 min, the

inorganic phosphate was quantified colorimetrically at 830 nm (Arion, 1989). Assays were performed by triplicate.

Statistical Analysis

Pyruvate tolerance test data were expressed as the mean glucose level ± standard error (SEM) at each time point on the curve. Area under curve (AUC) data were expressed as (mg/dl) × min ± SEM. One-way ANOVA with Tukey's *post hoc* tests were performed to compare glucose means among all the groups at each time point, the glucose means from each group versus the corresponding basal value, and the AUC values among the groups. *P*-values less than 0.05 were considered statistically significant. IC_{50} values were calculated by plotting concentration-response curves to find the best fitting regression model (linear or non-linear). Analysis was carried out in GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, United States).

RESULTS

Phytochemical composition of the tested plants was previously reported by our group (see references above). For reference purposes, we have included the HPLC-DAD profiles of the tested extracts that can be consulted in **Supplementary Material**.

In vivo Pyruvate Tolerance Tests

To evaluate gluconeogenesis as the major HGO source, rats were deprived of food for 18 h to deplete hepatic glycogen storage. Next, pyruvate was administered as the substrate for the inhibition assessment. As shown in **Table 1**, both intraperitoneal injection and oral administration of vehicle to normoglycemic (N) and hyperglycemic (H) control rats did not affect the blood glucose levels over the 2-h period of analysis (N and H groups), while pyruvate injection significantly increased these levels, as observed in the NP and HP groups. However, the effect was different between these two groups, since in the NP group, blood glucose levels significantly increased by approximately 60 mg/dl (57%) between 30 and 60 min after pyruvate administration, and almost returned to basal values after 2 h; in the HP group, pyruvate injection raised glucose levels by approximately 160 mg/dl (81%), which remained higher compared to the control group (H) and did not return to the initial value. In other words, even though pyruvate administration significantly increased the overall glucose in normo- and hyperglycemic rats compared to their controls (**Figure 1A**), a differential effect over time was observed due to the physiological state of each group.

For the treatments, metformin was effective in reducing blood glucose levels (HPM group; **Table 1**) since it was able to attenuate the rising glucose observed at 30 min in the HP group by approximately 51%. In addition, metformin had a significant hypoglycemic effect over the next 1 h and 30 min by decreasing the glucose levels beyond the basal level of the HPM group (15% lower).

The plant extracts did not completely block the rising levels of glucose by pyruvate administration since the basal glucose values were significantly lower than those at 30 min in all experimental groups. Although all of the extracts were able to reduce the hyperglycemic peak, only two plants showed a significant decrease. The experimental groups treated with the extracts of *A. petiolaris* and *R. mangle* exhibited similar behavior (HPAp and HPRm groups). These plant extracts were able to significantly reduce the hyperglycemic peak by approximately 20% at 30 min versus the HP control group. This significant difference was maintained during the remainder of the test, showing an inhibitory effect on gluconeogenesis.

The *S. moranensis* extract (HPSm group) decreased the hyperglycemic peak by 11%; however, the glucose values started to increase starting at 60 min. Similarly, the HPEm group reduced its hyperglycemic peak by approximately 10%; nevertheless, the *E. myriochaetum* extract maintained significantly lower glucose levels after 60 and 90 min. These outcomes showed that both extracts, at the doses used, exhibited a weak inhibitory effect on gluconeogenesis: the former lowered the hyperglycemic peak, and the latter showed an antihyperglycemic effect. No significant differences were found in gluconeogenesis after oral administration of the *B. karatas* extract (HPBk group) versus the

TABLE 1 | Plasma glucose values measured at the pyruvate tolerance test on STZ-NA induced hyperglycemic rats.

Glucose groups	T0 (mg/dl)	T30 (mg/dl)	T60 (mg/dl)	T90 (mg/dl)	T120 (mg/dl)
1. N	109 ± 4	111 ± 4	107 ± 3	104 ± 4	102 ± 3
	100%	102%	98%	96%	94%
2. NP	111 ± 3	173 ± 4^{a*}	176 ± 7^{a*}	149 ± 6*	132 ± 6
	100%	157%	158%	134%	119%
3. H	193 ± 4^{a}	213 ± 7^{ab}	200 ± 5^{ab}	198 ± 7^{ab}	189 ± 4^{ab}
	100%	110%	104%	103%	98%
4. HP	195 ± 8	354 ± 18*	373 ± 17*	365 ± 11*	362 ± 10*
	100%	181%	192%	188%	187%
5. HPM 500 mg/kg	190 ± 4	246 ± 6b*	212 ± 7b	189 ± 11b	160 ± 10b
	100%	130%	112%	100%	85%
6. HPAp 160 mg/kg	197 ± 5	288 ± 7b*	301 ± 8b*	317 ± 11b*	323 ± 9*
	100%	147%	153%	161%	165%
7. HPBk 218 mg/kg	189 ± 4	336 ± 10*	343 ± 12*	359 ± 9*	351 ± 10*
	100%	178%	181%	190%	186%
8. HPEm 330 mg/kg	191 ± 4	319 ± 14*	314 ± 12b*	319 ± 13b*	327 ± 14*
	100%	168%	166%	169%	173%
9. HPRm 90 mg/kg	194 ± 5	273 ± 14b*	279 ± 12b*	291 ± 11b*	295 ± 12b*
	100%	142%	145%	151%	154%
10. HPSm 80 mg/kg	199 ± 6	314 ± 12*	333 ± 13*	351 ± 10*	350 ± 12*
	100%	158%	168%	178%	178%

*The values represent the mean ± SEM. Letter "a" indicates significant difference versus normoglycemic (N) group at $p < 0.05$, letter "b" indicates significant difference versus hyperglycemic + pyruvate group (HP) at $p < 0.05$; * indicates significant difference with time 0 of the same group at $p < 0.05$. Change in blood glucose levels is represented as percentage. N, normoglycemic group; NP, normoglycemic + pyruvate group; H, hyperglycemic; HP, hyperglycemic + pyruvate group; HPM, hyperglycemic + pyruvate + metformin group; HPAp, hyperglycemic + pyruvate + Ageratina petiolaris group; HPBk, hyperglycemic + pyruvate + Bromelia karatas group; HPEm, hyperglycemic + pyruvate + Equisetum myriochaetum group; HPRm, hyperglycemic + pyruvate + Rhizophora mangle group; HPSm, hyperglycemic + pyruvate + Smilax moranensis group.*

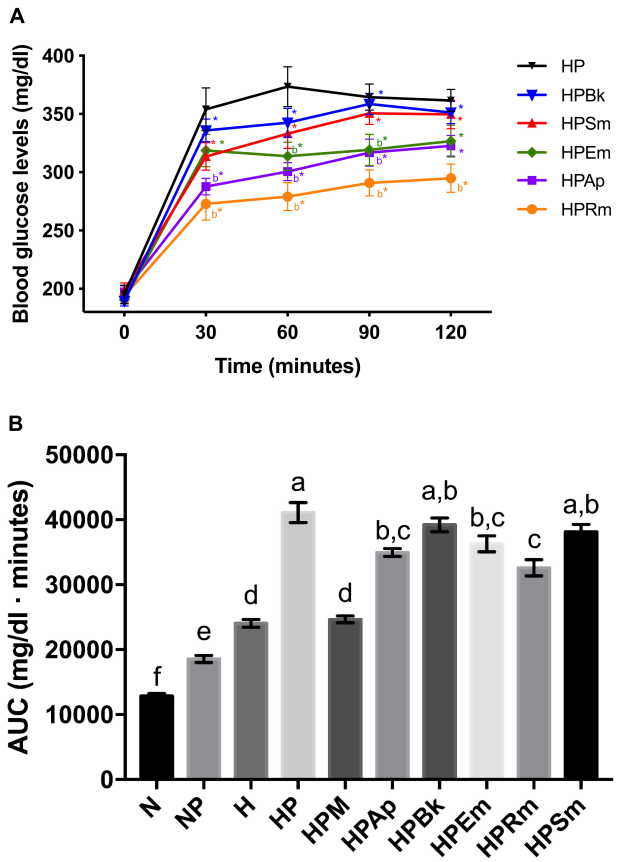

FIGURE 1 | Effect of plant extracts on pyruvate tolerance tests on STZ-NA induced hyperglycemic rats. **(A)** Comparison of plant extract curves. Letter "b" indicates significant difference versus hyperglycemic + pyruvate group (HP) at $p < 0.05$; *indicates significant difference with time 0 of the same group at $p < 0.05$. **(B)** AUC values. The bars represent the mean ± SEM. Different letters over bars indicate statistically significant differences at $p < 0.05$ (a > b > c > d > e > f). N, normoglycemic group; NP, normoglycemic + pyruvate group; H, hyperglycemic; HP, hyperglycemic + pyruvate group; HPM, hyperglycemic + pyruvate + metformin group; HPAp, hyperglycemic + pyruvate + *Ageratina petiolaris* group; HPBk, hyperglycemic + pyruvate + *Bromelia karatas* group; HPEm, hyperglycemic + pyruvate + *Equisetum myriochaetum* group; HPRm, hyperglycemic + pyruvate + *Rhizophora mangle* group; HPSm, hyperglycemic + pyruvate + *Smilax moranensis* group.

HP control group at any time point; therefore, this extract had no effect on glucose synthesis generated by pyruvate.

In addition to the comparation among extract effects on the pyruvate tolerance test (**Figure 1A**), an AUC analysis was performed for the evaluation of the effect of the plant extracts on blood glucose at a global level (**Figure 1B**). This analysis showed that *B. karatas* and *S. moranensis* had no effect on the overall glucose generated by pyruvate injection since their AUC values were not statistically significant versus the AUC of HP. However, *A. petiolaris*, *E. myriochaetum*, and *R. mangle* significantly decreased whole glucose levels over 120 min versus the HP group. Among these three extracts, *R. mangle* showed the greatest hypoglycemic effect due to gluconeogenesis inhibition by pyruvate administration since this AUC was significantly different from the others. In contrast to the extracts, the AUC of metformin was notably similar to the H group; that is, this hypoglycemic drug was able to restore glucose to the levels prior to pyruvate administration.

To summarize, administration of *A. petiolaris* and *R. mangle* extracts showed both higher and more stable HGO inhibition than *B. karatas*, *E. myriochaetum*, and *S. moranensis*.

In vitro G6Pase Activity

To evaluate the direct inhibition of G6Pase activity, concentration-response assays were performed, and the best-fitting regression model (linear or non-linear) to obtain the IC_{50} of each plant extract was chosen. CA was used as a positive control since it is a well-characterized inhibitor of G6Pase T1 translocase. As shown in **Table 2** and **Figure 2**, CA exerted the most potent inhibitory effect, followed by the extracts from *S. moranensis* and *R. mangle*. *A petiolaris* was the less effective; however, it exhibited a higher inhibitory effect than *B. karatas*. On the other hand, *E. myriochaetum* did not show an effect at any assayed concentration.

DISCUSSION

In Mexican traditional medicine, a common practice to treat T2D among patients is to drink an infusion of a medicinal plant between meals (fasting state) to control the disease (blood glucose levels). The fact that the plants are consumed in the fasting state associated with the statement that CA and other polyphenols have been related with the blockage of the enzyme G6Pase (because these compounds have been reported in several plants) led us to propose a link between the traditional use and the action mechanism. This link is not necessarily the best pharmacological option, but it explains the traditional use, considering the great number of plants that have been reported as hypoglycemic.

According to our results, the pyruvate-STZ-NA model was a good tool to assess the effect of plant extracts on HGO *in vivo* since pyruvate administration raised and maintained the glucose levels of fasting animals to approximately 200 mg/dl throughout the test. Additionally, the model was responsive to the hypoglycemic drug metformin. As shown, the glucose levels of the N group returned to a basal value at the end of the test because of an unaltered insulin secretion. On the other hand, the H group showed an increase in blood glucose, which never

TABLE 2 | IC_{50} values of plant extracts obtained from G6Pase system inhibition assay.

Plant extract	IC_{50} value
1. CA	63 μg/ml
2. *A. petiolaris*	223 μg/ml
3. *B. karatas*	1136 μg/ml
4. *E. myriochaetum*	–
5. *R. mangle*	99 μg/ml
6. *S. moranensis*	84 μg/ml

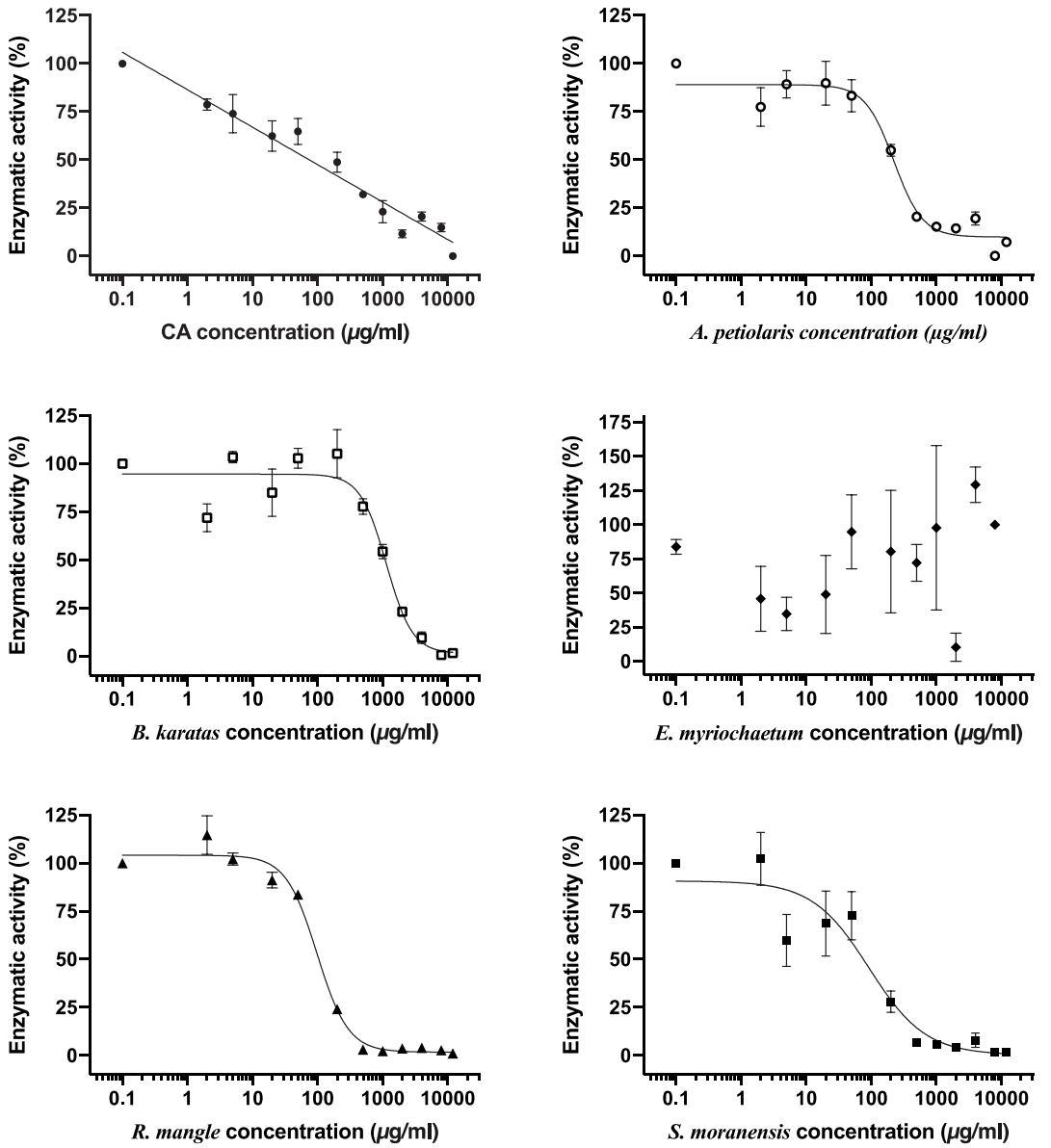

FIGURE 2 | Comparison of inhibitory concentration-response curves of chlorogenic acid and each plant extract on G6Pase system activity. Each point represents the mean of three replicates ± SEM. CA, chlorogenic acid.

returned to the initial values due to impaired insulin secretion, resulting in a low uptake of glucose by skeletal muscle and adipose tissue. Despite the damage to insulin secretory cells caused by STZ, the main effect of metformin was shown: its ability to reduce glucose levels via inhibition of gluconeogenesis (Tan et al., 2016).

The tested plant extracts were able to decrease the hyperglycemic peak after pyruvate administration. Based on the phytochemical composition of the extracts and previous reports in the international literature (see below), possible explanations for the observed effects could be: (1) the presence of CA in the extracts, which was not only able to inhibit G6Pase T1 translocase but also able to raise the phosphorylation levels of AMP-activated protein kinase (AMPK) (Ong et al., 2013); (2) inhibition of the G6Pase system by other compounds; (3) inhibition of another gluconeogenic enzyme; or (4) suppression of HGO via protein kinase B (Akt) or AMPK activation (**Figure 3**).

Rhizophora mangle was able to effectively inhibit the G6Pase system *in vitro*. In addition, the reduction of gluconeogenesis *in vivo* by the *R. mangle* extract can also be attributed to the decrease in the activity of other gluconeogenic enzymes such as PEPCK. Epicatechin, one of the main compounds found in *R. mangle*, has been shown to reduce the enzyme expression leading to diminished HGO in both NRK-52E and HepG2 cells. Additionally, this phenolic compound increased the total and phosphorylated protein levels of the insulin receptor (IR), insulin

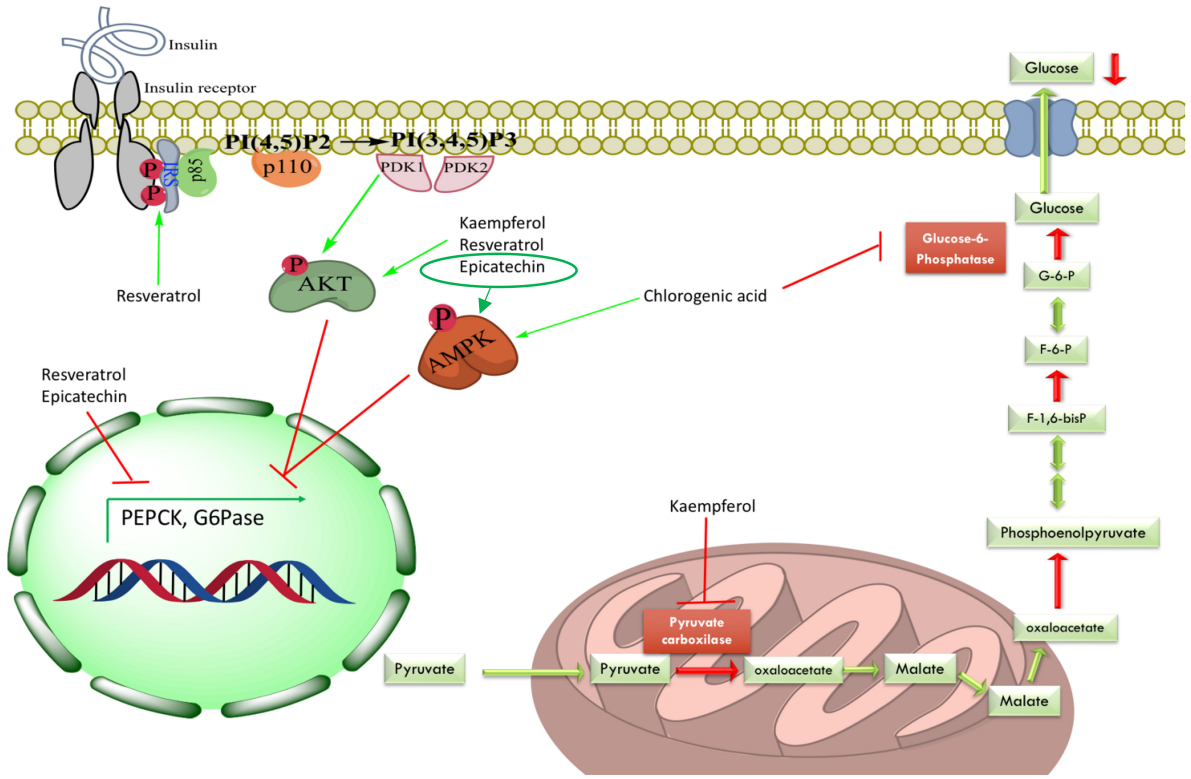

FIGURE 3 | Mechanisms of hepatic gluconeogenesis inhibition exerted by some identified compounds from evaluated plant extracts in this study. Active compounds could have two main ways to decrease HGO: (1) direct inhibition of gluconeogenic enzymes, for instance, inhibition of G6Pase system by CA and inhibition of PC by kaempferol; or (2) decreasing expression levels of gluconeogenic enzymes via Akt pathway (kaempferol, resveratrol and, epicatechin) or AMPK activation (CA and epicatechin). IRS, insulin receptor substrate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate; PDK1, phosphoinositide-dependent kinase 1; PDK2, phosphoinositide-dependent kinase 2; Akt, protein kinase B; AMPK, AMP-activated protein kinase; G6P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-bisP, fructose-1,6-bisphosphate; G6Pase, glucose-6-phosphatase.

receptor substrate-1 (IRS-1), and AMPK (Cordero-Herrera et al., 2014; Álvarez-Cilleros et al., 2018).

Ageratina petiolaris showed an inhibitory effect in the pyruvate tolerance test *in vivo* that could be correlated with its capacity of decreasing the activity of G6Pase system *in vitro* owing to its content of CA (Bustos-Brito et al., 2016). Furthermore, D-chiro-inositol, a polyalcohol present in many Fabaceae plants that has been chronically administered to high-fat diet STZ-treated Sprague Dawley rats, has induced a significant decreasing in fasting insulin levels, increasing hepatic glycogen, raising expression levels of glycogen synthase and GLUT4 genes, and increasing phosphorylation of hepatic Akt (Gao et al., 2016). Therefore, the isomer L-chiro-inositol identified in *A. petiolaris,* by our group, could have a similar ability to positively modulate the insulin signaling pathway, which would inhibit HGO in the pyruvate tolerance test or promote glycogen synthesis.

Smilax moranensis showed the most potent inhibitory effect on the G6Pase system, this could also be correlated with the content of CA and the presence of 3-O-caffeoylquinic acid, an isomer of CA (Romo-Pérez et al., 2019). However, it did not show a significant effect in the pyruvate tolerance test, which could be explained by its pharmacokinetics, either poor intestinal absorption (Ziberna et al., 2014) or metabolism of the G6Pase inhibitor before it can reach the target. *S. moranensis* contains *trans*-resveratrol, which has been associated with increased phosphorylation of both Akt and IRS-1; this activation involves the reduction of both insulin resistance and gluconeogenic enzyme expression (Szkudelski and Szkudelska, 2015).

Equisetum myriochaetum had a positive effect over the HGO in the pyruvate tolerance test but did not show G6Pase system inhibition, thus its mechanism of action could be related to directly inhibit another gluconeogenic enzyme. Some of the main isolated metabolites from *E. myriochaetum* were several types of kaempferols that have been shown to decrease pyruvate carboxylase (PC) activity with no change in protein expression levels due to an increase in hepatic Akt in high-fat diet-fed obese mice (Alkhalidy et al., 2018).

Although *B. karatas* showed a hypoglycemic effect in previous studies (Andrade-Cetto and Medina-Hernández, 2013), its aqueous extract did not show an inhibitory effect in the pyruvate tolerance test. Moreover, in the G6Pase inhibition assay, the IC_{50} for this extract was the highest one, suggesting that the mechanism of action of this extract is not related to the inhibition of HGO. However, further studies must be performed to determine how this hypoglycemic plant works.

Inhibition of G6Pase, which has been proposed to be a shared hypoglycemic mechanism of plants, presents two major problems as a therapeutic target in patients with T2D; (1) since this enzyme is present in both gluconeogenesis and glycogenolysis pathways, inhibitors may cause hypoglycemia; and (2) accumulation of intracellular G6P may induce the expression of lipogenic genes that could result in hepatic steatosis (Agius, 2007). Further studies are needed to prove if the extracts affect HGO in other ways, but a possible common mechanism of action for these plants could be found.

CONCLUSION

In summary, four of the tested plants showed an inhibitory effect on HGO by either decreasing gluconeogenesis in the pyruvate tolerance test or diminishing the HGO-related activity of G6Pase. This inhibition could be a shared mechanism that could be associated with the phytochemical composition of the plants, CA among other compounds, contributing to their hypoglycemic effect. These observations can be correlated to the traditional way of consumption of the plants.

The present work was a starting point approach to characterize the hypoglycemic effects related to HGO inhibition. Based on the known plant composition and literature review, further studies must be performed to test whether these extracts have an effect not only on other gluconeogenic enzymes but also on the signal transduction pathways related to gluconeogenesis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The protocol was approved by the Committee of Academic Ethics and Scientific Responsibility (CEARC) of the Faculty of Sciences, UNAM.

AUTHOR CONTRIBUTIONS

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

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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.2020.00215/full#supplementary-material

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SYSTEMATIC REVIEW
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Protective Effect and Possible Mechanisms of Astragaloside IV in Animal Models of Diabetic Nephropathy: A Preclinical Systematic Review and Meta-Analysis

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Astragaloside IV (AS-IV) has a variety of biological activities and is widely used to treat kidney diseases. We conducted a systematic review of 24 animal studies including 424 animals to evaluate the efficacy of AS-IV for diabetic nephropathy (DN); all current possible mechanisms were summarized. A search strategy was applied to eight databases from inception to June 2020. The CAMARADES 10-item quality checklist and Rev-Man 5.3 software were used to analyze the risks of bias of each study and data regarding outcome measures, respectively. The mean study quality score was 5.4 points (range 3–8 points). Meta-analyses data and comparisons between groups showed that AS-IV significantly slowed the progression of pathological signs in the kidney including glomeruli and tubules, increasing creatinine clearance rate, decreasing blood urea nitrogen, serum creatinine, 24-h urinary neutrophil gelatinase-associated lipocalin and N-acetyl-β-D-glucosaminidase, 24-h urinary albumin, 24-h urinary microalbumin and HbA1c. There were no significant differences between experimental and control groups with respect to mortality or levels of alanine aminotransferase and aspartate aminotransferase. In terms of the possible mechanisms of treatment of DN, AS-IV acts through antifibrotic, antioxidant, and antiapoptotic mechanisms, thereby alleviating endoplasmic reticulum stress, inhibiting mitochondrial fission, and increasing autophagic activity. Taken together, our findings suggest that AS-IV is a multifaceted renoprotective candidate drug for DN.

Keywords: astragaloside IV, rodent models, diabetic nephropathy, meta-analysis, systematic review

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INTRODUCTION

Diabetic nephropathy (DN) is defined as impairment of renal function arising from chronic hyperglycemia. It is among the most severe and common microvascular complications of diabetes mellitus (Saran et al., 2015; Flyvbjerg, 2017). It is characterized by proteinuria, hypertension, and progressive renal insufficiency resulting from compromise of the glomerular filtration barrier, eventually resulting in end-stage renal disease (ESRD) in 30–40% of patients (Bjornstad et al., 2014; Saran et al., 2015; Wang et al., 2016; Du et al., 2017). Management of hypertension and hyperglycemia is the most commonly used approach for treatment of DN. These have been shown to reduce the proportion of patients reaching ESRD (National Kidney Foundation, 2012), although with limited effects (Sharma et al., 2017). Unfortunately, no new therapies that specifically improve progression of DN have been translated into clinical use successfully (Fernandez-Fernandez et al., 2014). For this reason, new therapies are needed urgently to prevent progressive renal failure in these patients.

Astragalus membranaceus (Fisch.) Bunge is a perennial herbal plant of the Leguminous family that first appeared in the earliest complete Pharmacopoeia of China (Sheng Nong's herbal classic work). It was thought to promote Qi according to the theory of traditional Chinese medicine and therefore was widely used for various kidney diseases for thousands of years. Astragaloside IV (AS-IV, $C_{41}H_{68}O_{14}$, molecular weight = 784, **Figure 1**) is a small molecular saponin that is the active ingredient in *A. membranaceus* (Fisch.) Bunge. Recent studies demonstrated that AS-IV has several pharmacological activities *in vivo* and *in vitro*, including anti-inflammatory, antioxidative, antiapoptotic, antifibrotic, and immunoregulatory functions. AS-IV has also been reported to improve the prognosis of DN in streptozotocin-induced (STZ) diabetic rats *via* inhibition of renal inflammation (Gui et al., 2013a), inhibition of renal oxidative stress (Gui et al., 2013b), attenuation of podocyte apoptosis (Guo et al., 2016), and subsequent delay of DN progression (Sun et al., 2016).

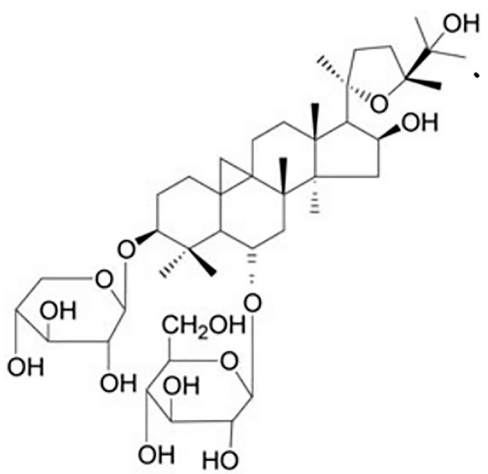

FIGURE 1 | Chemical structures of astragaloside IV.

Nevertheless, the efficacy of AS-IV for DN has not been evaluated systematically, and the mechanisms have not been summarized comprehensively. Therefore, the aim of the present study was to evaluate systematically research reports on the subject in order to summarize the significant outcomes on efficacy and mechanisms.

METHODS

Data Sources and Search Strategies

Animal experimental studies of AS-IV for DN were identified using a computerized literature search of Chinese Science and Technology Journal Database, WanFang, China National Knowledge Infrastructure, Chinese Biomedical Database, EMBASE, Cochrane library, PubMed, and Web of Science. All search strategies were performed from inception to June 2020. The following search terms were used in PubMed and were modified to suit other databases: "Astragaloside IV OR AS-IV OR Astragalus membranaceus" AND "Diabetes OR diabetic nephropathy". The reference lists of all the eligible studies were searched carefully to obtain additional studies.

Eligibility Criteria

The abstracts and titles of studies were screened and the full-text articles were subsequently reviewed for inclusion and exclusion by two authors (Hong Wang and Zhuang Zhuang) independently. Inclusion criteria were as follows (1) AS-IV (as monotherapy in any dose) to treat animal models of DN established in various ways; (2) nonfunctional and equal volumes of liquid (normal saline) or no treatment adopted for the control group; and (3) primary outcome measures were renal pathology, creatinine clearance rate (CCr), 24-h urinary albumin or microalbumin, 24-h urinary neutrophil gelatinase-associated lipocalin (NGAL) or N-acetyl-β-D-glucosaminidase (NAG), serum creatinine (SCr), blood urea nitrogen (BUN), HbA1c, or indicators of adverse reactions. The renoprotective mechanisms of AS-IV for DN were selected as secondary outcome measures. Exclusion criteria were as follows: (1) not *in vivo* studies (*in vitro* studies, clinical trials, review articles, case reports, comments, editorials, and abstracts); (2) treatment with AS-IV-based prescriptions or combinations with other drugs; (3) comparisons with other drugs with unclear efficacy; (4) no predetermined outcome index or available data; (5) not a DN model; (6) duplicate publication; and (7) no control group.

Data Extraction

Two authors (HW and Y-YH) independently evaluated all the eligible articles for data extraction in terms of: (1) the publication year of the study and the name of the first author; (2) details regarding the animals; (3) the methods to establish animal models, the criteria for modeling successfully and the use of anesthetics in the course of experiment; (4) the therapeutic regimen and the control group; and (5) primary, secondary outcomes, and intergroup differences. If the outcomes were displayed through gradient doses of drug therapy or multiple time points, only the data of the highest dose group and peak

time point group were included. The authors were contacted for specific data when the results were only rendered graphically. If a response was not received, the graph data were measured using Photoshop.

Risk of Bias in Individual Studies

For each included study, quality assessment was carried out by two authors (ZZ and Y-YH) independently using the CAMARADES 10-item quality checklist (Macleod et al., 2004) with minor modifications (modified sections: D: blinded induction of model [group randomly after modeling, or transgenic mice, or knockout mice]; F: use of anesthetic without significant renal protective activity or nephrotoxicity). Disagreements in selecting studies, extracting data, or assessing the quality of studies were resolved by consensus or arbitration by the correspondence authors (QZ and Y-LW).

Statistical Analysis

RevMan 5.3 software downloaded on the website (https://www.cochrane.org/) was used for data analysis where possible. When meta-analysis failed to run, comparisons between groups were performed for individual studies. The combined overall effect sizes of outcome measures were estimated by utilizing standard mean difference (SMD) with 95% confidence interval (CI). Heterogeneity was determined using the Cochrane Q-statistic test and the I2-statistic test (random effects model [I2 greater than 50%] or a fixed effects model [I2 less than or equal to 50%]). Sensitivity analyses were conducted when individual results deviated substantially. Bar graphs were drawn using Prism 6. When the probability value was less than 0.05, the difference was considered statistically significant.

RESULTS

Study Selection

The search strategy yielded 1,053 potentially relevant studies from the eight databases, of which 878 were duplicated or irrelevant studies. We excluded 91 non-animal studies after checking the titles and abstracts. Detailed inspection was performed for remaining 61 full-text studies; 37 of these were not considered because they presented at least one of the excluding criteria. Finally, 24 eligible studies were identified: 13 in English (Gui et al., 2012; Chen et al., 2014; Lu et al., 2015; Wang et al., 2015; Guo et al., 2016; Sun et al., 2016; Guo et al., 2017; Liu et al., 2017; Du et al., 2018; Lei et al., 2018a; Lei et al., 2018b; Fan et al., 2019; Ju et al., 2019) and 11 in Chinese (Chen and Chen, 2016; Huang et al., 2016; Li and Zhang, 2016; Ran and Ma, 2016; Wang et al., 2017; Han et al., 2018; He et al., 2018; Li, 2018; Liu et al., 2019; Ma et al., 2019; Song et al., 2020) (**Figure 2**).

Characteristics of Included Studies

Twenty-four studies with 424 animals were included. The sample size ranged from 12 to 28 animals in each study. Sprague Dawley (SD) male rats were used in 16 studies; male Wistar rats in two studies, male C57BL/6 mice in two studies, male db/db mice in three studies, and male KKAy mice in one study. The weight of SD or Wistar rats varied between 170 and 260 g, and the weight of mice varied between 16 and 24 g. Eighteen studies established the DN model by intraperitoneal injection of streptozotocin (STZ); two studies used feeding high-fat diet for several weeks and intraperitoneal injection of STZ; four studies used mutant or transgenic mice with spontaneous diabetes (four studies used db/db mice and one study used KKAy mice) that exhibits clinical and histological features of DN resembling those found in human DN (Sharma et al., 2003; Tesch and Lim, 2010). To induce anesthesia, anesthetics were unreported in 11 studies, pentobarbital sodium was reported in seven studies, chloral hydrate was used in four studies, urethane was used in one study, and ether was used in one study. Detailed information of AS-IV in each study is displayed in **Table 1**. Twenty-two studies implemented a dose gradient of AS-IV ranging from 3 to 1.08 $g{\bullet}kg^{-1}{\bullet}d^{-1}$ using oral or intragastric administration. Rats received 5 $mmol{\bullet}d^{-1}$ AS-IV by oral gavage in one study and normal standard diet supplemented with AS-IV (AS-IV: feed = 5 g:1 kg) was administered to rats by oral gavage in one study. In terms of outcome measures, renal pathology was utilized as primary outcome measure in 19 studies, BUN in 13 studies, SCr in 13 studies, 24-h urinary protein in 12 studies, 24-h urinary microalbumin in six studies, 24-h urinary NAG in three studies, 24-h urinary NGAL in two studies, blood glucose (BG) in 17 studies, glycated hemoglobin (HbA1c) in four studies; CCr in one study; alanine aminotransferase (ALT) in three studies, and aspartate aminotransferase (AST) in two studies. Secondary outcomes were as follows: superoxide dismutase (SOD) was reported in three studies; catalase (CAT) in two studies; malondialdehyde (MDA) in four studies; glutathione peroxidase (GSH-PX) in three studies; tumor necrosis factor-α (TNF-α) in one study; monocyte chemotactic protein-1 (MCP-1) in one study; caspase-3 in two studies; caspase-12 in one study; Bax and Bcl-2 in two studies; transforming growth factor-β1 (TGF-β1) in five studies; Smad2/3 in two studies; Smad7 in two studies; phosphothreonine kinase (p-Akt) in four studies; phosphorylated phosphatidylinositol-3-kinase (p-PI3K) in two studies; PKR-like eukaryotic initiation factor 2A kinase 1/2 (pERK-1/2) in three studies; PTEN-induced putative kinase (PINK) in one study; Jun N-terminal kinases (JUN) in two studies; forkhead box O1 (Fox O1) in one study; mammalian target of rapamycin (mTOR) in two studies; nuclear factor kappa B (NF-κB) in 2 studies; glucose-regulated protein 78 (GRP78) in three studies; sarco-endoplasmic reticulum calcium adenosine triphosphatase (SERCA) in two studies. The detailed characteristics of the included studies are displayed in **Table 2**.

Study Quality

The CAMARADES 10-item quality checklist was adopted to judge the risk of bias of each study and the number of criteria met varied from 3/10 to 8/10 with the average of 5.4. The review authors' judgments about each risk of bias item for each included study are summarized in **Table 3**.

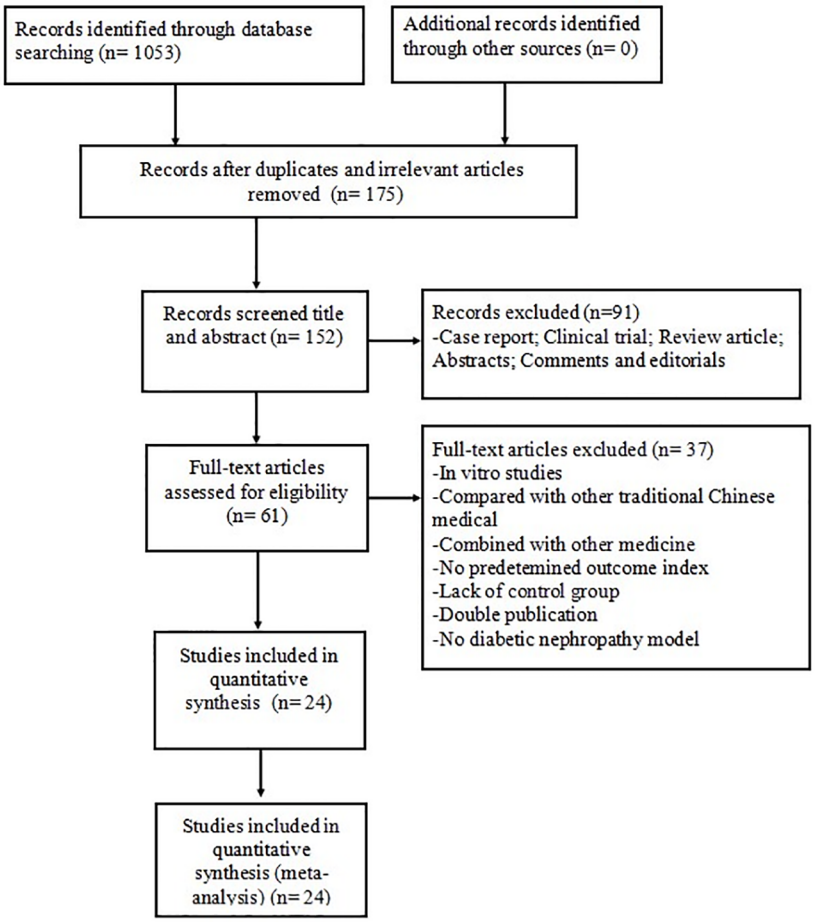

FIGURE 2 | Summary of the process for identifying candidate studies.

Effectiveness

Renal Pathology

Compared with the control group, AS-IV treatment inhibited podocyte apoptosis and ameliorate podocyte foot process effacement significantly in nine studies as assessed using electron or light microscopy. Treatment alleviated mesangial cell proliferation and the broadening of mesangial matrix significantly in 14 studies, but not to a significant degree in one study. There was inhibition of basement membrane thickening in four studies, narrowing the enlarged glomerular volume and alleviated the level of glomerular fibrosis in six studies, and alleviation of wall edema in proximal tubules and reduction of apoptosis of renal tubular epithelial cells in four studies.

CCr, BUN, and SCr

We found that CCr was reduced significantly by AS-IV compared with the control group ($P < 0.05$). Meta-analysis of 13 studies indicated BUN was reduced significantly by AS-IV compared with the control group (**Figure 3**). The publication bias funnel indicated that there is no substantial publication bias (**Figure 4**). Meta-analysis of 13 studies indicated SCr was decreased significantly by AS-IV (**Figure 5**). Sensitivity analyses of indicators of BUN and SCr were carried out, and these indicated that heterogeneity did not decline significantly after eliminating any study.

Levels of 24-h Urinary Albumin, Microalbumin, NAG, and NGAL

Meta-analysis of 12 and six studies reported that 24-h urinary albumin (**Figure 6**) and 24-h urinary microalbumin, respectively, were reduced significantly by AS-IV. After sensitivity analyses, one study was removed because the DN experimental model in that study was established using intraperitoneal injection of STZ with 125 *vs* ≤*65 mg/kg in other studies. Subsequently, heterogeneity of this indicator decreased substantially* (**Figure 7**). Meta-analysis of three and two studies indicated 24-h urinary NAG (**Figure 8**) and 24-h urinary NGAL (**Figure 8**), respectively, were reduced significantly by AS-IV.

TABLE 1 | Information of AS-IV of each study.

Study (years)	Specifications	Source	Purity (%)	Quality control reported
Gui et al., 2012	dry powder	Xi'an Sobeo Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC
Chen et al., 2014	dry powder	Xi'an Sobeo Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC
Lu et al., 2015	dry powder	Chengdu Jintaihe Pharmaceutical Chemical Technology Company, Limited	(98%)	Y-HPLC
Wang et al., 2015	dry powder	Unknown	Unknown	Unknown
Chen and Chen, 2016	dry powder	Shanghai Rongbai Biotechnology Company, Limited	(≥98%)	Batch number 14051502
Guo et al., 2016	dry powder	Shanghai Bogoo Biotechnology Company, Limited	(98%)	Unknown
Huang et al., 2016	dry powder	Shanghai Pharmaceutical Development Company, Limited	Unknown	Y-HPLC
Li and Zhang, 2016	dry powder	Unknown	Unknown	Unknown
Ran and Ma, 2016	dry powder	Dalian Meilun Biotechnology Company, Limited	(98%)	BR, batch number MB1955
Sun et al., 2016	dry powder	ChengDu ConBon Biotech Company, Limited	Unknown	Y-HPLC
Guo et al., 2017	dry powder	Shanghai Bogoo Biotechnology company, Limited	(98%)	Unknown
Liu et al., 2017	dry powder	ChengDu ConBon Biotech Company, Limited	Unknown	Y-HPLC
Wang et al., 2017	dry powder	American Sigma Company	(98%)	Y-TLC
Du et al., 2018	dry powder	ChengDu ConBon Biotech Company, Limited	(≥98%)	Y-HPLC
Han et al., 2018	dry powder	ChengDu Kangbang Biotechnology Company, Limited	(≥98%)	Y-HPLC
He et al., 2018	dry powder	American Sigma Company	(98%)	Y-TLC
Li, 2018	dry powder	Nanjing Zelang Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC, batch number ZL160305
Lei et al., 2018a	dry powder	Solarbio life sciences &technology Company, Limited	(≥98%)	Y-HPLC
Lei et al., 2018b	dry powder	Solarbio life sciences &technology Company, Limited	(≥98%)	Y-HPLC
Fan et al., 2019	dry powder	Dalian Meilun Biotechnology Company, Limited	(≥98%)	Unknown
Ju et al., 2019	dry powder	Nanjing Zelang Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC
Liu et al., 2019	dry powder	ChengDu Ruifensi Biotechnology Company, Limited	(≥98%)	Batch number 170768
Ma et al., 2019	dry powder	Nanjing Zelang Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC, batch number CY170925
Song 2020	dry powder	Xi'an Sobeo Pharmaceutical Technology Company, Limited	(≥98%)	Y-HPLC

HPLC, High performance liquid chromatography; TLC, thin Layer Chromatography.

BG and HbA1c

Seventeen studies used BG as an outcome measure, and all of these indicated that BG was decreased by AS-IV compared with the control group ($P < 0.05$). HbA1c in four studies failed to pool in the analysis because the ways of calculating HbA1c varied. Meta-analysis of the remaining three studies indicated that HbA1c level was significantly decreased by AS-IV (**Figure 9**).

Adverse Reactions

Three studies utilized ALT and three studies utilized AST as outcome measure to assess the adverse effects of AS-IV on the liver. Meta-analysis of these studies showed no significant effect of AS-IV on ALT (**Figure 10**) or AST (**Figure 10**). Two studies reported mortality as an outcome measure, and meta-analysis of both studies showed that there was no significant difference in mortality between the AS-IV group and the control group (**Figure 11**). Two studies reported external manifestations in animals (including, activity, and glossiness of fur) with the AS-IV + STZ group showing better results than the STZ group.

Renoprotective Mechanisms

Compared with control groups, meta-analysis of three studies and two studies showed that AS-IV increased SOD (**Figure 12**) and CAT (**Figure 12**), respectively. In three studies, there was increased GSH-px ($P < 0.05$). In four studies, there were decreased levels of MDA ($P < 0.05$). In one study, there were decreased levels of TNF-α and MCP-1. In two studies, there were decreased levels of caspase-3. In one study, there were decreased levels of caspase-12. In two studies, there were decreased levels of Bax and increased levels of Bcl-2. In five studies, there were decreased levels of TGF-β1. In two studies, there were decreased levels of Smad2/3. In two studies, there were increased levels of Smad7. In two studies, there were decreased levels of NF-κB. In three studies, there were decreased levels of GRP78.

Subgroup Analysis

Potential confounding factors (including various methods of modeling, different animal species, varying doses of AS-IV and duration of treatment) that may have increased the heterogeneity of outcome measure were explored using stratified analysis of the BUN and SCr. The subgroup analysis of Scr showed basically the same result as that of BUN (**Figures 13** and **14**). In the subgroup analysis of the various modeling methods, the effect size of the model induced by mutant mice showed a different result than that of the models induced by administration of STZ; the method of low dose STZ injection combined with high-fat diet (SMD −4.24 *vs.* SMD −1.89 *vs.* SMD −1.42 $P < 0.05$, **Figure 13**; SMD −0.1 *vs.* SMD −2.09 *vs.* SMD −3.04 $P < 0.05$, **Figure 14**), and the heterogeneity of the three groups decreased substantially. The group in which DN was induced in C57BL/6J mice showed greater effect size than in rats (SMD −3.62 *vs.* SMD −1.64, $P < 0.05$, **Figure 13**; SMD −6.73 *vs.* SMD −1.88, $P < 0.05$, **Figure 14**), and the heterogeneity of two groups decreased substantially. No difference was seen between the high-dose AS-IV group (>18 mg/kg, QD) and the low-dose group (≤18 mg/kg, QD) (SMD −1.96 *vs.* SMD −2.04, $P > 0.05$, **Figure 13**; SMD −3.17 *vs.* SMD −1.58, $P > 0.05$, **Figure 14**). Notably, the longer period of AS-IV

TABLE 2 | Characteristics of the 24 included studies.

(years)	Species (Sex, n = experimental/ control group)	Weight	Model (method)	The Criteria for modeling successfully	Anesthetic	Treatment group (Method to astragal sides)	Control group	Outcome index (time)	Intergroup differences
Gui et al., 2012	SD rats (male, 8/8)	180–200 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 300 mg/dl in 3 different times after 72 h of STZ injection	Pentobarbital sodium	By oral gavage of AS-IV (10 mg/kg, qd) in 2 weeks before STZ injection and lasted 14 weeks	By oral gavage of an equal volume of CMC in 2 weeks before STZ injection and lasted 14 weeks	1. Renal pathology 2. BUN and SCr 3. HbA1c 4. BG 5. ALT 6. SOD and MDA 7. CAT 8. Caspase-3 9. Bax and Bcl-2	1. $P < 0.05$ 2. $P > 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$ 8. $P < 0.05$ 9. $P < 0.05$
Chen et al., 2014	SD rats (male, 8/8)	180–200 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 300 mg/dl in 3 different times after 72 h of STZ injection	Pentobarbital sodium	By oral gavage of AS-IV (10 mg/kg, qd) in 2 weeks before STZ injection and lasted 14 weeks	By oral gavage of an equal volume of CMC in 2 weeks before STZ injection and lasted 14 weeks	1. Renal pathology 2. 24-h urinary protein 3. BUN and SCr 4. BG 5. ALT and AST 6. Integrin α3, β1 subunits and ILK	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$
Lu et al., 2015	SD rats (male, 14/14)	180–220 g	By intraperitoneal injection of STZ (125 mg/kg, qd) for 2 consecutive days	Rats with a blood glucose level over 16.7 mmol/L in 3 different times after 72 h of STZ injection	NM	By oral gavage of AS-IV (40 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of NS after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary microalbumin 3. BG 4. integrin α4 and β1 subunits and ILK	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$
Wang et al., 2015	SD rats (male, 14/14)	180–200 g	By intraperitoneal injection of STZ (50 mg/kg, qd) for 5 consecutive days	Rats with a blood glucose level over 300 mg/dl in 3 different times after 72 h of STZ injection	Pentobarbital sodium	By oral gavage of AS-IV (10 mg/kg, qd) in 2 weeks after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of NS in 2 weeks after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary protein 3. BUN and SCr 4. Kidney/body weight ratio 5. BG 6. Total and phosphorylated PERK, eIF2a, and JNK 7. GRP78 and ORP150	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$
Chen and Chen, 2016	SD rats (male, 10/10)	180–200 g	By intraperitoneal injection of STZ (40 mg/kg)	Rats with a blood glucose level over 16.6 mmol/L in 3 different times after 72 h of STZ injection	Chloral hydrate (35 mg/kg)	By oral gavage of AS-IV (1 mmol/L, 5 ml, qd) after STZ injection and lasted 30 days	By oral gavage of an equal volume of NS in after STZ injection and 30 days	1. Renal pathology 2. 24-h urinary protein 3. UCr 4. BG	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$
Guo et al., 2016	db/db mice (male, 10/10)	NM	BKS.cg-m +/+ Leprdb/J mice (Spontaneous diabetes)	NM	NM	By oral gavage of AS-IV (18 mg/kg, qd) at 8 weeks of age and lasted 8 weeks	By oral gavage of an equal volume of CMC at 8 weeks of age and lasted 8 weeks	1. Renal pathology 2. BUN and SCr 3. Kidney/body weight ratio 4. HbA1c 5. BG 6. HOMA-IR	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$

(Continued)

TABLE 2 | Continued

(years)	Species (Sex, n = experimental/ control group)	Weight	Model (method)	The Criteria for modeling successfully	Anesthetic	Treatment group (Method to astragal sides)	Control group	Outcome index (time)	Intergroup differences
								7. TNF-α 8. MCP-1 9. Serum insulin 10. SERCA	8. P < 0.05 9. P < 0.05 10. P < 0.05
Huang et al., 2016	Wistar rats (male, 10/10)	170–230 g	By intraperitoneal injection of STZ (60 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L and 24-h urinary protein level over 30mg/kg/d after 72 h of STZ injection	Urethane (100mg/kg)	By oral gavage of AS-IV (3 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of citrate buffer after STZ injection and lasted 8 weeks	1. 24-h urinary protein 2. BUN and SCr 3. BG 4. SOD, MDA, and GSH-Px 5. TGF-β1 mRNA 6. CAT mRNA	1.P<0.05 2.P<0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05
Li and Zhang, 2016	SD rats (male, 10/10)	NM	By intraperitoneal injection of STZ (60 mg/kg)	Rats with a blood glucose level over 13.9 mg/kg after 1 week of STZ injection	Pentobarbital sodium	By oral gavage of AS-IV (1.08 g/kg, qd) after STZ injection and lasted 6 weeks	By oral gavage of an equal volume of NS after STZ injection and lasted 6 weeks	1. Renal pathology 2. 24-h urinary microalbumin 3. Kidney/body weight ratio 4. BG 5. TGF-β1 6. Collagen I, II, III 7. α-SMA 8. TβRI, TβRII 9. Smad 2/3 and Smad 4	1. P < 0.05 2. P < 0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05 7. P < 0.05 8. P < 0.05 9. P < 0.05
Ran and Ma, 2016	SD rats (male, 10/10)	240–260 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	NM	By oral gavage of AS-IV (5 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of distilled water after STZ injection and lasted 8 weeks	1. Histological structure of kidney under transmission electron microscopy 2. 24-h urinary protein 3. 24-h urinary microalbumin 4. BG 5. Adiponectin	1. P < 0.05 2. P < 0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05
Sun et al., 2016	db/db mice (male, 8/8)	NM	BKS.Cg-Dock7^{m} +/+Lepr^{db}/ JNju) mice (Spontaneous diabetes)	NM	NM	By oral gavage of AS-IV (1 g/kg, qd) at 8 weeks of age and lasted 12 weeks	By oral gavage of an equal volume of NS at 8 weeks of age and lasted 12 weeks	1. Renal pathology 2. 24-h urinary protein 3. 24-h urinary NAG and NGAL 4.HbA1c 5.Serum insulin 6.ALT and AST 7.Urinary TGF-β1 8.p-Akt 9.β-actin 10.p-mTOR 11. p-NF-κB 12. p-ERK1/2 13. BG	1. P < 0.05 2. P < 0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05 7. P < 0.05 8. P < 0.05 9. P < 0.05 10. P < 0.05 11 .P < 0.05 12. P < 0.05 13. P < 0.05

(Continued)

TABLE 2 | Continued

(years)	Species (Sex, n = experimental/ control group)	Weight	Model (method)	The Criteria for modeling successfully	Anesthetic	Treatment group (Method to astragal sides)	Control group	Outcome index (time)	Intergroup differences
Guo et al., 2017	C57BL/6J mice (male, 12/12)	NM	By intraperitoneal injection of STZ (100 mg/kg, qd) for 2 consecutive days	Mice with a blood glucose level over 350 mg/dl after 1 week of STZ injection	NM	By oral gavage of AS-IV (12 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of NS after STZ injection and lasted 8 weeks	1. Renal pathology 2. BUN and SCr 3. GRP78 4. TF6 5. p-ERK1/2 6. p-eIF2α and p-IRE1α 7. CHOP 8. TRAF2 9. p-JNK 10. Caspase-12 11. SERCA	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$ 8. $P < 0.05$ 9. $P < 0.05$ 10. $P < 0.05$ 11. $P < 0.05$
Liu et al., 2017	db/db mice (male, 6/6)	NM	C57BL/KSJ mice (Spontaneous diabetes)	NM	Pentobarbital sodium (75 mg/kg)	By oral gavage of AS-IV (1 g/kg, qd) at 8 weeks of age and lasted 12 weeks	By oral gavage of an equal volume of NS at 8 weeks of age and lasted 12 weeks	1. Renal pathology 2. 24-h urinary protein 3. 24-h urinary NAG 4. Drp-1, Fis-1, and MFF 5. Mfn-1, Mfn-2,and OPA-1 6. PINK1, Parkin, p-Parkin (Ser 65), and LC-3II	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$
Wang et al., 2017	KKAy mice (male, 10/10)	22–24 g	By feeding with high-fat diet for 14 consecutive weeks before experiment and lasted 14 weeks	Mice with a blood glucose level over 13.9 mmol/L after 14 weeks of high-fat diet	Aether	By oral gavage of AS-IV (40 mg/kg, qd) after 14 weeks of high-fat diet and lasted 10 weeks	By oral gavage of an equal volume of NS after 14 weeks of high-fat diet and lasted 10 weeks	1. Renal pathology 2. BG 3. α-SMA 4. TGF-β1 5. Smad2/3	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$
Du et al., 2018	Wistar rats (male, 8/8)	180–220 g	By intraperitoneal injection of STZ (60 mg/kg)	Rats with a blood glucose level over 13.8 mg/kg after 72 h of STZ injection	NM	By oral gavage of AS-IV (16 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of NS after STZ injection and lasted 8 weeks	1. BUN and SCr 2. UCr 3. β2-MG 4. MDA, CAT and GSH-Px 5. T-AOC	1. $P< 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$
Han et al., 2018	C57BL/6J (male, 6/6)	NM	By intraperitoneal injection of STZ (200 mg/kg)	Mice with a blood glucose level over 16.7 mmol/L	NM	By oral of feed supplemented with AS-IV (AS-IV:feed = 5g:1kg) and lasted 8 weeks	By oral of an equal quality of standard feed and lasted 8 weeks	1.Histological structure of kidney under transmission electron microscopy 2. Renal pathology 3. 24-h urinary protein 4. 24-h urinary NAG and NGAL 5. Kidney/body weight ratio 6. p-Akt 7. p-mTOR	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$

(Continued)

TABLE 2 | Continued

(years)	Species (Sex, n = experimental/ control group)	Weight	Model (method)	The Criteria for modeling successfully	Anesthetic	Treatment group (Method to astragal sides)	Control group	Outcome index (time)	Intergroup differences
He et al., 2018	SD rats (male, 10/10)	180–220 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	NM	By oral gavage of AS-IV (10 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of CMC after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary protein 3. p-130Cas	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$
Li, 2018	SD rats (male, 11/10)	180–220 g	By intraperitoneal injection of STZ (55 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	Chloral hydrate (35 mg/kg)	By oral gavage of AS-IV (80 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of CMC after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary microalbumin 3. Kidney/body weight ratio 4. BUN and SCr 5. BG 6. MDA, SOD, and GSH-Px 7. Nrf2, NQO1, and HO1 8. TGF-β1	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$ 8. $P < 0.05$
Lei et al., 2018a	SD rats (male, 6/6)	200g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 300 mg/dl after 48 h of STZ injection	NM	By oral gavage of AS-IV (5 mg/kg, qd) after STZ injection and lasted 12 weeks	By oral gavage of an equal volume of citrate buffer after STZ injection and lasted 12 weeks	1. 24-h urinary protein 2. TUG1 level 3. The TRAF5 mRNA and protein level	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$
Lei et al., 2018b	SD rats (male, 6/6)	200–220 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 300 mg/dl after 48 h of STZ injection	Pentobarbital sodium	By oral gavage of AS-IV (5 mg/kg, qd) after STZ injection and lasted 12 weeks	By oral gavage of an equal volume of citrate buffer after STZ injection and lasted 12 weeks	1. BUN and SCr 2. TRAF5 3. miR-378	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$
Fan et al., 2019	SD rats (male, 6/6)	180-200 g	By intraperitoneal injection of STZ (65 mg/kg)	Rats with a blood glucose level over 300 mg/dl after 48 h of STZ injection	pentobarbital sodium	By oral gavage of AS-IV (10 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of CMC after STZ injection and lasted 8 weeks	1. Renal pathology 2. HbA1c 3. BUN and SCr 4. Nitric oxide 5. Endothelial nitric oxide synthase 6. Body weight 7. BG 8. 24-h urinary protein 9. Cell apoptosis rate	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$ 8. $P < 0.05$ 9. $P < 0.05$
Ju et al., 2019	SD rats (male, 8/8)	180–220 g	By feeding with high-fat diet for 6 consecutive weeks before modeling+ by intraperitoneal injection of STZ (35 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	Chloral hydrate	By oral gavage of AS-IV (80 mg/kg, qd) in 1 week after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of CMC in 1 week after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary microalbumin 3. BUN and SCr 4. Kidney/body weight ratio 5. Ratio of urine total protein to UCr 6. CCr	1. $P < 0.05$ 2. $P < 0.05$ 3. $P < 0.05$ 4. $P < 0.05$ 5. $P < 0.05$ 6. $P < 0.05$ 7. $P < 0.05$ 8. $P < 0.05$ 9. $P < 0.05$

(Continued)

TABLE 2 | Continued

(years)	Species (Sex, n = experimental/ control group)	Weight	Model (method)	The Criteria for modeling successfully	Anesthetic	Treatment group (Method to astragal sides)	Control group	Outcome index (time)	Intergroup differences
								7. BG 8. TG and TC 9. Bax, Bcl-2, and Bax/Bcl-2 10. Caspase-3 11. β-actin 12. GRP78	10. P < 0.05 11. P < 0.05 12. P < 0.05
Liu et al., 2019	SD rats (male, 10/10)	180-200 g	By intraperitoneal injection of STZ (50 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	NM	By oral gavage of AS-IV (60 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of distilled water after STZ injection and lasted 8 weeks	1. BG 2. Urine protein 3. BUN and SCr 4. p-PI3K 5. p-Akt 6. NF-κB 7. Renal hypertrophy index	1. P < 0.05 2. P < 0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05 7. P < 0.05
Ma et al., 2019	SD rats (male, 13/10)	180–220 g	By feeding with high-fat and high-suga diet for 6 consecutive weeks before modeling+ by intraperitoneal injection of STZ (35 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	Chloral hydrate	By oral gavage of AS-IV (80 mg/kg, qd) after STZ injection and lasted 8 weeks	By oral gavage of an equal volume of CMC after STZ injection and lasted 8 weeks	1. Renal pathology 2. 24-h urinary microalbumin 3. 24-h urinary protein 4. BUN and SCr 5. Kidney/body weight ratio 6. BG 7. p-Fox O1/Fox O1 8. p-PI3K/PI3K 9. p-Akt/Akt	1. P < 0.05 2. P < 0.05 3. P < 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05 7. P < 0.05 8. P < 0.05 9. P < 0.05
Song et al., 2020	SD rats (male, 8/8)	200–220 g	By intraperitoneal injection of STZ (58 mg/kg)	Rats with a blood glucose level over 16.7 mmol/L after 72 h of STZ injection	NM	By oral gavage of AS-IV (10 mg/kg, qd) after STZ injection and lasted 12 weeks	By oral gavage of an equal volume of distilled water after STZ injection and lasted 12 weeks	1. Renal pathology 2. 24-h urinary protein 3. BUN and SCr 4. Kidney/body weight ratio 5. BG 6. Desmin	1. P < 0.05 2. P < 0.05 3. P > 0.05 4. P < 0.05 5. P < 0.05 6. P < 0.05

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BG, blood glucose; BUN, blood urea nitrogen; β2-MG, β2-microglobulin; Cas, crk-associated substrate; CAT, catalase; CCr, creatinine clearance rate; CHOP, CCAAT/enhancer-binding protein homologous protein; CMC, carboxymethyl cellulos; COl-IV, type IV collagen; Cr, creatinine; Drp-1, dynamin-related protein 1; Fis-1, mitochondrial fission protein 1; Fox O1, forkhead box O1; HbA1c, glycated hemoglobin; GRP78, glucose-regulated protein 78; GSH-PX, glutathione peroxidase; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; HDL, high density lipoprotein; ILK, integrin-linked kinase; IL-1β, interleukin-1β; JNK, Jun N-terminal kinases; LDL, low-density lipoprotein; LN, laminin; MCP-1, monocyte chemotactic protein-1; MDA, malondialdehyde; MFF, mitochondrial fission factor; Mfn-1, rabbit antimitofusin 1; Mfn-2, rabbit antimitofusin 2; mTOR, mammalian target of rapamycin; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; NM, not mentioned; NQO1, nad(p)h quinnone dehydrogenase 1; Nrf2, nuclear factor E2 related-factor 2; NS, normal saline; OPA-1, mouse anti-optic atrophy 1; ORP150, 150 kDa oxygen-regulated protein; PERK, PKR-like eukaryotic initiation factor 2A kinase; p-PI3K, phosphorylated phosphatidylinositol-3-kinase; PINK, PTEN-induced putative kinase; SCr, serum creatinine; SD rats, Sprague Dawley rats; SERCA, sarco endoplasmic reticulum calcium adenosine triphosphatase; SOD, superoxide dismutase; STZ, Streptozotocin; T-AOC, total anti-oxidative capacity; TC, Serum total cholesterol; TG, Triglyceride; TP, Total protein; TNF-α, tumor necrosis factor-α; TRAF2, TNF receptor associated factor 2; UCr, urinary creatinine.

TABLE 3 | Risk of bias of the included studies.

Study	A	B	C	D	E	F	G	H	I	J	Total
Gui et al., 2012	√	√				√			√	√	5
Chen et al., 2014	√	√	√			√			√	√	6
Lu et al., 2015	√		√							√	3
Wang et al., 2015	√	√				√				√	4
Chen and Chen, 2016	√	√	√			√				√	5
Guo et al., 2016	√	√	√	√					√	√	6
Huang et al., 2016	√	√	√			√				√	5
Li and Zhang, 2016	√	√	√	√		√				√	6
Ran and Ma, 2016	√	√	√						√	√	5
Sun et al., 2016	√	√	√	√					√	√	6
Guo et al., 2017	√	√	√							√	4
Liu et al., 2017	√	√	√	√		√			√	√	7
Wang et al., 2017	√	√	√	√		√			√	√	7
Du et al., 2018	√		√			√			√	√	5
Han et al., 2018	√	√	√	√						√	5
He et al., 2018	√	√	√							√	4
Li et al., 2018		√	√	√		√			√	√	6
Lei et al., 2018a	√	√		√					√	√	5
Lei et al., 2018b	√	√	√			√			√	√	6
Fan et al., 2019	√	√		√		√			√	√	6
Ju et al., 2019	√	√	√	√		√	√		√	√	8
Liu et al., 2019	√	√	√	√						√	5
Ma et al., 2019	√	√	√	√		√			√	√	7
Song et al., 2020	√	√	√							√	4

Studies fulfilling the criteria of: A: peer reviewed publication; B: control of temperature; C: random allocation to treatment or control; D: blinded induction of model (group randomly after modeling or transgenic mice or knockout mice); E: blinded assessment of outcome; F: use of anesthetic without significant renal protective activity or nephrotoxicity; G: appropriate animal model (aged, hyperlipemia or hypertensive); H: sample size calculation; I: compliance with animal welfare regulations (including three or more of the following points: preoperative anesthesia, postoperative analgesia, nutrition, disinfection, environment temperature, environment humidity, circadian rhythm, and euthanasia); J: statement of potential conflict of interests.

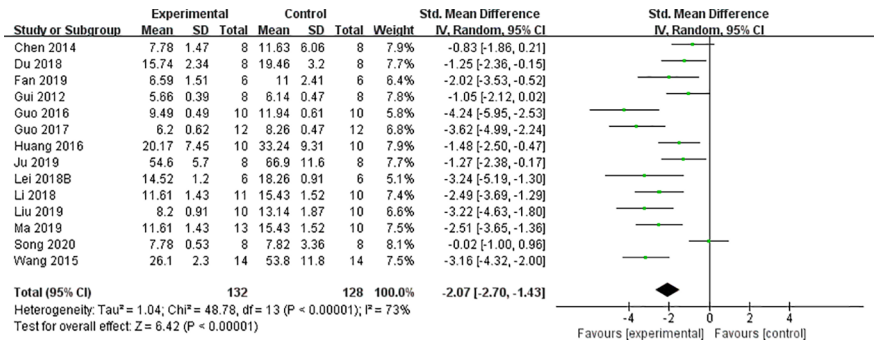

FIGURE 3 | Effect of astragaloside IV on blood urea nitrogen *vs* control.

treatment showed a smaller effect size than the shorter treatment lasting 8 weeks or less with a small drop in the heterogeneity of the two groups (SMD −1.23 *vs.* SMD −2.26, P < 0.05, **Figure 13**; SMD −0.92 *vs.* SMD −2.04, P < 0.05, **Figure 14**).

DISCUSSION

Summary of Evidence

This first-ever preclinical systematic review including 24 studies with 424 animals measured the efficacy of AS-IV for treatment of DN. The quality of included original studies was moderate. Our findings suggest that AS-IV is a multifaceted renoprotective candidate drug for treatment of DN.

Limitations

There are some limitations of this meta-analysis and systematic review: (1) though blinding induction of model was reported in 10 studies, most of the studies had flaws with respect to blinding assessment of outcome and sample size calculation (Moher et al., 2015); (2) selection bias was unavoidable because only eight frequently-used databases were searched for English and Chinese language articles; (3) the absence of negative studies might have led to the true effect

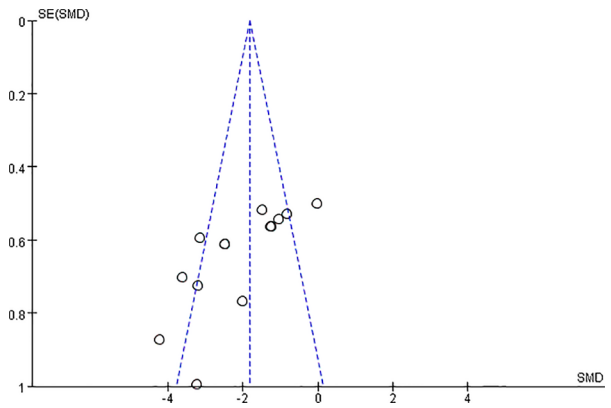

FIGURE 4 | The funnel plot: effect of astragaloside IV on blood urea nitrogen.

of AS-IV being overestimated (Franco et al., 2014); (4) though animal welfare regulations were observed in 14 studies, no study reported disinfection when the experimenter performed intrusive procedures such as intraperitoneal injection, subcutaneous injection, and blood glucose measurement, all of which are regarded as crucial steps in animal models, especially in diabetic models; and (5) no study utilized animals with relevant comorbidities.

Implications

It is commendable that the blinding protocols were reported in ten studies; nevertheless, the average score of the included studies was moderate. According to the ARRIVE guidelines (Kilkenny et al., 2010), lack of crucial standards in study design (Moher et al., 2015) such as sample size estimation and blinded assessment of outcome is the primary defect. In future animal studies, the ARRIVE guidelines should be followed; sample size estimations and blinded assessments of outcome should be emphasized. Specific methods of these two points could be referred to in the following two studies (Hayes and Bennett, 1999; Hooijmans et al., 2014). Use of experimental animals with comorbidities such as advanced age, obesity, hypertension, hyperlipidemia, or other risk factors may be more in line with the physiology of diabetic patients and may be helpful for the clinical translation of experimental results (Brosius et al., 2009).

Different animals display varying sensitivities to STZ. C57BL/6 and CD-1 mice are reliably sensitive to STZ (Like and Rossini, 1976; Rossini et al., 1977) as are Wistar and SD rats. Indeed, rats rather than mice tend to be selected to establish diabetic models because their greater size is conducive to monitoring of renal physiology, access to sufficient renal tissue, and repeated blood sampling for analysis. It is difficult but necessary to strictly control the dosage of STZ to avoid problems of high mortality and low modeling rate due to the high sensitivity of rats to STZ. The subgroup analysis indicated that the group in which DN

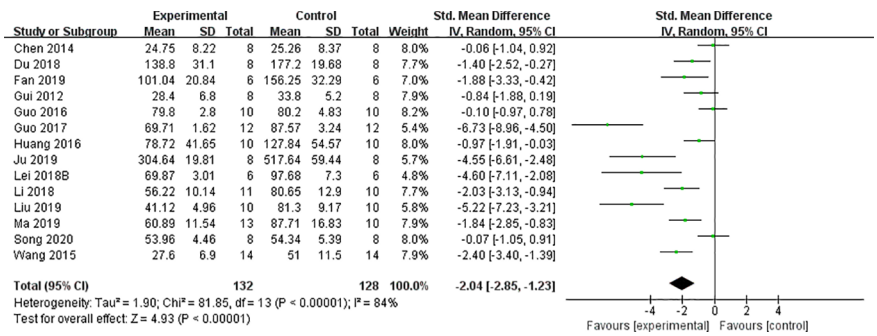

FIGURE 5 | Effect of astragaloside IV on serum creatinine *vs* control.

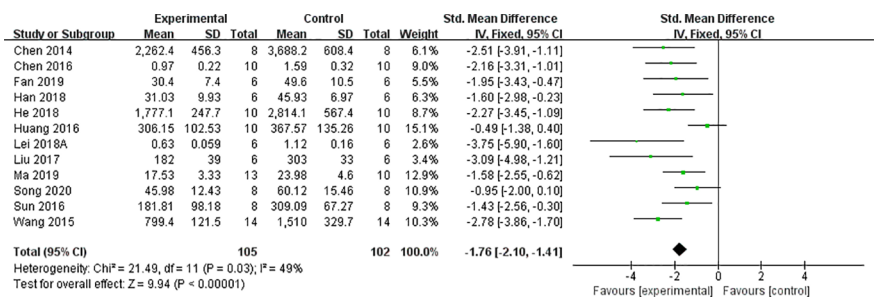

FIGURE 6 | Effect of astragaloside IV on 24-h urinary albumin *vs* control.

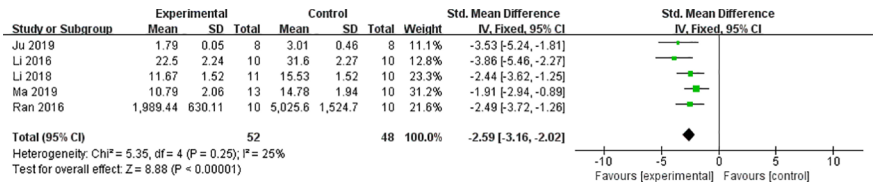

FIGURE 7 | Effect of astragaloside IV on 24-h urinary microalbumin *vs* control.

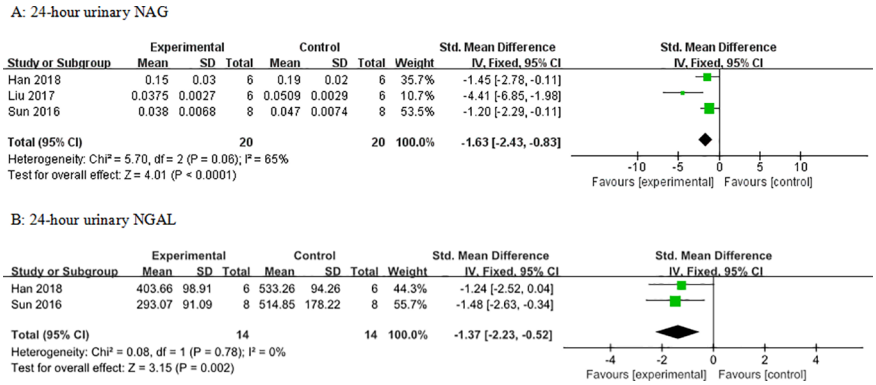

FIGURE 8 | Effect of astragaloside IV on **(A)** 24-h urinary N-acetyl-β-D-glucosaminidase (NAG) and **(B)** neutrophil gelatinase-associated lipocalin (NGAL) *vs* control.

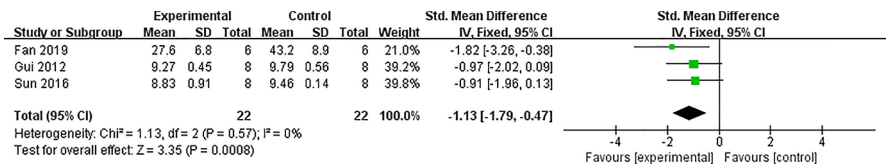

FIGURE 9 | Effect of astragaloside IV on HbA1c *vs* control.

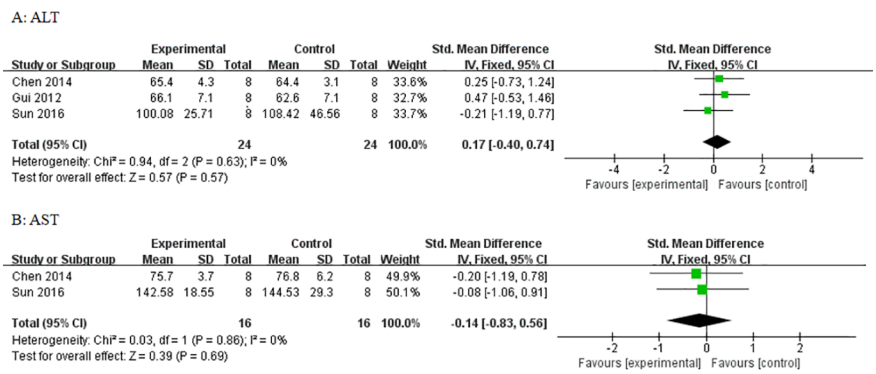

FIGURE 10 | Effect of astragaloside IV on **(A)** alanine aminotransferase (ALT) and **(B)** aspartate aminotransferase (AST) *vs* control.

FIGURE 11 | Effect of astragaloside IV on mortality of rats *vs* control.

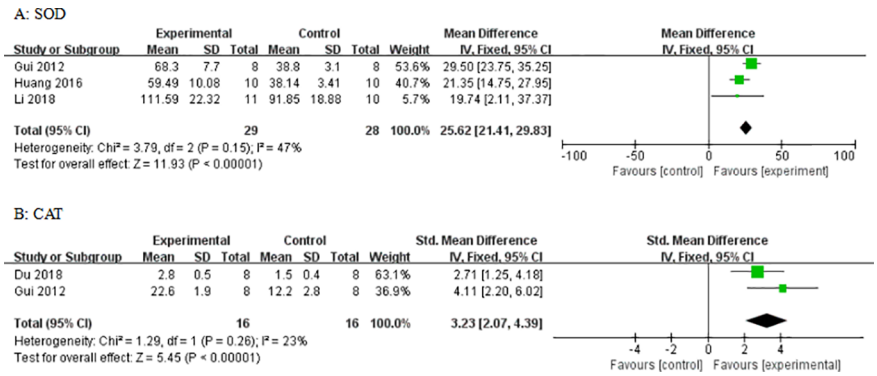

FIGURE 12 | Effect of astragaloside IV on **(A)** superoxide dismutase (SOD) and **(B)** catalase (CAT) *vs* control.

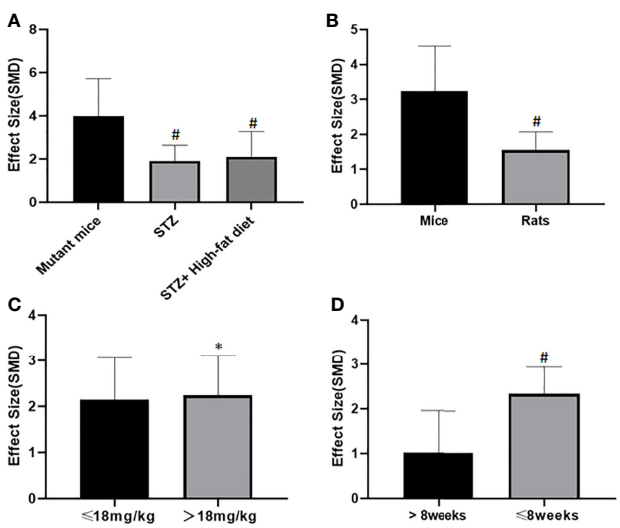

FIGURE 13 | Effect of astragaloside IV on blood urea nitrogen in subgroups. **(A)** Induction type; **(B)** Species; **(C)** AS-IV dose; **(D)** Duration of treatment. #P < 0.05 *vs* control; *P > 0.05 *vs* control.

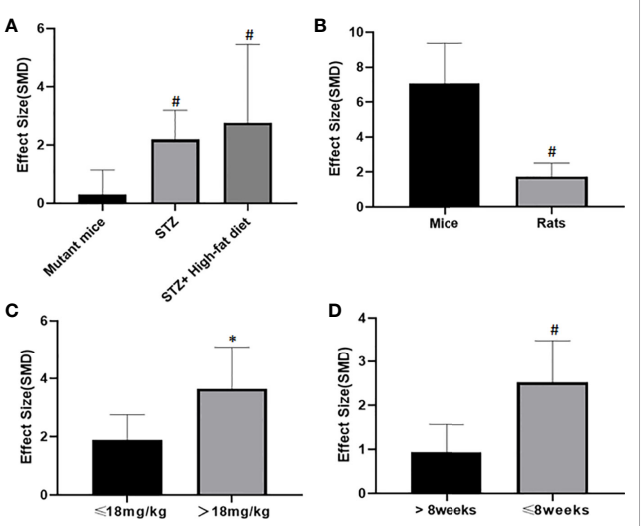

FIGURE 14 | Effect of astragaloside IV on serum creatinine in subgroups. **(A)** Induction type; **(B)** Species; **(C)** AS-IV dose; **(D)** Duration of treatment. #P < 0.05 *vs* control; * P > 0.05 *vs* control.

model was induced in the C57BL/6J mice showed greater effect size than that of rats though the dosage of STZ in the mice group (100 mg/kg, IP, QD for 2 d), much larger than that of the rat group (≤65 mg/kg), suggesting that high doses of STZ may cause less β-cell damage in mice. Although high-proficiency operational skills and high-quality nursing are required, replacing rats with mice less sensitive to STZ may be beneficial to increase the tolerance to STZ and to reduce the mortality of experimental animals. Furthermore, the included studies used three methods to imitate the characteristics of type 1 diabetes or

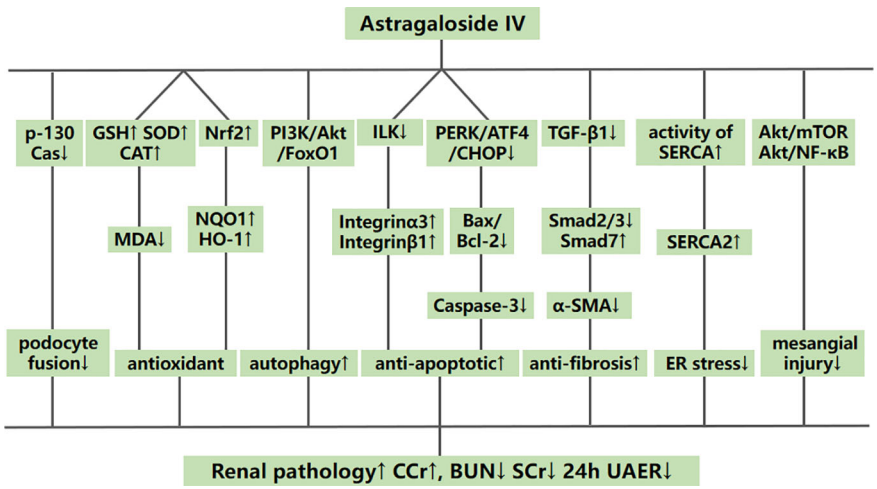

FIGURE 15 | A schematic representation of possible renoprotective mechanisms of astragaloside IV for diabetic nephropathy.

type 2 diabetes to establish DN model according to varying purposes and laboratory conditions: 18 studies established the DN model using STZ; two used low-dose STZ injection combined with high-fat diet; and four studies used mutant or transgenic mice. In our subgroup analysis, the effect size of AS-IV for decreasing BUN and SCr in various models induced by different methods showed significant differences, suggesting that this factor may be the source of the high degree heterogeneity.

Although no difference was seen between the high- and low-dose AS-IV treatment groups, this subgroup analysis was carried out to determine the sources of high heterogeneity rather than its dose–effect relationship given that it is a comparison under different experimental conditions. We perused all included studies that were designed to study the effects of different doses of AS-IV in DN in the same experiments. Of these, two studies reported that the levels of BUN were markedly greater in the DN-vehicle group than in the control group, both of which were significantly reduced by AS-IV treatment in a dose-dependent manner (3–12 mg/kg). Six studies reported that varying doses of AS-IV (0.75–80mg/kg) decreased the levels of BUN, though not significantly ($P > 0.05$). Such a large oral dose range yields similar results suggesting that there may be problems in AS-IV absorption or reaction to receptor binding. Actually, previous studies have reported that the high molecular weight and low lipophilicity of AS-IV may limit its passive transport in the intestine which directly results in low permeability and low bioavailability of AS-IV (the absolute bioavailability in rat: 2.2% only) (Gu et al., 2004; Huang et al., 2006). Up to now, various strategies have also been studied to solve this problem. Absorption enhancers of AS-IV that can open the tight junction are one of the major directions to explore the absorption enhance strategy of AS-IV (Gu et al., 2004). Special solvent such as self-microemulsifying drug delivery system was explored to enhance absorption of AS-IV (Zhang et al., 2019). In addition, the development and application of astragaloside injection may effectively solve the intestinal absorption problem of AS-IV (Chen et al., 2019). In the present study, it still stays at the stage to explore whether the drug is effective, and its mechanisms and the results show that AS-IV has renoprotective functions in DN. The relationship between dose and efficacy remains uncertain probably due to low bioavailability of AS-IV at present. Thus, we suggested that the methods of appeal to promote the bioavailability of AS-IV should be applied more in future experiments to explore the optimal dose range of AS-IV in DN. Furthermore, the longer period of AS-IV treatment suggested poorer efficacy than the shorter treatment lasting 8 weeks or less, suggesting that the duration of treatment may be a source of the high degree of heterogeneity. We attribute this to the fact that DN is a progressive and irreversible disease, and extended treatment time of AS-IV merely delayed the progression of DN rather than reversing it.

Systematic reviews of preclinical studies are thought to be valuable tools to determine mechanisms and to provide important insights into the designed animal studies (Van et al., 2013). The possible mechanisms of AS-IV that mediated kidney protection in the included studies are summed up as follows: (1) alleviating renal fibrosis by inhibiting the expression of TGF-β1 in renal tissues, further decreasing Smad2/3 and enhancing Smad7 levels to reduce the expression of α-SMA; (2) antioxidant action by increasing GSH, SOD, and CAT to reduce the release of MDA and enhancing Nrf2 to upregulate the expression of NQO1 and HO-1; (3) inhibiting apoptosis by downregulating the PERK-ATF4-CHOP pathway, Bax/Bcl-2 and the expression of caspase-3; in addition, inhibiting apoptosis in podocytes specifically by downregulating ILK and by upregulating integrins α3 and β1; by enhancing TUG1 and miR-378 and downregulating the expression of TRAF5; and by up-regulating APN; (4) reducing the proliferation of mesangial cells by regulating the Akt/mTOR pathways; (5) alleviating endoplasmic reticulum stress by enhancing the activity of SERCA and the expression of SERCA2, and reducing the activation of the PERK/eIF2α and IRE1/JNK pathways; (6) reducing the damage to mesangial cells by downregulating the Akt/NF-κB pathway; (7) inhibiting mitophagy by downregulating the PINK1/Parkin pathway; (8)

inhibiting mitochondrial fission by reducing the expression of renal dynamin-related protein 1 (Drp-1), mitochondrial fission protein 1 (Fis-1), and mitochondrial fission factor (MFF); (9) inhibiting fusion of podocyte processes by downregulating p-130Cas; and (10) increasing autophagic activity of renal tissue cells by downregulating the PI3K/Akt/FoxO1 pathway (**Figure 15**).

CONCLUSION

Preclinical *in vivo* evidence suggests that AS-IV has renoprotective functions in DN, probably *via* antifibrotic, antioxidant, and antiapoptotic actions, resulting in alleviation of endoplasmic reticulum stress, inhibition of mitochondrial fission, and increased autophagic activity. Taken together, the findings suggest that AS-IV is a renoprotective candidate drug for treatment of DN.

AUTHOR CONTRIBUTIONS

HW, ZZ, and Y-YH contributed equally to this work. QZ, HW, and Y-LW designed the study; HW, YJ, X-JL, and Z-ZZ collected the data; HW, Y-YH, and ZZ performed all analyses. All authors contributed to the article and approved the submitted version.

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REVIEW
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Involvement of TGF-β and Autophagy Pathways in Pathogenesis of Diabetes: A Comprehensive Review on Biological and Pharmacological Insights

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Despite recent advancements in clinical drugs, diabetes treatment still needs further progress. As such, ongoing research has attempted to determine the precise molecular mechanisms of the disorder. Specifically, evidence supports that several signaling pathways play pivotal roles in the development of diabetes. However, the exact molecular mechanisms of diabetes still need to be explored. This study examines exciting new hallmarks for the strict involvement of autophagy and TGF-β signaling pathways in the pathogenesis of diabetes and the design of novel therapeutic strategies. Dysregulated autophagy in pancreatic β cells due to hyperglycemia, oxidative stress, and inflammation is associated with diabetes and accompanied by dysregulated autophagy in insulin target tissues and the progression of diabetic complications. Consequently, several therapeutic agents such as adiponectin, ezetimibe, GABA tea, geniposide, liraglutide, guava extract, and vitamin D were shown to inhibit diabetes and its complications through modulation of the autophagy pathway. Another pathway, TGF-β signaling pathway, appears to play a part in the progression of diabetes, insulin resistance, and autoimmunity in both type 1 and 2 diabetes and complications in diabetes. Subsequently, drugs that target TGF-β signaling, especially naturally derived ones such as resveratrol, puerarin, curcumin, hesperidin, and silymarin, as well as Propolis, *Lycopus lucidus*, and *Momordica charantia* extracts, may become promising alternatives to current drugs in diabetes treatment. This review provides keen insights into novel therapeutic strategies for the medical care of diabetes.

Keywords: diabetes, autophagy, TGF-β, natural agents, systematic review

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HIGHLIGHTS

- The involvement of autophagy in the development of diabetes is corroborated by affecting the physiology and role of pancreatic β cells and the homeostasis of glucose.
- The prominent role of autophagy signaling pathway was supported by the alteration of autophagy markers in patients and animal models of T1DM, T2DM, and gestational diabetes.
- Several anti-diabetic strategies including adiponectin, ezetimibe, liraglutide, taurine, adipose tissue-derived stem cells (ADSCs) and even exercise as well as natural products such GABA tea, geniposide, guava extract, vitamin D have been shown to target autophagy.
- TGF-β, especially TGF-β1 as an uppermost isoform of TGF-β superfamily, may play a very essential role in the development of insulin resistance and obesity and finally, diabetes.
- Anti-diabetic drugs such as metformin and rosiglitazone have been reported to act *via* modulation of the TGF-β signaling pathway.
- Natural agents including compounds as resveratrol, puerarin, curcumin, hesperidin and silymarin, and extracts of propolis, *Lycopus lucidus*, and *Momordica charantia* have been shown to combat diabetes *via* modulation of TGF-β signaling pathway.

INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent metabolic diseases worldwide. It is characterized by hyperglycemia and defective production and/or secretion of insulin and complications in the heart, kidney, and neural system leading to death, which have drawn notable attention to the management of diabetes. Among 451 million patients with diabetes, 5 million deaths were considered in the 20–99 years age range in 2017 (Cho et al., 2018). Despite recent achievements in the treatment of diabetes, it is important to continue deducing the molecular mechanisms of diabetes pathogenesis and shed light on new horizons for the complete treatment. Among several molecular mechanisms, autophagy and transforming growth factor–β (TGF-β) signaling pathways may play a causal role in the induction and progression of diabetes.

The involvement of dysregulated autophagy and TGF-β signaling pathways in the pathogenesis of diabetes and arising complications including cardiomyopathy, retinopathy, and nephropathy, has been reported in several studies (Jung et al., 2008; Levine and Kroemer, 2008; Al-Mulla et al., 2011; Bartolomé et al., 2014). The integrity of the autophagy pathway is a requisite to normal regulation of cells (Ebato et al., 2008). Autophagy induction is usually known as a protective mechanism to degrade unwanted components and proteins in cells (Klionsky and Emr, 2000). In the absence and/or dysregulation of autophagy, the accumulation of destructed proteins and components leads to deficits in cells. Activation of the TGF-β pathway is commonly associated with cell cycle arrest and induction of apoptosis, whereas altered signaling of TGF-β has been shown to play a substantial role in tumorigenesis (Zhao et al., 2018). However, one of the most conspicuous trends in recent years has been to evaluate the impact of TGF-β in the pathogenesis of other disorders such as diabetes (Wilson et al., 2017; Yadav et al., 2017). In addition, the potential cross-talk between autophagy and TGF-β has received a great deal of attention.

In light of this evidence, it is of interest to investigate studies regarding the involvement of autophagy and TGF-β signaling pathways and their cross-talk in the progression of diabetes to provide an impetus for identifying therapeutic strategies in the management of hyperglycemia and subsequent complications. For this aim, electronic databases including "Scopus," "PubMed," and "Cochrane Library" were searched using the keywords ("Autophagy" OR "mTORC" OR "LC3" OR "ATG" OR "TGFβ (TGF-beta)" OR "SMAD") [all field] AND ("diabetes" OR "hyperglycemia") [title/abstract/keyword]. Data was gathered from the inception date until January 2019. Among them, English language papers were solely included. Two independent investigators evaluated primarily obtained papers. From a total of 6,532 results, 4,336 papers were excluded because of duplication, 1,074 being irrelevant on the title and/or abstract, 920 reports for being reviews, and 16 because of language restriction. Among 281 retrieved papers, 26 were excluded according to their full text, and 95 were excluded because of the involvement of TGF-β and autophagy in diabetes complications including nephropathy, retinopathy, and neuropathy. One-hundred sixty articles were finally included in this systematic review. **Figure 1** discloses a flow chart of the study design.

BIOLOGICAL AND PHARMACOLOGICAL ASPECTS OF AUTOPHAGY SIGNALING

Autophagy is a catch-all, self-eating process in which intracellular components are recruited to the lysosome for degradation. Macroautophagy, microautophagy, and chaperone-mediated autophagy, as the three main classes of autophagy, are involved in the lysosome-associated degradation of cytoplasmic constituents as well as unfolded proteins. Macroautophagy (hereafter known as autophagy) is an evolutionarily conserved mechanism characterized by double-membrane autophagosomes from cytoplasmic components that are fused with the lysosome to form an autophagolysosome for degradation of engulfed components with lysosomal enzymes (Klionsky and Emr, 2000). Consequently, an outstanding role of autophagy in multicellular organelles is the perseverance of homeostasis in metabolic, functional, and structural processes. While autophagy provides a driving force in the regulation of cell viability and function, it is also considered as the second type of programmed cell death in which the accumulation of autophagosomes distinguishes itself from apoptosis (Boya et al., 2005; Tsujimoto and Shimizu, 2005). Recent studies have

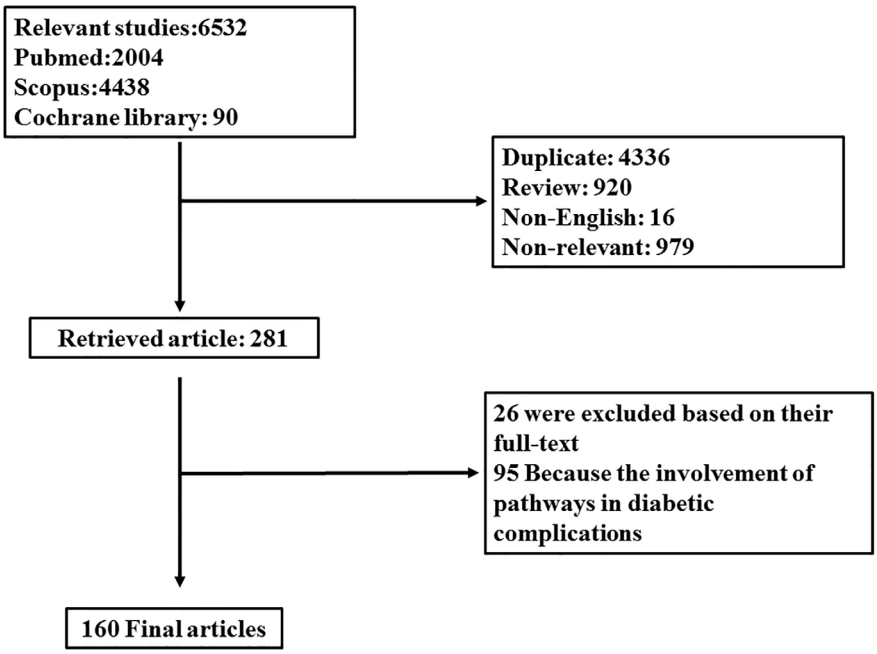

FIGURE 1 | Flow diagram related to selection process of papers.

contributed to a significant understanding of the cellular initiation and activation of autophagy signaling pathway (Hurley and Young, 2017; Yu et al., 2018). Specifically, the detailed molecular pathway behind the cellular function of autophagy and involved factors in mammals have recently been explored by a recent review article (Yu et al., 2018). As outlined in this review article, signaling factors such as ATG (AuTophaGy related proteins) family proteins, Unc-51 like autophagy activating kinase (ULK) complex, phosphatidyl inositol-3 phosphate (PI3P) and its producing complex, Ras-associated binding (Rab), 1A/1B-light chain 3 (LC3), 5′ adenosine monophosphate-activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), sulfiredoxin (SRX), WD-repeat protein interacting with phosphoinositides (WIPI), and soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins implicated in the autophagy pathway (Yu et al., 2018).

The molecular and cellular mechanisms of autophagy are summarized in **Figure 2**. There are accumulating evidence emphasizing on the significance of autophagy's role in the maintenance of cellular homeostasis (Eltschinger and Loewith, 2016). However, in spite of extensive studies over the past several years, the mechanism of autophagy remains only partially understood. It has been proposed that autophagy acts as a protective mechanism to retinue cells from the production of aggregative proteins and activation of inflammatory responses (Levine and Kroemer, 2008). The role of autophagy in metabolism management is supported by activation of the autophagy mechanism in a starvation state through inhibition of mTOR. Additionally, the impact of autophagy on metabolism was examined through the development of $Atg7^{+/-}$ haploinsufficient mice in the normal condition where no disorders were observed. Yet, in crossed $Atg7^{+/-}$ with ob/ob mice (deficiency of leptin), there were increased diabetogenic symptoms relative to ob/ob mice (Lim et al., 2014). From literature, dysfunctional autophagy leads to depreciated fat mass, increased degradation of hepatic lipid, and plunges in lipid levels in hepatocytes through activation of fibroblast growth factor 21 (FGF21), eventually leading to insulin resistance (Shibata et al., 2009; Singh et al., 2009; Zhang et al., 2009; Kim et al., 2013). The primary role of autophagy in the pathogenesis of diabetes is further corroborated by several studies on the physiology and function of pancreatic β cells (Kaniuk et al., 2007; Ebato et al., 2008).

Role of Autophagy Signaling in Diabetes

Under physiological conditions, autophagy may be upregulated as a cellular defense mechanism. As mitochondria play a pivotal role in cellular endogenous ROS production and insulin biosynthesis and secretion, regulation of mitochondrial quality and quantity control through the selective engulfment of excessive or damaged mitochondria *via* autophagosomes promotes β cell health and is of paramount importance in preventing the progression of diabetes. In this line, an enhanced increase in mitochondrial oxidative stress and HbA1C levels resulted in mild hyperglycemia in patients with prediabetes, newly diagnosed type 2 diabetes mellitus (NDT2DM) and advanced duration of type 2 diabetes mellitus (ADT2DM). This increasing glycemic burden enhanced mitochondrial dysfunction in patients with T2DM. With the increased levels of oxidative stress, the aggregation of dysfunctional mitochondria was occurred in cells due to

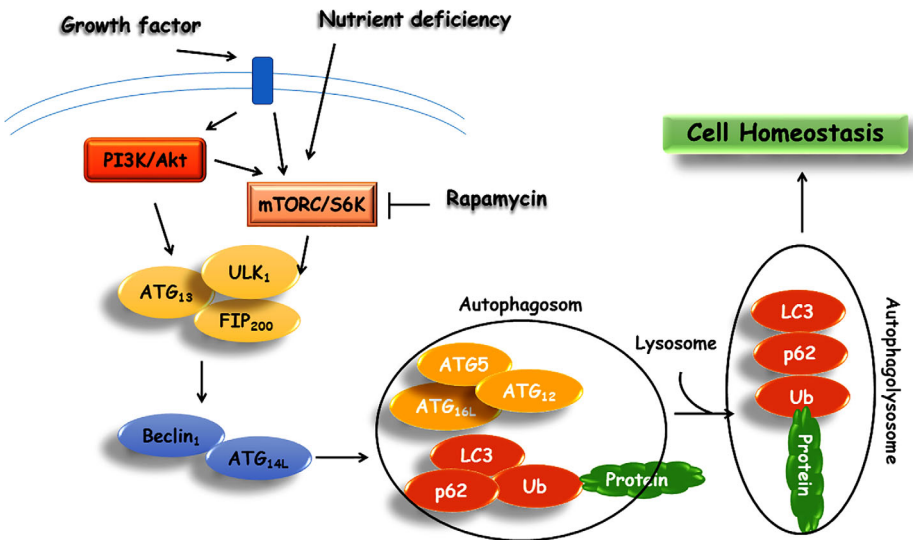

FIGURE 2 | Schematic representation of autophagy signaling pathway.

autophagy dysregulation (Scherz-Shouval and Elazar, 2007), resulted in increased insulin resistance, and acceleration T2DM disease progression (Petersen et al., 2003).

Examination of the classical autophagy markers, LC3II and LAMP2, revealed a remarkable rise in LC3-II and LAMP-2 mRNA expression in subjects with prediabetes (Rovira-Llopis et al., 2015), while patients with NDT2DM and ADT2DM showed the significant reduction in both LC3II and LAMP2 mRNA and protein expression. Mitochondrial autophagy (mitophagy) by clearing cells of damaged mitochondria *via* autophagosomes contributes to the improvement of prediabetic and diabetic symptoms. Mitophagy is regulated by several numbers of factors such as PTEN induced putative kinase 1 (PINK1), PARKIN, microtubule-associated protein light chain 3 (LC3), and lysosome-associated membrane protein2 (LAMP-2) and mitophagy receptors NIP3 like protein X (NIX) and mitofusin2 (MFN2) (Ding and Yin, 2012). Prediabetic subjects have been shown to possess an increased level of mitophagy biomarkers and mitochondria as compared to T2DM patients. It has also been demonstrated that the expression levels of Mitofusin-2 (MFN2), NIX, PINK1, and PARKIN were augmented in prediabetes in comparison with healthy ones. On a protein level, though NIX and PINK1 levels were comparable to the controls, MFN2 showed a significantly increased expression.

Among T2DM patients, a comparison between ADT2DM and NDT2DM showed a significantly decreased level of LAMP2 in patients with ADT2DM in comparison with NDT2DM, indicating the suppression of mitophagy induced by oxidative stress, resulting in further deterioration of survival. Moreover, a recent study by Møller et al. (2017) reported a decreased level of LC3II in muscle cells from T2DM patients as compared to the control subjects. Attenuation of LAMP-2 expression results in reduced autophagy, leading to β cell dysfunction and insulin resistance (Liu et al., 2013; Bhansali et al., 2017). In patients with NDT2DM and ADT2DM, researchers have been found a significantly reduced mRNA and protein expression of MFN2, NIX, PINK1, and PARKIN. In this line, oxidative stress-mediated by moderate to severe hyperglycemia was shown to be associated with a decreased level of these genes leading to impairment of mitophagy. Specifically, augmented oxidative stress in T2DM patients leads to impairment in PINK1 and PARKIN-mediated mitophagy, as delineated by the reduced content of LC3II and LAMP2 proteins, resulting in aggregation of disturbed mitochondria.

Binding of insulin to the insulin receptor (IR) is associated with phosphorylation of downstream targets including IRS-1 and IRS-2 (Withers and White, 2000) and subsequent activation of phosphatidylinositol 3-kinase (PI3K) signaling pathway, which is associated with activation of several processes such proliferation, cell growth, and glucose uptake. Suppression of insulin signaling pathway by ER stress is resulted in phosphorylation of IRS-1/2 by JNK and insulin resistance (Özcan et al., 2004). Preventing IR processing or reducing its expression has also been shown to cause insulin resistance and diabetes in humans (Yoshimasa et al., 1988; Formisano et al., 1993; Iwanishi et al., 1993). In 3T3-L1 adipocytes, it has been observed that ER stress through induction of autophagy results in down-regulation of the IR protein level (Özcan et al., 2004; Nakatani et al., 2005). This reduction in IR levels is accompanied by a decrease in IR downstream signaling and inducing insulin resistance in 3T3-L1 adipocytes. Specifically, the autophagy inhibitor 3-methyladenine (3-MA) utilization in 3T3L1 adipocytes has been shown to reveal the function of autophagy in triggering ER stress-induced IR degradation (Zhou et al., 2009). In addition, in 3T3-L1 adipocytes, glucose uptake under insulin stimulation is suppressed by ER stress suggesting that a reduced number of IR medicated by ER stress seems to play a causal role

in insulin sensitivity (Zhou et al., 2009). However, normal glucose tolerance in IR (+/-) heterozygous knockout mice (Joshi et al., 1996), indicating that less IR may still exhibit expression in the range of normal. This is further complicated by research showing that inhibition of autophagy to ameliorate ER stress was not successful in 3T3L1 adipocytes; proposing that under stressful ER conditions, IR has been disturbed. Although, *in vitro* models of obesity, it has been demonstrated that chemical chaperones and/or overexpression of ER chaperone ORP150 may potentially alleviate insulin signaling and insulin sensitivity (Ozawa et al., 2005; Özcan et al., 2006).

Studies have documented that autophagy may act as an early event in experimental diabetes. streptozotocin (STZ)-induced diabetes leads to activation of VMP1-mediated autophagy in pancreatic β cells after 3 h administration (Grasso et al., 2009). The early detection of VMP1 and autophagic signals in STZ-treated rats and cells indicates that increased autophagy expression and related signals in β cells may play an important role as the recognized biomarkers of diabetes development. However, this supposition requires further evidence with future experiments (Grasso et al., 2009). Further validity was obtained through studies examining specific autophagic factors in mutagen and/or knockout animal models, namely, the Atg7-knockout mice ($Atg7^{\Delta\beta cell}$) model (Jung et al., 2008; Fujitani et al., 2009). In one study, Ebato and colleagues clarified that the altered expression of autophagy factors, namely autophagy-related gene 7 (Atg7) knockout in mice fed a high-fat regimen, plays the causal regulatory role in maintaining the normal structure and function of β cells, leading to destruction of β cells and eventual insulin resistance (Ebato et al., 2008). In this line, dysregulated autophagy seems to play an important role in the pathogenesis of both T1DM and T2DM as well as their arising complications (Fierabracci, 2014). It is proposed that in β cells lacking autophagy, autoantigens are embarked on major histocompatibility complex (MHC) class I and are consequently recognized by T cell receptors on $CD8^+$ T cells. This results in T cell activation and β cell destruction, where β and T lymphocytes are recruited to these inflammatory sites, such as pancreatic islets, with the production of autoantibodies (Lam-Tse et al., 2002). Development of the autoimmunity process leads to further activation of β and T cells, promoting huge lysis of β cells and insulitis. As a key to illuminate the crucial role of autophagy in diabetes progression, immunoblotting analysis unraveled the altered expression of some autophagic signals including light chain IIIB (LCIIIB), Beclin I, ATG12, and p62 proteins in non-obese diabetic (NOD) mice (Fierabracci, 2014).

Insulin resistance as a feature of T2DM is commonly associated with a progressive decrease in the β cell function and the emergence of hyperglycemia (Fujimoto, 2000; Kahn, 2000), mediating oxidative stress that hinders cell-reparative process like autophagy downstream. On the other hand, to maintain the β cell function and survival as well as insulin sensitivity at target sites, the involvement of autophagy seems to be crucial (Marchetti and Masini, 2009; Gonzalez et al., 2011; Kruse et al., 2015). It has been reported that decreased number of autophagosomes in β cells from ob/ob mice, implying the inhibition of autophagic degradation in insulin resistance (Abe et al., 2013). Furthermore, aggregation of polyubiquitinated proteins due to increased oxidative stress in the β cells of Zucker diabetic rats was shown to be mediated by autophagic dysfunction (Kaniuk et al., 2007). Moreover, a reduced level of IL-10 mRNA level has been reported in PBMCs from T2DM patients. Several studies have demonstrated that mTOR activation has a direct relationship with the expression of IL-1β and TNF-α but has a negative relationship with IL-10 through the IKKβ (Laplante and Sabatini, 2012; Shigihara et al., 2014). Notably, evaluation of the link between inflammation and autophagy revealed that autophagy play the main role in regulating inflammation (Ma and Blenis, 2009; Menzies et al., 2011) in macrophages (Kaushik and Cuervo, 2012), keratinocytes (Menon et al., 2014), hypothalamus (Hardie, 2003), adipocytes (Rivera et al., 2014), and peripheral blood mononuclear cells (PBMCs) (Lempiäinen and Halazonetis, 2009). In PBMCs of T2DM and non-diabetic (ND) subjects, mRNA expression of BECN1, LAMP2, and LC3B decreased as the protein level of p62/SQSTM1 was increased (Inoki et al., 2003), meaning that the autophagic process decreased. The decline in protein levels of LC3B-II is accompanied by the rise in TNF-α and IL-1β expressions, while the increase in the protein level of p62 parallels the reduction in them. Thus, It seems that decreased autophagy in PBMCs of T2DM may be due to hyperglycemia (Egan et al., 2011; Guillén and Benito, 2018). However, the inhibitory effect of mTOR on autophagy pathway may be another motive (Sancak et al., 2007; García-Aguilar et al., 2016) as its activation attenuates the Beclin 1 gene expression in PBMCs. AMPK also plays the premier role in the regulation of autophagy (García-Aguilar et al., 2016), albeit with inconspicuous involvement relative to mTOR (Guillén and Benito, 2018).

It is a fact that T2DM could be characterized by chronic inflammation in which leukocytes are impaired in function (Hernandez-Mijares et al., 2013; Rovira-Llopis et al., 2014). Hyperglycemia-induced oxidative stress and ER stress are well-known mechanisms involved in the progression of T2DM (Gonzalez et al., 2011; Scherz-Shouval and Elazar, 2011). A large cohort study indicated that autophagy is enhanced in the leukocytes of T2DM patients. It has been shown that regulation of autophagy in diabetes is different depending on cell types, namely, autophagy is activated in the pancreatic β cells and inhibited in the liver of T2DM mice. In the leukocyte of T2DM patients, an increase in autophagy marker, Beclin 1, is in accordance with the increase in intracellular ROS level (Rovira-Llopis et al., 2015). Although autophagy should control this ROS accumulation by removal of damaged mitochondria, leukocytes from T2DM patients indicate that autophagy activation is not sufficient to reduce ROS production (Hubbard et al., 2010). In addition to oxidative stress, ER stress upregulates autophagy *via* inhibition of the AKT/TSC/mTOR pathway by mediating LC3 lipidation to LC3-II in conjunction with the activity of PERK/eIF2a phosphorylation and by transcriptional activation of autophagy-related genes (Gonzalez et al., 2011).

In several *in vitro* and *in vivo* studies to evaluate the role of an amyloidogenic protein in β cells, human islet amyloid polypeptide (hIAPP) was shown to play the causal role in the pathogenesis of T2DM. Furthermore, a reciprocal interplay was found between the clearance of hIAPP and the activation of autophagy, where autophagy deregulation led to failed clearance of hIAPP and subsequently aggregated hIAPP is associated with decreased activity of the autophagy system (Kahn et al., 1999; Morita et al., 2011; Rivera et al., 2011; Shigihara et al., 2014). Neuronal dysfunction and cognitive impairment in dementia have also been shown to have a strong association with β cell failure, such that AD is known as the type 3 diabetes in literature (Ott et al., 1996; Steen et al., 2005; Moroz et al., 2008; Chen and Zhong, 2013). The role of autophagy in the development of memory dysfunction was scrutinized in STZ-induced diabetic rats, where expression of $A\beta_{1\text{-}42}$ and autophagic markers, LCII and beclin 1, was increased in the hippocampus. Adversely, the reduced expression of lysosome factors, such as LAMP1 and LAMP2, illustrated the involvement of lysosome dysfunction in the neurodegenerative impact of diabetes. These results endorse the impact of autophagy and lysosome deregulation in neuronal loss in diabetic conditions (Ma et al., 2017). As another example, it was shown that high fat-fed, STZ-induced diabetic mice are vulnerable to cognitive dysfunction through altered expression of autophagy signaling factors including LCII/I, p62, and beclin1 (Guan et al., 2016) (**Figure 3**).

Drugs Targeting Autophagy Signaling for Therapeutic Benefits in Diabetes

Adiponectin

Adiponectin is a metabolic hormone secreted by adipose tissue. It has been shown that adiponectin acts as a diabetes regulating hormone (Cheng et al., 2014) that activates the autophagy pathway in insulin target cells and decreases in metabolic diseases. Adiponectin mediates direct metabolic effects and improves insulin sensitivity by the autophagic cellular mechanism (Jahng et al., 2015; Xu and Sweeney, 2015). For example, the mechanism of adiponectin demonstrated the involvement of the AMPK signaling pathway in improving insulin sensitivity and glucose tolerance in db/db mice or mice fed a high-fat diet (Okada-Iwabu et al., 2013). Moreover, insulin resistance in L6 skeletal muscle cells (Huang et al., 2002b) revealed that the increase in expression of a GRP78 promoter-dependent fluorescent reporter and IRE1 phosphorylation (pIRE1), pPERK, and ATF6 is induced under treatment of high insulin/glucose (HIHG) and is paralleled by ER-stress induction (Ahlstrom et al., 2017), while adiponectin treatment alleviated ER stress in an autophagy-dependent manner *via* activation of AMPK signaling factor.

Ezetimibe

Mounting evidence indicates a direct relationship between metabolic disorders, such as obesity, liver diseases and insulin resistance (Clark, 2006; Byrne, 2012). Owing to the correlation between abnormal cholesterol metabolism and the development of metabolic diseases, it appears that the same drugs could be beneficial for both types of diseases. Ezetimibe treatment is a good example of both decreasing intestinal cholesterol incorporation by blocking Niemann-Pick C1-like (NPC1L1) protein (Altmann et al., 2004; Garcia-Calvo et al., 2005) and as described in previous studies, improving glycemic control, leading to an increase in bioactive GLP-1 and pancreatic β cell mass in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Yang et al., 2011). Furthermore, the expression of ATG5, ATG6, and ATG7 in the liver was considerably increased in ezetimibe-treated OLETF rats (Chang et al., 2015), demonstrating the efficacy of ezetimibe treatment in

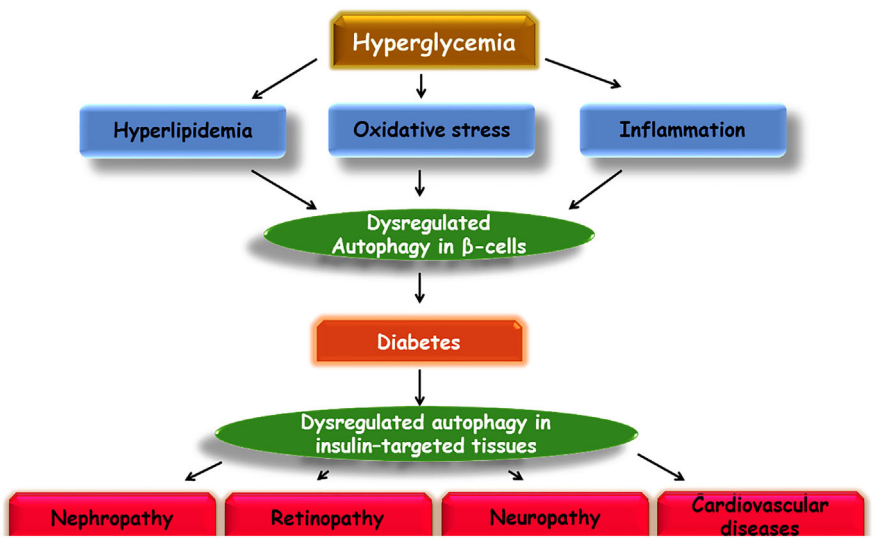

FIGURE 3 | Schematic illustration of a proposed model of autophagy involvement in diabetes and its complications.

improving impaired autophagy process and increased ER stress in insulin resistance disease.

Liraglutide

Liraglutide is a synthetic peptide with 97% sequence homology to native human GLP-1 and is a promising anti-diabetic dug (Drucker and Nauck, 2006; Lovshin and Drucker, 2009). Injection of liraglutide, which can cross the blood-brain barrier, may prove as a neuroprotective agent in animal models with neurologic disorders, such as cerebral ischemia (Zhu et al., 2016), traumatic brain injury (Li Y. et al., 2015), stroke (Sato et al., 2013), and Alzheimer's complications (Han et al., 2013). As a result, liraglutide may attenuate DM-induced cognitive decline in mice.

Cognitive impairments, DM-induced hippocampal neuronal injuries, and synaptic ultrastructure degradation were shown to decrease in STZ-induced diabetic mice under liraglutide treatment, which recruits the AMPK/mTOR pathway to elevate the autophagic process (Qi et al., 2016). Additionally, improvements in lesions in neuronal morphology and density in the hippocampal CA1 region have also been observed after chronic liraglutide administration. It has been suggested that liraglutide did not have protective effects in STZ-induced diabetic mice. Similarly, earlier data from preclinical and clinical studies also indicated that GLP1 and its analogues had no significant improvement in the glucose levels or body weight (Zhao et al., 2013; Frandsen et al., 2015; Dejgaard et al., 2016; Dietrich et al., 2016; Hernández et al., 2016; Zanotto et al., 2017).

Taurine

Taurine is a free amino acid and natural compound that shows promising results for improving impaired glucose metabolism by enhancing the low levels of PPARγ and mTORC2 expression induced by inorganic arsenic (iAs) in the liver of mice and HepG2 cell line. In fact, taurine administration may ameliorate iAs-induced insulin resistance through activation of PPARγ-mTORC2 signaling and subsequent inhibition of hepatic autophagy. Although autophagy activation contributed to the relief of insulin resistance with treatment by various compounds (Shi et al., 2015; Li et al., 2017), taurine effectiveness in insulin resistance is obtainable through autophagy inhibition.

Natural Products Targeting Autophagy Signaling in Diabetes

GABA Tea

Several mechanisms including decreased γ-aminobutyric acid (GABA) neurotransmission, oxidative stress, and apoptosis have been considered for the pathogenesis of encephalopathy in diabetes. Therefore, several studies have carried out experiments on the effects on diabetic encephalopathy through the administration of GABA tea (Hininger-Favier et al., 2009; Zhao et al., 2011). Reduced GABA uptake (Duarte et al., 2000) and extracellular GABA were reported in hyperglycemia, which attributed to increased neuronal disorders. Consequently, it was observed that the administration of GABA tea with enriching GABA neurotransmitters in diabetic animals exerts hypoglycemic and anti-apoptotic effects on rat brain cerebral cortex.

GABA tea also known as Gabaron, developed for the first time in Japan, is a new form of tea in which during a fermentation process, GABA is accumulated in the leaves of tea. The common use of GABA tea is in the amelioration of blood pressure (Omori et al., 1987; Abe et al., 1995). Further examination of the mechanism whereby GABA tea ameliorates the neurodegenerative symptoms of diabetes in the brain of STZ-induced DM rats revealed that blood glucose levels increase in STZ-induced diabetes rats and improvement of hyperglycemia is achieved by deactivation of the cortical Fas ligand, Fas-associated death domain protein (FADD), caspase-8, Bid, and t-Bid levels—all of which increased following 4 weeks of STZ-induced diabetes. Moreover, signaling factors, such as Bax, cytochrome c, activated caspase 9, and activated caspase 3 in the cerebral cortex of STZ-induced diabetes rats, were significantly increased compared with non-diabetic rats. In addition, GABA tea exposure was associated with suppression of the apoptotic pathways mediated by diabetes. Another cellular process affected by GABA tea in STZ-induced diabetes is autophagy as seen by decreases in related protein levels including Beclin 1, ATG7, ATG12, LC3-I, and LC3-II following treatment with GABA tea.

Geniposide

One potential drug for the regulation of abnormal signaling pathways in diabetes is geniposide, an iridoid compound isolated from *Gardenia jasminoides* J.Ellis with anti-inflammatory, anti-angiogenesis, and anti-tumor activities (Lee et al., 1995; Koo et al., 2004; Koo et al., 2006). Furthermore, it is significantly capable of promoting glucose uptake (Guo et al., 2012). When HepG2 cells in IR were treated with 62.5 mg/L geniposide, the decreased levels of glucose were shown in the supernatant in a time-dependent manner. Consequently, it is thought that geniposide promotes autophagy in insulin resistance HepG2 cells, leading to the inhibition of NF-κB signaling factor and reversing the inhibitory impact of NF-κB on the expression of GLUT-4, thereby increasing mRNA and protein expression levels of GLUT-4. As such, geniposide may aid in insulin resistance treatment.

Guava Extract

Guava, *Psidium guajava* L. (Myrtaceae), is a tropical fruit with anti-oxidative, anti-inflammatory, and anti-diabetic attributes (Li P. Y. et al., 2015) because of an increased level of vitamin C, flavonoids, and polyphenolic ingredients (Flores et al., 2013). In particular, the effectiveness of guava extracts in the reduction of ROS, protection against inflammation in the kidney, DM-induced sclerotic injury, and cell arrangement in the pancreas has been observed (Lin and Yin, 2012). Moreover, the impact of guava leaf extracts/trehalose treatment on T2DM was confirmed in some studies (Eidenberger et al., 2013; Lin et al., 2016). Trehalose as a disaccharide found in almost all of the organisms has various therapeutic effects on T2DM (Bartolomé et al., 2010). With this in mind, the effect of trehalose in combination with guava extract was observed as a potent scavenger of intracellular ROS in STZ-induced diabetic mice. Along with T2DM-enhanced renal ROS, three types of

programmed cell death including apoptosis, autophagy, and pyroptosis were significantly diminished using guava juice and trehalose exposure. While autophagy in diabetic patients has mainly been proved to be a protective process against ER-stress, guava juice and trehalose affect DM positively by reducing autophagy leading to cell death (Lin et al., 2016).

Vitamin D

There is the enormous evidence for considering vitamin D deficiency as one of the main causes of T1DM development (Wolden-Kirk et al., 2011; Wranicz and Szostak-Wegierek, 2014; Grant, 2015). The protective role of vitamin D against diabetes has been suggested in several studies (Sørensen et al., 2012; Dong et al., 2013). For example, pre-treatment with $1,25(OH)_2D3$ promoted insulin secretion in STZ-treated β cells, confirming this notion. Examination of the precise underlying mechanisms of vitamin D in STZ-induced T1DM mouse model and mouse insulinoma 6 (MIN6) β cells revealed that vitamin D increases the expression of LC3 and Beclin 1, autophagic signaling factors that may influence the promotion and development of diabetes (Lee, 2014; Ding and Choi, 2015). Furthermore, enhanced expression of Bcl-2 in STZ-treated mouse and MIN6 cells may serve as a good indicator for showing the decrease in the apoptotic rate of pancreatic β cells. Indeed, vitamin D may induce autophagy and suppresses apoptosis, therefore accelerating the regeneration of organelles under hyperglycemic conditions (Wang et al., 2016).

BIOLOGICAL AND PHARMACOLOGICAL ASPECTS OF TGF-BETA SIGNALING

Transforming growth factor-beta (TGF-β) was identified during the early 1980s. Subsequently, TGF-β signaling was elucidated in the middle of the 1990s with the identification of SMAD proteins and TGF-β receptors. Since the first identification of TGF-β, research is in progress to expound the TGF-β signaling pathway and its pathophysiological importance. TGF-β signaling has been studied in detail at the cellular and molecular level by various scientists. Particularly, numerous cross-talks have emerged regarding the relationship of TGF-β with other pathways as well as the role of the TGF-β signaling pathway in the development of various diseases. TGF-β is grouped in a family of peptide growth factors that are involved in the developmental processes, homeostasis of adult tissues, and a wide range of other cellular functions. The impairment in TGF-β signaling has been reported in several diseases including cancer, diabetes, and cardiovascular diseases. The basic components of the TGF-β signaling consist of a receptor complex with membrane-associated receptors type I and type II and SMAD proteins, which play a role in downstream transcriptional factors. Type I and type II TGF-β receptors are similar in composition. The receptors are composed of a cytoplasmic kinase domain, a single transmembrane segment, and an ectodomain that is glycosylated and disulfide-rich. Serine/threonine kinase activity and induced phosphorylation of tyrosine are characteristic features of kinase domains in TGF-β receptors. There are seven different types of type I TGF-β receptors, which are referred to as activin receptor-like kinases (ALKs). Specifically, ALK5 or TGF-βRI is responsible for TGF-β signaling in all types of mammalian cells. For example, ALK5 works alongside ALK1 and ALK2 in endothelial or other various cell types (Yan and Zhang, 2018; Zhang, 2018).

In TGF-β signaling, ligand binding allows the receptor complexes to assemble and activated type II TGF-β receptors may phosphorylate type I receptor's glycine-serine-rich (GS) domain. This leads to the activation of type I receptors. Then, the activated type I receptors phosphorylate the SMADs at their carboxyl terminus, aiding SMADs to enter the nucleus to regulate gene expression. Different SMADs are encoded in the mammalian genomes and possess different features. Examples include SMAD 2/3 for ALK5 and SMAD 1/5/8 for ALK1/2, which act as direct substrates for their cognate receptor kinases. By virtue of this ability, they are also referred to as receptor-specific, or R-SMADs. Further downstream signal transducers, such as small guanosine triphosphatases (GTPases), MAPKs, Janus kinase/signal transducer and activator of transcription (JAK/STAT), and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) are also activated by TGF-β receptors. When activated, these various signal transducer pathways perform specific functions, such as converging to SMADs to influence the output of TGF-β signaling. Thus, TGF-β signaling not only has its specific role in the body but influences other pathways as well; thereby explaining why dysregulation in TGF-β signaling is involved in several diseases. Non-SMAD signaling transducers can also be activated by TGF-β. One of the most important non-SMAD effectors is ubiquitin E3 ligase tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 produces activation of downstream p38 MAPK, c-Jun N-terminal kinase (JNK), and transforming growth factor beta-activated kinase 1 (TAK1) (Yan and Zhang, 2018; Zhang, 2018). As such, non-SMAD pathways may also shed light on the variety of proteins that TGF-β receptors interact with them. This serves as a greater option for cells to influence downstream responses in accordance with pathological and physiological demands (**Figure 4**).

ROLE OF TGF-β SIGNALING IN DIABETES

TGF-β is a cytokine with several numbers of functions inside the body consisting of apoptosis, immune response in several cells, differentiation, cell proliferation, and wound healing. Recent experimental research has pointed out that TGF-β may play a very essential role in the development of insulin resistance and obesity (Beaudoin et al., 2014). There is evidence that TGF-β1 (a predominant isoform of the superfamily of TGF-β) may be involved in the development of diabetes (Flores et al., 2004). Additionally, TGF-β plays an important role in the immune system. It is involved with differentiation, chemotaxis, survival, and lymphocyte proliferation. Regulation of leukocyte function is an important role played by TGF-β, and dysregulation can lead to autoimmune diseases, such as type T1DM. In T1DM, the

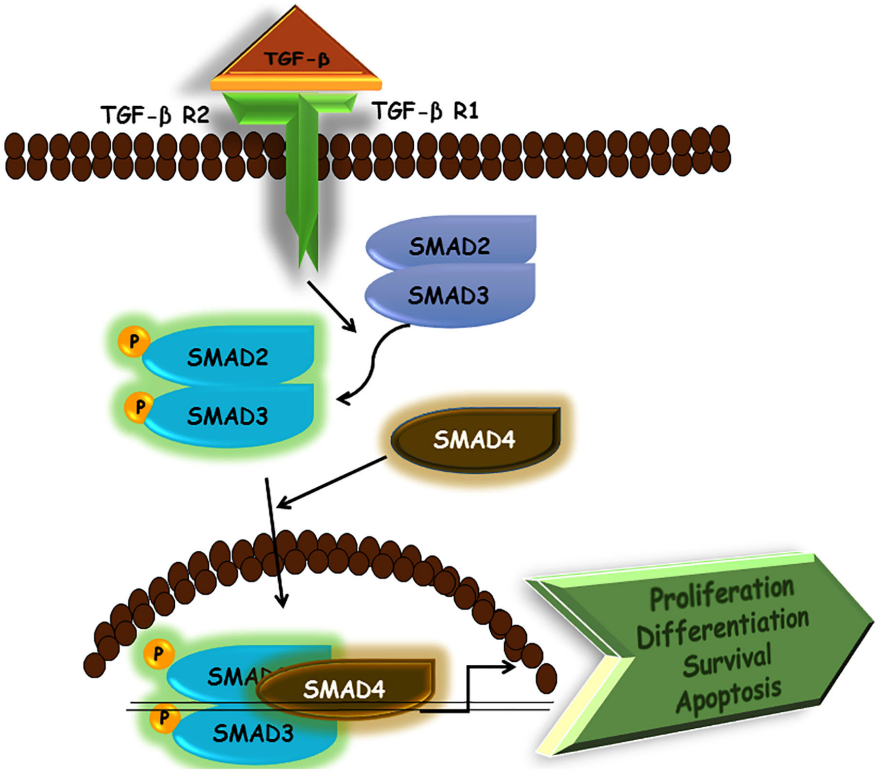

FIGURE 4 | Schematic representation of TGF-β signaling pathway.

destruction of insulin-producing β cells takes place and is mediated by T cells, important targets of TGF-β1. Blocking of TGF-β signaling in mice has led to an autoimmune phenotype, involving activation and differentiation of T cells. In β cells, TGF-β1 is expressed under the influence of insulin promoters and inhibits T1DM from developing (Chen et al., 2008) (**Figure 5**).

A study using human subjects evaluated changes in the levels of TGF-β2 and nerve growth factor in T1DM patients compared with normal subjects and T2DM patients. It was found that levels of TGF-β2 were significantly lower and levels of nerve growth factor were higher in T1DM patients when compared to healthy controls and T2DM patients (Azar et al., 1999). In another study, TGF-β1 levels were evaluated in women with a previous history of gestational diabetes mellitus (GDM) due to increased risk of insulin resistance, obesity and endothelial dysfunction later in life and early development of atherosclerosis. Therefore, this study consisted of women with a prior history of GDM (pGDM), women with T2DM, and the third group of healthy women. The results showed that women with pGDM had significantly higher levels of TGF-β1 than healthy women, but lower levels of TGF-β1 than the T2DM group. The study indicated that age, postprandial glucose levels, and BMI all affected TGF-β1 levels. The elevation in TGF-β1 levels may be a result of the inflammatory response that is produced against insulin resistance and hyperglycemia (Yener et al., 2007). In another study carried out to study oxidation, glycation, and TGF-β1 levels, TGF-β1 levels were measured in children suffering from type I diabetes mellitus and healthy children. It was observed that parameters related to oxidation and glycation were considerably augmented in diabetic children compared to healthy children, such as TGF-β1 levels. Furthermore, correlation existed between the TGF-β1 levels and the age of the children and the duration of type I diabetes mellitus. However, no correlation existed between parameters for oxidation and glycation and levels of TGF-β1 (Jakuš et al., 2012).

Dysregulation of TGF-β pathway is specially associated with progression of various complications associated with DM, such as diabetic neuropathy, and delayed wound healing. Features of diabetic neuropathy include glomerular sclerosis, tubulointerstitial fibrosis, extracellular matrix (ECM) alterations, and mesangial expansion. TGF-β1 is an important regulator of fibrosis associated with diabetic nephropathy as indicated by increased renal TGF-β1 expression in persistent hyperglycemic conditions of human patients and animal models of diabetes. The effects of TGF-β1 are produced as a result of binding to TGF-β1 type II receptors (TβRII) and subsequently, induces activation of TGF-β1 type I receptor (TβRI) kinase. Consequently, this results in phosphorylation and activation of SMAD 2/3. Oligomeric

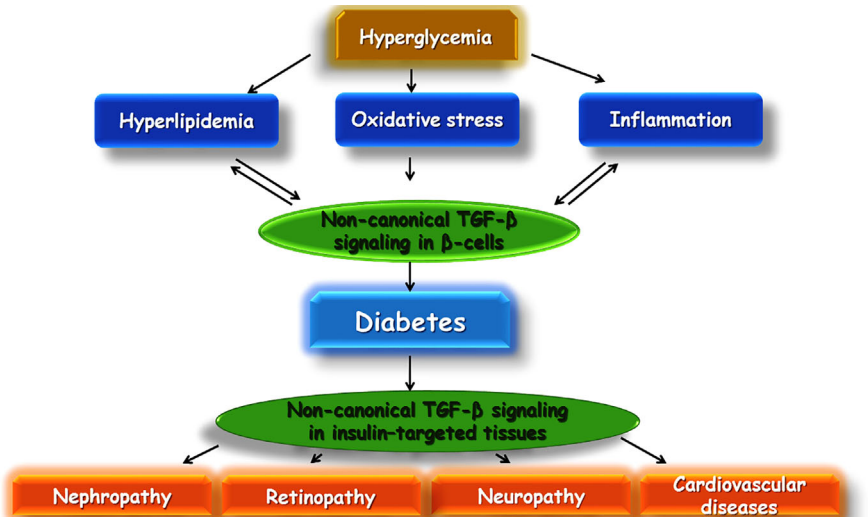

FIGURE 5 | Schematic illustration of a proposed model of TGF-β involvement in diabetes and its complications.

complexes of activated SMAD2/3 are then formed with SMAD4 and translocated to the nucleus, leading to the expression of target genes, such as extracellular matrix (ECM) proteins as well as the production of tubulointerstitial and glomerular fibrosis. Thus, diabetic renal fibrosis may be treated by inhibiting the TGF-β1/SMAD pathway (Wu et al., 2015).

The deregulated vascular system in the eyes and kidneys from T1DM patients has been suggested to be affected by several factors including the duration of diabetes and deregulation of several signaling factors such as vascular endothelial growth factor (VEGF), TGF-β1, and angiogenin (Zorena et al., 2009). Further, TGF-β1 participation in vasculature and wound healing has been proved *via* the promotion of extracellular matrix proteins formation (Maheshwari et al., 2011). The measurement of TGF-β1 serum levels in children and adolescents revealed a positive relationship between the duration of T1DM and complications in the vascular system (Zorena et al., 2013). Augmentation of pro-inflammatory cytokines secreted by the peripheral blood mononuclear cells (PBMCs) is associated with atherosclerotic damages in T2DM (Ebato et al., 2008; Zoncu et al., 2011; Lin et al., 2012) and results in resistance to insulin and abnormality in β cell.

TGF-β serves as an important regulator of wound healing as it is released by platelets at an early stage and plays many roles downstream. For example, TGF-β is involved with chemotaxis of immune and inflammatory cells to the wound site and formation of granulation tissue and deposition of ECM. TGF-β also plays an important role in the end stages of tissue remodeling in wound healing by aiding replacement of collagen type III with collagen type I. TGF-β also plays an important role in wound healing by promoting epithelialization of wound. Thus, therapeutic benefits may be achieved in diabetes by either producing inhibitory or stimulatory effects on the TGF-β pathway depending on what effect is needed (Hozzein et al., 2015). An animal study was conducted to study the defective resolution of inflammation and impaired TGF-β signaling in delayed wound healing in a female rat model of T2DM. This clarified that wound healing was delayed due to elevated tumor necrosis factor (TNF)-α/NF-κB activity and decreased estrogen levels, which leads to decreased TGF-β/SMAD signaling and impaired inflammation resolution. However, PEGylated soluble tumor necrosis factor receptor type 1 (PEG-sTNF-RI) therapy and estrogen treatment produced amelioration in the above-mentioned defects (Al-Mulla et al., 2011).

A study was carried out to measure urinary TGF-β1 levels in patients suffering from DM, due to the role of TGF-β1 in enhancing renal fibrosis in diabetic neuropathy (DN). In a study, urinary levels of TGF-β1 were measured in groups consisting of healthy controls and patients suffering from DN. The levels of TGF-β1 were found to be higher in diabetic patients suffering from diabetic neuropathy compared to those belonging to the normal control group (Tsapenko et al., 2013). Another study on the role of TGF-β in DN utilized subjects that were grouped into those who had a fast development of DN, those with the slow development of DN, and healthy humans serving as the control. In this study, cultured skin fibroblasts of the subjects were evaluated for messenger ribonucleic acid (mRNA) expression levels for latent TGF-β binding protein-1 (LTBP-1), thrombospondin-1, TGF-β type II receptor (TGF-β RII), and TGF-β1. Measurements were collected using real-time RT-PCR. The results concluded that mRNA expression of LTBP-1 was reduced in patients belonging to the slow development of DN compared to both patients with the fast development of DN and control subjects. Additionally, thrombospondin-1, TGF-β RII, and TGF-β1 mRNA expressions were found to be similar in all the groups. Low levels of LTBP-1 may point towards a genetically

determined protective effect against DN. The study also suggests that LTBP-1 may be involved in the development of DN *via* regulation of TGF-β activity (Huang et al., 2002a).

Drugs Targeting TGF-Beta Signaling for Therapeutic Benefits in Diabetes

Drugs that target the TGF-β signaling may be potential candidates for therapeutic benefits in DM. Among them, rosiglitazone is conventionally used as an agonist for the proliferator activated receptor-gamma (PPAR-γ) for the treatment of DM. In a recent study, the effects of rosiglitazone were studied on the TGF-β/SMAD signaling pathway in Zucker diabetic fatty (ZDF) male rats. One group of rats received a chow diet and rosiglitazone treatment while the other group received chow diet without rosiglitazone treatment. The treatments were given for the duration of six weeks and rosiglitazone was administered in a dose of 100 mg/kg. Excision of retroperitoneal white adipose tissues (rpWAT) and subcutaneous white adipose tissues (scWAT) was performed to evaluate protein content/phosphorylation. It was found that in both scWAT and rpWAT, the protein content of mitochondria and glucose tolerance was found to be increased. However, the protein content of fatty acid handling enzymes was only shown to be elevated in the scWAT of animals that received rosiglitazone. Specifically, there was an elevation in the expression of SMAD4, TGF-β receptor I and II, and anchor of SMAD for activation of the receptor. Additionally, administration of rosiglitazone elevated levels of E3 ubiquitin ligase SMURF2 and inhibitory SMAD7 as well as reduced the phosphorylation of SMAD2 and SMAD3. These results indicate that rosiglitazone specifically inhibits signaling produced by SMAD2 and SMAD3 in scWAT. Besides, the SMAD7 and SMURF2 mechanisms induced by rosiglitazone are most likely responsible for decreasing phosphorylation of SMAD2 and SMAD3. A feedback mechanism is formed by activation of the SMAD signaling factors to oppose rosiglitazone induced synthesis of lipid in scWAT (Beaudoin et al., 2014).

Metformin has also been used as an antidiabetic agent for a long time. However, the exact mechanism of metformin is not clear. Yet, certain researchers have found that TGF-β1 may be a target for the action of metformin. A surface plasmon resonance-based assay was used by researchers to explore the effect of metformin on TGF-β1. It was found that metformin showed direct binding with TGF-β1 and inhibited binding of TGF-β1 with its receptor. Binding of TGF-β1 with metformin at the receptor-binding domain of metformin was demonstrated in the molecular dynamic and molecular docking studies. Additionally, metformin suppresses the dimerization of type II TGF-β1 receptor upon binding TGF-β1, which is essential for downstream signal transduction in the TGF-β pathway (Xiao et al., 2016). In a study on diabetic rats, it was shown that vitamin D produced improvement in TGF-β and insulin like growth factor 1 (IGF-1) levels in their intervertebral disc. However, the administration of vitamin D (calcitriol) had a protective effect against degenerative changes produced in the intervertebral disc of diabetic rats and improved IGF-1 and TGF-β levels. Thus, by increasing the levels of TGF-β1 and IGF-1, vitamin D may be helpful in the prevention and treatment of intervertebral disc degeneration in patients suffering from diabetes (An et al., 2017). A study was conducted to observe the effect of undenatured camel whey protein in enhancing wound healing in diabetic mice. Researchers found that whey protein increased the expression of TGF-β, fractalkine (CX3CL1), KC (keratinocyte-derived chemokine), MIP (macrophage inflammatory proteins)-2, and MIP-1α levels in diabetic mice treated with whey protein. Additionally, levels of IL (interleukin)-6, IL-10, TNF-α, and IL-1β were restored to normal levels through whey protein treatment. Thus, the actions of whey protein exerted a beneficial effect on wound closure in diabetic mice (Badr et al., 2012).

Natural Products Targeting TGF-β Signaling in Diabetes

Natural products may be used for targeting TGF-β signaling and inducing therapeutic benefits in diabetes. The advantage of these natural products is that they may be safer alternatives in the treatment of diabetes in comparison to allopathic drugs. By targeting TGF-β signaling, they could play a novel role in the treatment of diabetes in comparison to standard drugs. Some of the natural products that may be used in the targeting of TGF-β signaling for therapeutic benefits in diabetes are as follows:

Resveratrol

Resveratrol (3, 5, 4′-trihydroxystilbene) is a phenolic compound with very important health benefits (Yeung et al., 2019). Resveratrol is found in several plants and is effective in the treatment of a number of age-dependent, metabolic and chronic diseases such as cancer, Alzheimer's, diabetes, inflammation, bacterial and viral infections (Pawar et al., 2017; Moosavi et al., 2018). In a study, resveratrol improved diabetic neuropathy in STZ-induced diabetic rats. Resveratrol exhibited an effect on diabetic neuropathy by inhibiting the TGF-β/SMAD and extracellular signal-regulated kinase (ERK)1/2 signaling. Streptozotocin was administered in a dose of 65 mg/kg body weight. Induction of diabetes was confirmed by diabetic symptoms in rats, such as polyphagia, polydipsia, and fasting blood glucose of ≥300 mg/dL. Resveratrol was administered to animals at a dose of 0.75 mg/kg body weight for 8 weeks and three times per day. Animals were divided into groups in the following manner: normal animals receiving only normal saline, diabetes rats administered resveratrol treatment, and diabetic rats not administered resveratrol treatment. After the test, animals were sacrificed and histology of their kidney was examined by using microscopy. Biochemical and other important parameters were measured to evaluate the effect of resveratrol in diabetic rats. It was shown that glomerular hypertrophy and urinary albumin excretions as well as, expression of collagen-IV, fibronectin, and TGF-β in the glomeruli, were reduced in rats that received resveratrol treatment. Specifically, the thickness of the glomerular basement membrane was reduced to original thickness *via* resveratrol treatments while the expression of nephrin was enhanced to normal levels in diabetic rats administered

resveratrol. In the kidneys of diabetic rats, phosphorylation of SMAD2, SMAD3, and ERK1/2 was shown to be inhibited by resveratrol. Therefore, this study suggests that resveratrol reduces early glomerulosclerosis in diabetic nephropathy through inhibition of ERK1/2 and TGF-β/SMAD. Furthermore, resveratrol reduces podocyte injuries in diabetic rats (Chen et al., 2011).

Lycopus lucidus Turcz. ex Benth.

Lycopus lucidus Turcz. ex Benth. is a medicinal plant used in Chinese herbal medicine. It has a traditional phytomedicine with anti-inflammatory, antioxidant, antimicrobial, anti-allergic, anti-osteoclastogenesis, anti-cancer, and anti-diabetic properties (Shin et al., 2005; Yu et al., 2011; Yao et al., 2013; Lu et al., 2015; Jeong et al., 2019). The aqueous extract of *L. lucidus* was used in a study to improve renal damage in STZ-induced diabetic rats with diabetic nephropathy. Two models were used in this study for determining renal fibrosis: an *in vivo* model in which STZ was used for inducing diabetic nephropathy in rats and an *in vitro* model where renal fibrosis was determined by treating fibroblasts with recombinant TGF-β1 (rhTGF-β1). Results showed that the aqueous extract of *L. lucidus* suppressed the activation of ERK1/2 and SMAD2 by rh-TGF-β1. The aforementioned effect also downregulated the expression of SMAD7, SMAD4, TGF-βRII, and TGF-βRI in SV40MES13 cells without any inhibitory effect on cell viability. In the *in vivo* rat model, *L. lucidus* reduced the serum levels of blood urea nitrogen (BUN) and serum creatinine (SCr) along with activity of superoxide dismutase. In glomerular tissues, *L. lucidus* ameliorated the expansion of the mesangial area. Furthermore, *L. lucidus* reduced mRNA levels of TGF-β1 and phosphorylation of SMAD2. Thus, the above study confirmed that *L. lucidus* may be a potential new candidate in inhibiting renal fibrosis by blocking the signaling pathway of TGF-β. The study also points towards a protective effect of *L. lucidus* in preventing renal damage in STZ-induced diabetic rats by (Yao et al., 2013).

Puerarin

Puerarin is an isoflavonoid found in many plants and a common adjuvant therapy in China in the alleviation of diabetes, cardiac fibrosis, angina pectoris, and cardiovascular diseases (Zhang et al., 2015; Yuan et al., 2016; Jin et al., 2017). In a study, the effect of puerarin on renal damage was observed in STZ-induced diabetic Wistar rats. The animals were grouped into normal control group, untreated diabetes group, two diabetes groups treated with two doses of puerarin at 140 and 200 mg/kg, respectively and a standard drug-treated group. It was found that the diabetic untreated group compared to the normal control group showed increased levels of total cholesterol, triglyceride, blood glucose, IFN (interferon)-γ, IFN-γ/IL-4, malondialdehyde (MDA), and kidney index while the levels of catalase (CAT), superoxide dismutase (SOD), fasting blood insulin (FPI), body weight, IL-4, glutathione peroxidase (GSH-Px), and nitric oxide (NO) were decreased in the untreated diabetic group. Additionally, the untreated diabetic group displayed increased glomerular extracellular matrix (relative area), Scr, urine protein (UP), and BUN in comparison to the control group. Protein and mRNA expressions of SMAD2, TGF-β1, connective tissue growth factor (CTGF), and fibronectin (FN) were measured using western blot analysis and real-time fluorescence quantitative polymerase chain reaction analysis (RT-FQ-PCR). All these parameters were found to increase in diabetic rats without any treatment as compared to the normal control group. In the puerarin treated group, the elevated parameters were decreased and the decreased parameters were increased in comparison to the diabetes untreated group. Also, there was an improvement in the renal functions in the puerarin treated group as compared to the diabetic untreated group. There was also the downregulation of CTGF, FN, SMAD2, and TGF-β1 mRNA and protein expressions. Thus puerarin exerted its antidiabetic action through inhibitory effects on the TGF-β1/SMAD2 pathway (She et al., 2014).

Propolis

Propolis is a natural mixture is found in plants and is produced by bees through the mixing of salivary enzymes and wax with plant material. Mounting evidence has been supported the anti-cancer, anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, immunomodulatory and hepatoprotective properties of propolis (Banskota et al., 2001; Gülçin et al., 2010; Sawicka et al., 2012; Chan et al., 2013). A study was conducted by researchers on the effect of propolis in enhancing the healing of cutaneous wounds in STZ-induced diabetic mice. When untreated diabetic mice were compared with non-diabetic mice, it was shown that a delay in wound closure was found in diabetic mice in comparison to the non-diabetic mice. Additionally, the levels of TGF-β1 were found to be decreased in diabetic mice in comparison to untreated mice. Moreover, the levels of inflammatory cytokines and matrix metalloproteinase 9 (MMP9) were enhanced in the wound tissues in diabetic mice in comparison to the untreated mice. This corresponds with decreased production of collagen and phosphorylation of SMAD2 and SMAD3 in wound tissues of diabetic mice. However, when the propolis treated diabetic group was compared to the untreated diabetic mice, it was found that propolis treatment significantly increased closure of diabetic wounds. Importantly, the levels of TNF-α, IL-6, IL-1β, and MMP9 were found to be at normal levels in propolis treated diabetic mice. The propolis treated group also showed an enhanced formation of collagen by promoting TGF-β1/SMAD2,3 signaling in comparison to the untreated diabetic group (Hozzein et al., 2015).

Momordica charantia L.

Momordica charantia L. (MC) or bitter gourd ointment is a traditional medicine with an ethnobotanical survey in India and the Caribbean commonly used in the management of diabetes (Semenya et al., 2012; Talukdar and Hossain, 2014). For instance, MC was used in a study to evaluate its effect on enhancing the healing of wounds in diabetic Sprague-Dawley rats. Streptozotocin was used to induce diabetes, and the rats were divided into normal control group (non-diabetic), diabetic rats (untreated), diabetic rats treated with MC powder, diabetic rats treated with an ointment containing MC, diabetic rats treated with povidone ointment, and diabetic rats treated with ointment

base. The excision wound model was used in the study. All the treatments were given for 10 days. The healing of the wound was determined by total protein content, TGF-β expression, histological observations, and the rate of closure of the wound. Results showed that the closure of the wound was delayed in the diabetic groups as compared to the control group. In comparison to the untreated diabetic group, MC ointment treated group showed a significantly faster rate of closure of wound. Also on day 10, MC ointment treated group showed the best wound closure rate in comparison with other diabetic groups that were given treatment. A high level of total protein content and intense expression of TGF-β1 were shown by MC ointment treated group. The diabetic wound healing potential of MC ointment in the study was suggested to be due to its ability to enhance the expression of TGF-β (Hussan et al., 2014).

Curcumin

Curcumin is a polyphenol extracted from *Curcuma longa* L. (Turmeric) as a yellow pigment component which used as a hepatoprotective drug in traditional Chinese medicine (Kumar and Sakhya, 2013). It is known to possess multiple therapeutic actions and has the potential to become an important antidiabetic agent. Protection against diabetic neuropathy through curcumin induced by inhibiting collagen IV, fibronectin and TGF-β1. Curcumin has also shown the potential to reduce diabetic cardiomyopathy. Fibrosis in tissues of heart was found to be decreased by administration of curcumin in a dose of 300 mg/kg/day for 16 weeks in STZ-induced diabetic rats. Also in a clinical study, diabetic patients received 66.3 mg of curcumin per day for 2 months. No changes were made in the medications that they were taking. Results of the trials showed that curcumin did not produce any changes in the lipid or glucose profile, but levels of urinary IL-8 and TGF-β were found to be reduced. TGF-β and IL-8 are involved in the formation of diabetic kidney disease. Thus, curcumin showed potential in lowering complications associated with diabetes (Rivera-Mancía et al., 2018).

Hesperidin

Hesperidin (3,5,7-trihydroxy flavanone-7-rhamnoglucoside) is a flavanone glycoside as an active ingredient of citrus fruits with anti-oxidant, neuroprotective, and anti-inflammatory properties (Parhiz et al., 2015) Hajialyani et al., 2019). In a study on the wound healing potential of hesperidin, streptozotocin was used to induce diabetes in Sprague Dawley rats in a dose of 55 mg/kg. The wound was made in the hind paw of rats, and when this wound was stabilized, hesperidin was administered in doses of 25, 50, and 100 mg/kg p.o. for the time duration of 21 days. Several histopathological, molecular, and biochemical parameters were evaluated in the wound tissues of rats. The study showed that hesperidin treatment enhanced vasculogenesis and angiogenesis through upregulation of the Ang1/Tie-2, TGF-β1, SMAD-2/3, and VEGF-c mRNA expression to result in acceleration of healing of the wound in rats (Li et al., 2018).

Silymarin

The flavonoid silymarin is a cocktail of flavonolignans obtained from the plant *Silybum marianum* and is used to treat a wide variety of liver problems and diabetes (Rasool et al., 2014; Ferenci, 2016; Belwal et al., 2020; Singh et al., 2020). A study was conducted to study the potential of silymarin in improving diabetic cardiomyopathy by inhibiting TGF-β1/SMAD signaling. In one study, the treatment of diabetic rats with silymarin down-regulated the levels of blood glucose and produced improvements in collagen deposition and cardiac fibrosis in diabetic rats. Cardiac dysfunction in diabetic rats was decreased by silymarin as detected from the results of echocardiography. Silymarin produced a decrease in the levels of TGF-β and p-SMAD2/3 and enhanced the levels of SMAD7 in comparison to the untreated diabetic rats. Thus, this study points towards the potential of silymarin in improving diabetic cardiomyopathy by inhibiting TGF-β1/SMAD signaling. Silymarin can be a potential new candidate for the treatment of diabetic cardiomyopathy (Meng et al., 2019).

IS THERE ANY CROSS-TALK BETWEEN TGF-β AND AUTOPHAGY SIGNALING PATHWAYS IN DIABETES?

TGF-β by using several numbers of signaling pathways except to SMADs can regulate a wide array of cellular processes. As yet, the cross-talk between TGF-β and autophagy has not been studies in diabetes. However, some studies conducted to unravel the involvement of two signaling pathways in progression of diabetic complications such as liver and kidney fibrosis. Combinatory interactions of type I and type II TGF-β receptor serine/threonine kinases induce receptor-activated SMADs and its downstream signaling pathways (Derynck and Zhang, 2003) or directly activate many receptor pathways in a SMAD-independent manner. The PI3K-AKT-mTOR axis is activated directly by TGF-β ligand in a SMAD-independent way, resulting in the phosphorylation of numerous substrates, such as S6 kinase by mTORC1 and AKT by mTORC2, which are important for malignant progression (Ao et al., 2006; Lamouille and Derynck, 2007; Lamouille et al., 2012) and pathological bone metastases. As mTOR activity is tightly associated with many aspects of tumorigenesis, its regulation may be effective in the prevention of tumorigenesis (Zoncu et al., 2011).

TGF-β-miR-96 signaling pathway in an SMAD-dependent manner regulates the activation of mTOR activity, which is under the direct effect of the TGF-β pathway. Indeed, the AKT substrate modulated by mTORC2, AKT1S1 (also known as PRAS40) (Sancak et al., 2007; Vander Haar et al., 2007), is targeted by microRNAs activated in response to TGF-β ligand. Consequently, mTORC1 kinase will protect from the inhibitory effect of AKT1S1 as phosphorylation levels of S6K increase. In addition to AKT1S1, there are several tumor suppressors, such as FOXO1 and FOXO3a (Lin et al., 2010), that are negatively regulated by miR-96. In this line, miR-96 was considered as a metastamir and/or oncomir in the progression of cancer.

Specifically, autophagy targeting of misfolded proteins or damaged organelles improves cell survival (Massagué, 2000; Gozuacik and Kimchi, 2004). However, excessive autophagic

activity may lead to type II programmed cell death, which completely differs from apoptosis or type I programmed cell death (Ding et al., 2010). In mouse mesangial cells (MMC), serum deprivation induces autophagy, which eventually results in apoptosis. Yet, treatment with TGF-β results in the induction of the autophagy pathway, suppressing apoptosis activation. In primary MMCs cultured in serum deprivation circumstances, TGF-β signals through the TGF-β-activated kinase 1 (TAK1) and triggers the activation of several downstream cell signaling cascade, including MKK3/6-p38 MAPK and the PI3K-Akt-mTOR-S6K signaling axis (Ninomiya-Tsuji et al., 1999; Wang et al., 2001). The recruitment of the PI3K-Akt pathway (Chen et al., 2007; Gingery et al., 2008; Ding et al., 2010) leads to the induction of autophagy, which inhibits caspase 3 activity. Moreover, under the influence of the PI3K-Akt pathway, the G1/S cell cycle progress through the upregulation of cyclin D1 (Diehl et al., 1998) and downregulation of p27KIP1 (p27) levels through ubiquitination-dependent proteolysis (Murillo et al., 2001). In addition, Blocking TGF-β by autophagy inhibitor in MMC decreases the p27 protein level, suggesting that p27 levels were regulated through autophagy.

Liver fibrosis is a chronic liver disease with augmentation of ECM proteins, especially collagen, in liver tissues (Wu et al., 2017). The process of liver fibrosis is initiated by the activation of hepatic stellate cells (HSCs) (Li et al., 2016). When various factors, such as mechanical stimulation and inflammatory cytokines, especially TGF-β1, activate the quiescent HSCs, HSCs could not preserve the balance between ECM production and degradation (De Minicis et al., 2007). Indeed, TGF-β1 ligand secreted by KCs and HSCs promotes continuous activation of HSCs and interacts with TGF-β receptors (TβRs) of HSCs to phosphorylate SMAD3 and promote the translocation of phospho-SMAD3 (p-SMAD3) to the nucleus, leading to the production of ECM components that facilitate fibrosis pathogenesis (Shi and Massagué, 2003; Su et al., 2014).

The secretion of TGF-β1 could be regulated by the nuclear factor-kappa B (NF-κB) signaling pathway (Hayden and Ghosh, 2008) and activation of NF-κB could be modulated by TGF-β ligand. In other words, binding of IκBα to NF-κB subunits forms the IκBα/p50/p65 complex, which blocks NF-κB translocation into the nucleus (Luedde and Schwabe, 2011). TGF-β1 induced the degradation of IκBα, resulting in the enhancement of NF-κB (13) and also promoted the activation of NF-κB by TGF-β–activated kinase TAK1 and the IκB kinase (Sakurai et al., 1999; Attisano, 2001). In turn, NF-κB induces the transcription of TGF-β1 to promote the activation of HSCs ECM for the development of liver fibrosis (Feng et al., 2015). Furthermore, TGF-β1/SMAD3 pathway can lead to the induction of Beclin-1, which plays a critical role in the nucleation of the autophagy process (Gordy and He, 2012; Li et al., 2016), contributing indigestion of lipid droplets and supplies energy for the promotion of HSCs, thereby developing liver fibrosis (Hernández-Gea et al., 2012). Regarding the pivotal role of the TGF-β1/SMAD3 signaling pathway in activated HSCs, it seems that inhibition of this pathway could be beneficial in treatment of liver fibrosis. In a recent study, salidroside in combination with rat mesenchymal stem cell transplantation showed an efficiency in the treatment of liver fibrosis (Ouyang et al., 2010). In another study, bleomycin-induced lung fibrosis was improved by the administration of salidroside through modulation of the NF-κB and TGF-β1/SMAD2/3 pathways (Tang et al., 2016). In addition, utilizing salidroside was reported to be associated with reduced levels of TGF-β1 in KCs and HSCs *via* suppression of the NF-κB pathway, indicating that reduced autophagy in HSCs was performed by downregulation of the TGF-β1/SMAD3 pathway (Feng et al., 2018).

Fucoidan is another drug which functions similar to salidroside so that by inhibition of TGF-B -β1, reduces phosphorylation of SMAD2/3 and impedes the transferring of SMAD2/3 from the pulp to the nucleus to combine with specific DNA sequences of Beclin-1 gene which is a component of the class III phosphatidylinositol 3-kinase (PtdIns 3-kinase) complex. Therefore, Beclin-1 could not promote the autophagosome biogenesis through interaction with PI3K, which induces the conversion of LC3-I to LC3-II (Gordy and He, 2012; Nikoletopoulou et al., 2013) and consequently hinders the formation of autophagosomes.

In kidney injury and fibrosis induced by a unilateral ureteral obstruction (UUO), deficiency of autophagic protein LC3 and Beclin 1 leads to increased mature TGF-β levels and collagen deposition. In contrast, through autophagic degradation, the mature TGF-β1 protein levels are regulated, and kidney fibrosis induced by UUO will be suppressed (Pang et al., 2016).

Another key mechanism involved in the pathogenesis and progression of kidney fibrosis is the deregulation of epithelial-mesenchymal transition (EMT) (Koesters et al., 2010; Hernández-Gea et al., 2012). It has been shown that autophagy mediates the effect of TGF-β on the induction of EMT (Böttinger and Bitzer, 2002; Masszi et al., 2004; Zheng et al., 2009; Pang et al., 2016). Indeed, TGF-β induces autophagy degrading E-cadherin, resulting in β-catenin release. Following the β-cathenin release, autophagy activates Src in order to phosphorylate β-catenin, which by translocation to nucleus and binding to SMAD2 or SMAD3 as a co-activator increases the gene expression of ILK signaling factor which contributes in EMT. However, it has been demonstrated that both ILK and p-B-catenin/pSMAD2 are able to induce EMT and fibrosis, individually (Kim et al., 2009; Ulsamer et al., 2012; Tian et al., 2013).

It is conceivable that the regulation of autophagy by TGF-β signaling was done in a context-specific manner. Namely, autophagy may be possessed angiogenic or anti-angiogenic effects under various conditions. TGF-β has been connected to autophagy induction through TAK1 and JNK in epithelial and tumor cells, whereas activation of AKT and mTOR mediated by TGF-β has been shown to strongly inhibit autophagy in fibroblasts. In the endothelial cells, TGF-β suppresses transcription of beclin1 mediating PIP3-dependent recruitment of additional ATG proteins through the recruitment of SMAD2. Indeed, SMAD2 functions as an inhibiting factor of Beclin 1 in the autophagy process. In addition, it has been shown that beclin1 gene expression is controlled by several

transcription factors such as FoxO3, NFκB, HIF1α, c-Jun, and E2F1 signaling factors.

Extensive studies over the past several years have revealed that autophagy acts as a cytoprotective effector in a response to increased stress so dysregulation of autophagy is found in the development of human disorders (Mizushima et al., 2008; Choi et al., 2013). The culmination of these observations shed light on the importance the interplay of two pathways' cross-talk and development of several diseases, while further studies is required to uncover the involvement of TGF-β and autophagy cross-talk in pathogenesis of diabetes and rising complication so that paved the way to discover novel therapeutic strategies.

CONCLUDING REMARKS AND PERSPECTIVE

Current strategies for the management of diabetes include the prevention of glucose absorption as well as the inhibition of related metabolic pathways and factors such as gluconeogenesis and α-glucosidase. Moreover, various signaling pathways are involved in the regulation of metabolic disorders, leading to more recent studies to investigate the role of signaling pathways in the normal function of β cells and insulin-responsive cells. Specifically, the main effective signaling pathways, namely, autophagy and TGF-β1/SMAD signaling cascades have been considered as the useful therapeutic strategies in the overcome DM.

Thus, the prominent roles of both autophagy and TGF-β1/SMAD signaling pathways in diabetes and its complications were further supported by altered expression and activity of signals. Furthermore, therapeutic strategies aiming to regulate TGF-β1/SMAD signaling and autophagy present promising remedies in the treatment of diabetes.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this article.

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GLOSSARY

3-MA	3-methyladenine
AD	Alzheimer disease
adFNDI	autosomal-dominant familial neurohypophyseal diabetes insipidus
ADSCs	Adipose tissue-derived stem cells
ADT2DM	advanced duration of type 2 diabetes mellitus
ALK	activin receptor-like kinase
AMPK	5′ adenosine monophosphate activated protein kinase
ATF6	activating transcription factor-6
ATG	AuTophaGy related proteins
Atg7	autophagy-related gene 7
Bcl2	B-cell lymphoma 2
BUN	blood urea nitrogen
CAT	catalase
CNS	central nervous system
CTGF	connective tissue growth factor
DDIT3	DNA-damage inducible transcript 3
DM	Diabetes mellitus
DN	diabetic neuropathy
DRP1	Dynamin-related protein 1
ECM	extracellular matrix
EGFR/MAPK	epidermal growth factor receptor/mitogen activated protein kinase
EIF2AK3	eukaryotic translation initiation factor 2 α kinase 3
EMT	epithelial mesenchymal transition
ER	endoplasmic reticulum
ERN1/Ire1α	endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme-1α
Ex-4	exendin-4
FADD	Fas-associated death domain protein
FGF21	fibroblast growth factor 21
FPI	fasting blood insulin
FIP200	family interacting protein of 200 Kd
FN	fibronectin
GABA	γ-aminobutyric acid
GABARAPL1	GABA A receptor-associated protein like 1
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLUT 4	glucose transporter 4
GS	glycine-serine-rich
GSH-Px	glutathione peroxidase
GSK3	Glycogen synthase kinase 3
GTP	guanosine triphosphatases
hIAPP	human islet amyloid polypeptide
HIF1α	hypoxia inducible factor 1-α
HIHG	high insulin/glucose
HSC	hepatic stellate cell
GDM	gestational diabetes mellitus
iAs	Inorganic arsenic
IR	insulin receptor
pIRE1	IRE1 phosphorylation
JNK	c-Jun N-terminal kinase
LC3	light chain 3
LTBP-1	latent transforming growth factor beta binding protein -1
LAMP-2	lysosome associated membrane protein2
LC3	1A/1B-light chain 3
LCIIIB	light chain IIIB
MHC	major histocompatibility complex
MAPK	mitogen-activated protein kinase
MC	*Momordica charantia*, MDA, malondialdehyde
MFN2	Mitofusin-2
MIN6	mouse INsulinoma 6
MMC	mouse mesangial cells
MMP9	matrix metalloproteinase 9
mRNA	messenger ribonucleic acid
mTOR	mTORC2, rapamycin complex 2
mammalian target of rapamycin	
NAC	N-acetylcysteine
NAFLD	nonalcoholic fatty liver disease
ND	non-diabetic
NF-kB	nuclear factor kappa-B light-chain-enhancer of activated B cells
NDT2DM	newly diagnosed type 2 diabetes mellitus
NO	nitric oxide
NOD	nonobese diabetic
NPC1L1	Niemann-Pick C1-like
OLETF	Otsuka Long-Evans Tokushima Fatty
PEG-sTNF-RI	pegylated soluble tumor necrosis factor receptor type 1
PPARγ	peroxisome proliferator-activated receptor γ
PBMCs	peripheral blood mononuclear cells
PKA	protein kinase A
pGDM	prior history of gestational diabetes mellitus
PERK	endoplasmic reticulum kinase
PI3P	phosphatidyl inositol-3 phosphate, Rab, Ras-associated binding
RT-FQ-PCR	real time fluorescence quantitative polymerase chain reaction analysis ROS, reactive oxygen species
rpWAT	retroperitoneal white adipose tissues
scWAT	subcutaneous white adipose tissues
SCr	serum creatinine
SNARE	SolubleN-ethylmaleimide-sensitive factor-attachment protein receptor; reactive oxygen species
SOD	superoxide dismutase
STZ	Streptozotocin
SRX	sulfiredoxin
T2DM	type 2 diabetes mellitus
TAK1	beta-activated kinase 1
TEM	transmission electron microscopy
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor-α
TGN	trans-Golgi network
TRAF6	Tumor necrosis factor receptor-associated factor 6
TUDCA	tauroursodeoxycholic acid
ULK1	Unc-51-like kinase 1
UP	urine protein
UPR	unfolded protein response
UUO	unilateral ureteral obstruction
VEGF	vascular endothelial growth factor
Vps34	Vacuolar Protein Sorting Protein 34. WIPI, WD-repeat protein interacting with phosphoinositides.

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