ALTERNATIVE AND COMPLEMENTARY METHODS FOR THE CONTROL OF INFECTIOUS DISEASES IN ANIMALS

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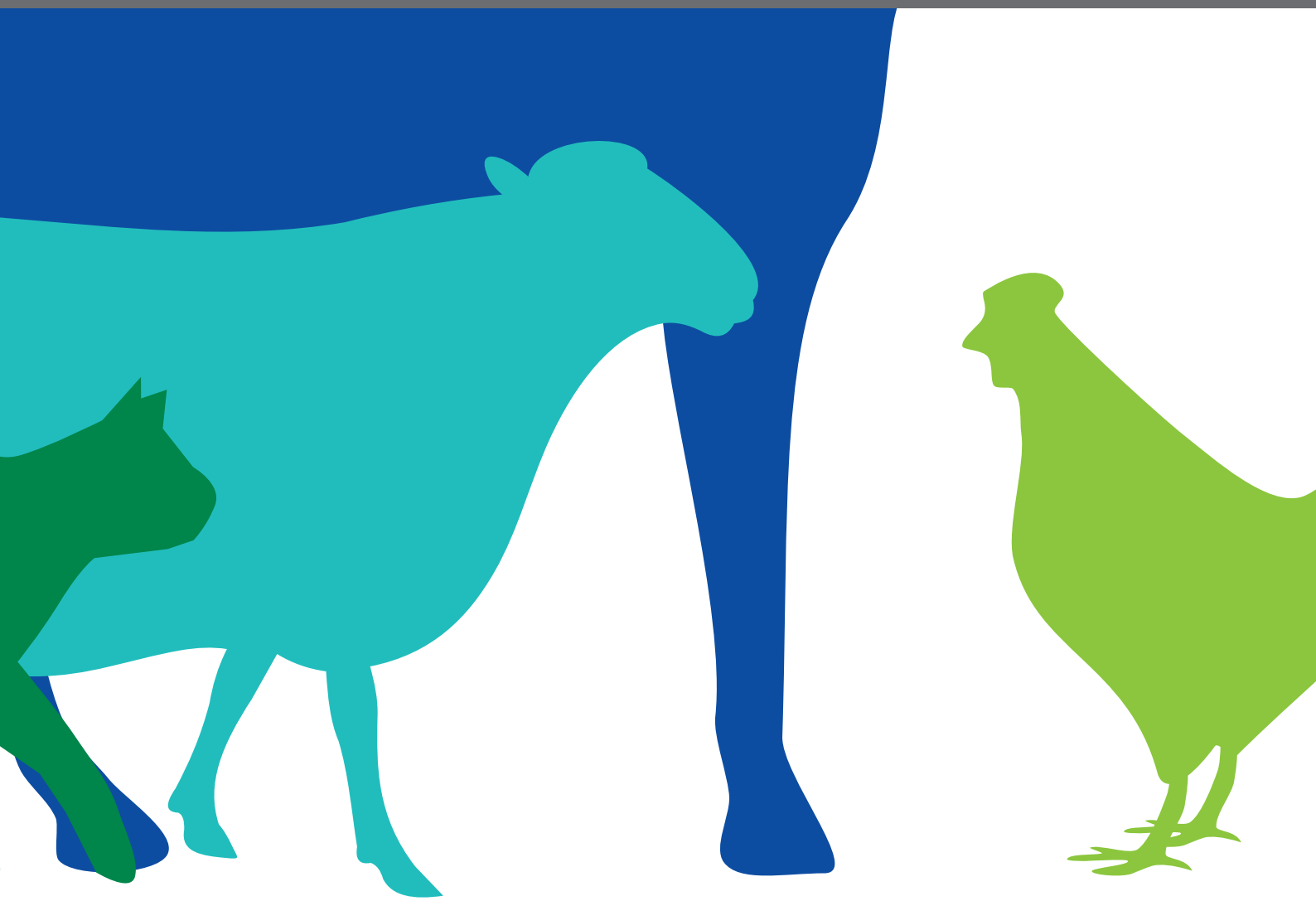

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ALTERNATIVE AND COMPLEMENTARY METHODS FOR THE CONTROL OF INFECTIOUS DISEASES IN ANIMALS

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Editorial: Alternative and complementary methods for the control of infectious diseases in animals

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Editorial on the Research Topic
Alternative and complementary methods for the control of infectious diseases in animals

Animal production contributes significantly to the food security, income and wellbeing of humans. However, infectious diseases in production, companion and wild animals often cause serious losses to the economy, in addition to exacerbating food insecurity (1). Conventionally, livestock farmers, animal health clinics and zoos often rely on the extensive use of synthetic chemicals, including antibiotics, anthelmintics and feed supplements to improve meat production and animal welfare (2). Long-term use of chemical drugs has led to serious problems, such as the rapid development of drug-resistant infectious agents and negative consequences for human and animal health (3, 4). As a result, there is a paradigm shift toward the search for alternative or complementary options and more "natural" ways of managing animal health and production (5–7). Alternative methods of animal husbandry and medication are increasingly being recognized for their potential usefulness in contributing to animal welfare, particularly at the level of primary animal health care. It remains pertinent that researchers continuously explore treatments that involve the application of biological control, vaccines, management practices, plants and natural products (1).

Against this background, the current Research Topic brings together different academic disciplines to offer new knowledge regarding alternative and complementary methods useful against infectious diseases in animals. We present a collection of 10 articles, including original research (70%), reviews (20%) and perspectives (10%).

In the face of the multifarious dynamic nature of animal production in Europe, which is partly attributed to political and economic factors, research by Mattalia et al. compared current and past ethnoveterinary practices, and identified trajectories in ethnoveterinary knowledge in northern and southern Eastern Europe. The results identified some patterns common to several countries and to veterinary medicine promoted during the time of the Soviet Union. Using the Netherlands as a case study, Groot et al. provided an insightful perspective on how to further reduce the application of veterinary antibiotics to safe-guard public health. Recommendations include the potential of plant-based products for farm health plans and the importance of monitoring antibiotic use to identify non-registered applications and to set benchmarks. In addition, the need to develop new analytical strategies to monitor the use of antibiotics on farms was emphasized.

Foot-and-mouth disease is recognized as a dangerous infectious disease in livestock. The study by Kass et al. focused on how Estonia addressed the two outbreaks of foot-and-mouth disease in 1952 and 1982. Based on diverse engagements, including written and archival sources as well as interviews with 29 experts, the study explored interesting historical methods used to manage foot-and-mouth disease as well as the practicability of such methods in today's world. Moreover, the importance of plant-based remedies for managing cattle diseases in South Africa was the focus of the review by Chakale et al. An inventory of 310 plants that are known as herbal medicine against 10 categories of cattle diseases in South Africa was provided. The authors revealed that only 21% of these plants have been screened for biological activities (mainly antibacterial effects) and putative safety. The identified gaps in the review open new opportunities for research necessitating urgent attention to promote and explore the benefits of plants in ethnoveterinary medicine.

There is a need to discover new approaches to combat the proliferation of multiple antibiotic resistant pathogens in the dairy sector. The increasing incidence of such pathogens poses a great threat to food sustainability and security, and the health of the public. Bovine mastitis is a major health problem in the dairy industry that results in significant economic losses. The review by Ajose et al. entailed a critical appraisal on how ethnoveterinary medicine can be explored as a viable alternative for combating bovine mastitis in the face of increasing global antimicrobial resistance. The predominant pathogens implicated in causing bovine mastitis include *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus dysgalactiae* and coliform bacteria. The review addressed diverse aspects relating to bovine mastitis in terms of the risk factors, pathogenesis, management, the molecular identification of causative agents, as well as the potential of ethnoveterinary medicine as an alternative therapy. Furthermore, research by Jiang et al. focused on determining rational regimens of cefquinome for the treatment of bovine mastitis caused by *Staphylococcus aureus*. This entailed extensive *ex vitro* pharmacokinetic and pharmacodynamic investigations of cefquinome, which was administered as three consecutive intramammary doses at 75 mg/gland. The authors established that the CO_{PD} for cefquinome against *S. aureus* was 2 μg/ml at the recommended dose of 55 mg/gland/12 h. The findings could assist in the treatment of clinical mastitis and reduce the prevalence of drug-resistance, thereby enhancing animal welfare and human health.

The use of plant-based remedies for the treatment of poultry diseases caused by various pathogens remains popular. Olawuwo et al. screened six selected medicinal plants against planktonic and biofilm forms of poultry pathogens (bacterial and fungal strains). In addition, the cellular safety of the plant extracts was assessed. Based on the potential *in vitro* antimicrobial effect and safety, the authors identified *Morinda lucida* extract as a promising candidate for development of an alternative feed additive or supplement in poultry production. In a similar vein, Qinghao Powder (QHP) is a herbal medicine made from the Chinese medicinal plant *Artemisia annua*. In an effort to explore the potential of this plant-based remedy, Wang, Guo et al. investigated the efficacy and safe dose of the petroleum ether (PE) extract of QHP on broiler chickens with coccidiosis induced using *Eimeria tenella* oocysts (Guangdong strain). The authors established that QHP at 0.30 g/kg would be an effective and safe therapy for intermittent treatment of *E. tenella*-infected chicks. In another study, Jambwa et al. evaluated the potential development of a poultry phytogenic feed additive from *Senna singueana*. Bioassay-guided fractionation led to the isolation and identification of luteolin. The authors investigated the antibacterial, anti-lipoxygenase and antioxidant activity, as well as the *in vitro* safety of fractions and isolated compounds. Based on the promising bioactivity and low cytotoxicity, *S. singueana* was recommended as a promising candidate for the development of poultry phytogenic feed additives.

The residue effect of commonly used antibiotics in the environment, including water bodies, remains a major concern globally (4). Polymyxins including colistin (polymyxin E) are last-resort antibiotics for treatment of multidrug-resistant Gram-negative bacterial infections (Wang, Lv et al.). However, the large quantity of colistin released *via* animal feces into the environment has resulted in increasing incidence of the novel *mcr* mobile colistin resistant gene. As a means of combating this problem, research by Wang et al. revealed that ferrate (VI) oxidation is a highly effective and environment-friendly strategy to degrade colistin in water bodies.

In summary, this Research Topic highlights exciting new research dealing with a range of alternative and complementary methods for the control of infectious diseases in animals. From dairy cows to poultry, several studies provide evidence that there is some light at the end of the tunnel of antibiotic resistance. Reports emphasize that a wide range of medicinal plants are used traditionally to combat various diseases in livestock and this is a largely untapped resource for further investigation. The

rising threat of diseases caused by drug resistant pathogens lends further support to the urgent investigation of other means for controlling such pathogens without relying on the development of new antibiotic molecules against which resistance is certain to emerge within a short period.

An approach encompassing various complementary methods for combating infectious animal diseases will certainly assist in improved production animal farming, leading to better food security and animal welfare as well as lowering the risk of spreading zoonoses. Companion animals also stand to benefit from a more holistic approach to animal health, in this way promoting human wellbeing as an added advantage. It is hoped that this Research Topic motivates further investigations in this critical area of exploration, leading to scientific validation of a host of potential alternative and complementary approaches to controlling animal infectious diseases. This will contribute to a multitude of areas involving animal and human health and wellbeing.

Author contributions

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

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Ethnoveterinary Knowledge and Biological Evaluation of Plants Used for Mitigating Cattle Diseases: A Critical Insight Into the Trends and Patterns in South Africa

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Cattle farming is a traditional agricultural system that contribute to the rural economic, social and cultural values of the communities. Cattle as common with other livestock, are affected by many diseases that cause mortality and economic losses. In many rural households, the use of plants and associated knowledge are popular for managing cattle diseases especially in areas experiencing challenges with conventional veterinary medicine. Evidence on the documentation of indigenous knowledge and biological evaluation of plants used against cattle diseases remain understudied and fragmented. The aim of the review is to collate and analyse the ethnoveterinary knowledge and biological evaluation of plants used against cattle diseases in South Africa. Different scientific databases were systematically explored to extract data from 37 eligible studies. A total of 310 medicinal plants from 81 families used to treat 10 categories of cattle diseases across seven (7) provinces in South Africa. Leguminosae (Fabaceae), Compositae (Astereceae), Asparagaceae, and Xanthorrhoeaceae were the most frequently used plant families. Common plant parts used were leaves and roots. Twenty-seven (27) combination remedies involving 2–6 plants were identified as treatment regimes against cattle diseases. Common preparation methods were infusion and decoction while the administration mode was predominantly unspecified (52%) while oral and topical contributed 26 and 22%, respectively. In terms of diseases, the most treated ones were general systems infection, reproduction disorders and gastrointestinal problems. Currently, an estimated 21% of the 310 plants have been evaluated for diverse biological activities using relevant bioassays related to cattle diseases. Antibacterial activity remained the most studied biological activity. Evidence from the review revealed the significance of ethnoveterinary medicine against cattle diseases especially in rural areas of South Africa. Nevertheless, the use of plants for cattle diseases among other ethnic groups, particularly in the Northern Cape and Western Cape, remain under-studied.

Keywords: animal health, ethnobotany, food security, livestock, antibacterial, retained placenta

INTRODUCTION

Cattle farming is the backbone of the rural sector and contribute to social and cultural values such as ancestral rituals, *lobola* (bridal) payment, cleansing and sustainable rural livelihoods (1–3). Particularly, cattle are part of livestock farming and a catalyst to enhance household food security and alleviating poverty in small-scale cooperative farming areas. In South Africa, there are about 14 million cattle, which make up 1.6 million dairy cattle (604,781 cows in milk) and 12.5 million beef cattle. Furthermore, ∼53 and 47% are in commercial and subsistence systems, respectively (4). However, cattle are often affected by many diseases that cause mortality and economic losses (5–7). Preventing and managing cattle diseases remain a major concern in South Africa as well as in other African countries (8). Therefore, ethnoveterinary medicine (EVM) has become a program that is used to protect and manage animal health and diseases (9–12).

Rural communities often utilize EVM and associated practices to maintain health of wide range of cattle populations (12–14). In South Africa, the use of medicinal plants for treating human diseases have been extensively documented in literature (15–18). However, the neglect relating to ethnoveterinary especially the botanical recording of medicinal plants used to treat animal diseases remain a major concern (19). The need for treatment possibilities is rapidly becoming a key aspect of basic health care within various communities (10, 20). The need to record indigenous knowledge of plants to mitigate their lost due to rapid urbanization and acculturation cannot be over-emphasized (21).

Global interest in EVM practices has increased in the last decade, leading to extensive work especially in Africa (10, 11, 22–24); Asia (25–30); North and South America (31, 32); as well as Europe (33–37). Interest in EVM research is due to readily availability, ease of preparation and administration as well as affordability (13, 38–40). Increasing evidence strongly suggests that EVM has the potential to improve agricultural productivity of local communities (11, 30, 41–43). The current review provides a critical appraisal on the trends and patterns for traditional knowledge and biological evaluation of plants used against cattle diseases in South Africa. It is anticipated that the review will identify existing knowledge gaps and may serve as a reference material for future research efforts in the field of EVM.

MATERIALS AND METHODS

Selection of Scientific Publications

This review was based on the ethnoveterinary studies conducted in South Africa until May 2021. The information on traditional/indigenous knowledge on plants used against cattle diseases in South Africa was extracted from published scientific journals, books, reports from national, and regional, dissertation, theses, conference papers, and reports in South African universities websites/libraries (electronic data repositories), conference proceedings, regulatory and non-governmental organizations. Literature was searched using specific search terms in international online databases such as PubMed, JSTOR, Science Direct, Scopus, and Google Scholar. In the review process, the following search terms were included (singular or plural forms when necessary) in conjunction with South Africa: ethnoveterinary medicine, indigenous knowledge, cattle health care, local cattle husbandry, traditional cattle medicine, animal health anthropology, ethnomedicinal, plant, ethnopharmacology, folk medicine, herbal remedies for cattle diseases, and ethnobotanical papers containing information on plants which was unambiguously linked to a veterinary use. Research articles were also searched by examining bibliographies.

Selection Criteria

For any article/study to be included in the review, it must include and indicate details of a specific EVM plants relative to its use for treating cattle diseases within the research period (i.e., up to May 2021). For each study, the following information was collected: Latin name of plant used, plant parts, diseases or condition treating, dosage, preparation and mode of administration, the classification of cattle diseases or conditions or therapeutic use of plants. Articles that were excluded were review articles, those solely concerned with modern medicines, or those which cattle were not subject matter. Furthermore, letters, case-reports, manuals, and guidelines, and those reporting only human studies were excluded for this review (**Figure 1**). The selection of articles was done in four steps. Step one, the relevance of studies was checked based on their title. In the second step, abstracts were evaluated to match to the inclusion criteria. If primary inspection of an abstract of a paper did not give adequate information to make an informed judgment, the full paper was searched in the third step and reviewed by the authors prior to deciding on their inclusion in the review. Finally, those that met the inclusion criteria were retrieved for extra appraisal (**Figure 1**). All scientific plant names were cross-checked with The Plant List (www.theplantlist.org), while the common names were confirmed using PlantZAfrica (www.pza.sanbi.org).

RESULTS AND DISCUSSION

South African Ethnoveterinary Medicine Studies Based on Cattle Healthcare

In South Africa, most rural community farmers depend on conventional health practices to preserve and improve their livestock health by preventing and managing diseases (44). Cattle diseases have an influence on the economy and have an impact on cultural practices (45). Ethnoveterinary practices play a greater role in the welfare of cattle as an alternative or an integral part of traditional veterinary practices in rural communities. The use of medicinal plants and indigenous methods/practices for the treatment of diseases is not only limited to humans, but also applies to the treatment of different diseases in cattle (46). Farmers believe that indigenous practices and plants are easy to use/apply, affordable and have less side effects on their livestock. One of the earliest evidence on the use of the EVM was indicated in the work of Gerstner (47). Further studies have been undertaken toward increasing the database of therapies for animal diseases and conditions (**Table 1**). Studies have been undertaken throughout South Africa with a view to recording indigenous community knowledge of cattle health care, but many

TABLE 1 | An overview of reviewed literature on ethnoveterinary studies on plants used against cattle diseases in South Africa.

Reference	#Province	Area/region	Ethnic group	Number of plant species	Number of plant families	Diseases/ conditions	Voucher specimen deposited	Preparation method	Administration mode	Characteristic of participants	Methodological framework (data collection and analysis, techniques)
Gerstner (47–49)	KZN	Unspecified	Zulu	14	10	9	Unspecified	Unspecified	Unspecified	Unspecified	Ethnobotanical book
Doke and Vilakazi (50)	KZN	Unspecified	Zulu	1	1	1	Unspecified	Unspecified	Unspecified	Unspecified	Ethnobotanical book
Hulme (51)	KZN	Unspecified	Zulu	4	3	4	Unspecified	Yes	Unspecified	Unspecified	Ethnobotanical book
Watt and Breyer-Brandwijk (15)	Southern and Eastern Africa	Unspecified	Unspecified	29	19	10	Unspecified	Yes	Yes	Unspecified	Ethnobotanical book
Bryant (52)	KZN	Unspecified	Zulu	2	1	2	Unspecified	Unspecified	Unspecified	Unspecified	Ethnobotanical book
Pujol (53)	South Africa	Unspecified	Unspecified	2	2	1	Unspecified	Yes	Unspecified	Unspecified	Ethnobotanical book
Roberts (54)	South Africa	Unspecified	Unspecified	3	3	5	Unspecified	Yes	Yes	Unspecified	Ethnobotanical book
Mabogo (55)	LP	Venda	Venda	2	2	3	Unspecified	Yes	Unspecified	Community members	Ethnobotanical book
Hutchings (56)	KZN	Unspecified	Zulu	40	20	20	Unspecified	Yes	Yes	Unspecified	Ethnobotanical book
Masika et al. (57)	EC	Victoria East, Keiskammahoek, Middledrift, Zwelitsha, Mdantsane, Peddie, and Stutterheim	Xhosa	11	10	2	Unspecified	Unspecified	Yes	Farmers	Semi-structure interview, group interview and Rapid Rural Appraisal (RRA)
Masika et al. (58)	EC	Mnqaba-Kulile, Gqumashe, Gwaba, Upper Gxulu, Dongwe, Feni and Fair View, Kubusi, and Kwezana	Xhosa	11	11	7	Yes	Yes	Yes	Farmers, herbalist	In-depth semi-structured interview, convenience sampling, group interview and observation
Dold and Cocks (59)	EC	Ebenezer, Penryn, and Victoria Post	Xhosa	32	26	13	Yes	Yes	Yes	Households, farmers	Questionnaire
Van der Merwe et al. (60)	NW	Madikwe	Tswana	40	21	25	Yes	Unspecified	Unspecified	Farmers, extension officers, traditional healers, knowledgeable elders	RRA, group interview, observation and field walk
Getchell et al. (61)	NW	Seboana, Kromdraai, Vryhof, Setlagole, Kraaipan, and Madibogo	Tswana	4	4	1	Yes	Unspecified	Unspecified	Farmers	Participatory research model
Masika and Afolayan (62)	EC	Unspecified	Xhosa	30	26	8	Yes	Yes	Yes	Farmers and herbalists	RRA, field walk, semi-structure interview guide

(Continued)

TABLE 1 | Continued

Reference	#Province	Area/region	Ethnic group	Number of plant species	Number of plant families	Diseases/ conditions	Voucher specimen deposited	Preparation method	Administration mode	Characteristic of participants	Methodological framework (data collection and analysis, techniques)
Luseba and Van der Merwe (63)	LP	Greater Giyani municipality	Tsonga	15	8	15	Yes	Yes	Yes	Farmers and traditional healers	RRA and interviews
Mahlo (64)	LP	Basani	Unspecified	5	5	4	Unspecified	Yes	Yes	Farmers	Interview, and literature
Moyo (65)	EC	Qolora by-Sea and Nontshinga	Xhosa	3	3	1	Yes	Yes	Yes	Farmers and herbalists	Stratified randomly sampling, interviews
Soyelu and Masika (66)	EC	Amatola Basin	Xhosa	12	10	1	Yes	Yes	Yes	Farmers and community members	structured questionnaires, Snowball sampling
Matlebyane et al. (42)	LP	Ga-Mphahlele, Ga-Dikgale, and Moletjie	Pedi	6	6	7	Yes	Unspecified	Unspecified	Farmers	Semi-structured questionnaires
Luseba and Tshisikhawe (44)	LP	Mutale, Thohoyandou, Nzhelele, and Pundamaria	Venda, Tsonga, and Pedi	26	20	15	Yes	Yes	Yes	Farmers	Open-ended questions, field walks, student's participation in the form of assignments
Beinart and Brown (8)	NW, GP, FS, EC	Mafikeng, Mabeskraal, Garankuwa-Mabopane-Winterveld, QwaQwa, Koppies, Mbotyi—-Mpondoland, Andrew Ainslie, Vimbai Jenjezwa, and Mike Kenyon	Tswana, Sotho, Xhosa, Afrikaners	65	31	24	Unspecified	Yes	Yes	Farmers and community members	interviews
Magwede et al. (67)	LP	Vhembe district	Venda	27	14	2	Yes	Yes	Yes	Farmers, elders and community members	Sem-structured questionnaire, systematic sampling
Kambizi (68)	EC	Pondoland	Xhosa	20	15	10	Unspecified	Unspecified	Unspecified	Herbalists and villagers	Field survey
Mphahlele (69)	LP	Blouberg Municipality	Pedi	5	2	1	Yes	Unspecified	Unspecified	Farmers	Purposeful sampling, Semi-structured interviews
Ramovha and Van Wyk (70)	LP	Vhembe district	Venda	18	9	1	Yes	Yes	Unspecified	Farmers, herders, traditional healers, anthropologists, agriculture extension officers	RRA approach, field surveys, Semi-structured interviews, and observations

(Continued)

TABLE 1 | Continued

Reference	#Province	Area/region	Ethnic group	Number of plant species	Number of plant families	Diseases/ conditions	Voucher specimen deposited	Preparation method	Administration mode	Characteristic of participants	Methodological framework (data collection and analysis, techniques)
Mogale (71)	LP	Tshebela and Ga-Mogano	Pedi	7	7	6	Yes	Yes	Yes	Farmers	Semi-structured interview guide, focus groups discussions, interpretive phenomenological approach
Chitura et al. (72)	LP	Mutale	Venda	9	9	8	Yes	Yes	Yes	Farmers	Purposive sampling, structured questionnaire
Shiba (73)	MP	Chief Albert Luthuli Municipality	Tsonga	7	5	1	Yes	Yes	Yes	Farmers	Questionnaire
Mongalo and Makhafola (74)	LP	Blouberg	Pedi	9	4	Unspecified	Yes	Unspecified	Unspecified	Traditional healers, herbalists	Random sampling, structured questionnaire, s field walks
Mthi et al. (75)	EC	Upper Gqumeya, Ciko, and Goso	Xhosa	6	6	2	Yes	Yes	Yes	Community households	Purposive sampling, semi-structured questionnaire and field observations, analysis
Ndou (76)	NW	Lokaleng, Mogosane, Lokgalong, and Masutlhe	Batswana	24	13	17	Yes	Yes	Yes	Farmers, traditional healers, and community members	Snowball sampling, semi-structured questionnaire, group interviews
Semenya et al. (77)	LP	Ga-Mphahlele	Pedi	30	23	10	Yes	Yes	Yes	Community members	Random sampling, semi-structured questionnaires, field observations
Khunoana et al. (78)	MP	Mnisi/ Bushbuckridge	Tsonga	11	7	7	Yes	Yes	Yes	Farmers, animal health technician, herders, herbalists	Rapid Rural Appraisal, semi-structured interview
Moichwanetse et al. (12)	NW	Dinokana	Batswana	25	18	17	Yes	Yes	Yes	Farmers and herders	Semi-structured interviews, SPSS
Mthi et al. (79)	EC	Upper Gqumeya, Ciko, and Goso	Xhosa	9	8	3	Yes	Yes	Yes	Extension officers, community elders and local authorities	Semi-structured questionnaire, descriptive statistical analysis
Mthi and Rust (80)	EC	Upper Gqumeya, Ciko, and Goso	Xhosa	6	6	1	Yes	Yes	Yes	Community members	Cross-sectional survey using semi-structured questionnaire, purposive sampling

#Province, EC, Eastern Cape, LP, Limpopo Province, MP, Mpumalanga Province, NW, North West; GP, Gauteng Province; FS, Free State; KZN, KwaZulu-Natal; RRA, Rapid Rural Appraisal.

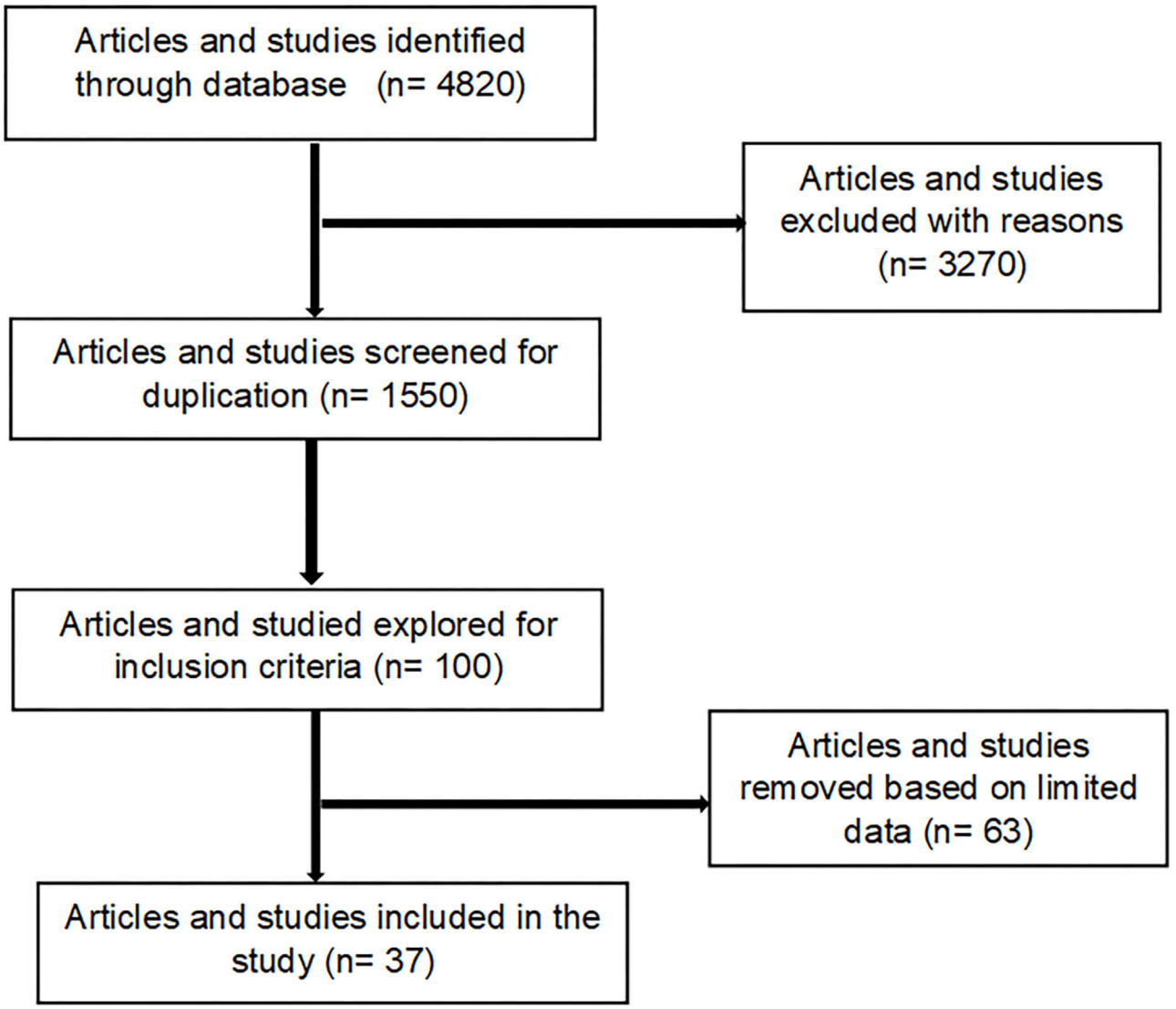

FIGURE 1 | Process used for the selection of articles included in the generated inventory (**Supplementary Table S1**).

rural communities have limited documentation. This justifies the need for the continuation of work in the rest of the country in order to complete the documentation of the EVM used against diseases in cattle.

Based on inclusion and exclusion criteria, a total of 37 studies on EVM plants used against cattle disease conducted throughout South Africa were identified (**Figure 1**). In the last 10 years, we observed an increase in publications related to EVM plants used against cattle diseases, indicating an increasing interest in the field. In terms of the geographical distribution of the studies (**Table 1**), Limpopo province dominated accounting for 32% of the total number of articles. This is due to the province's rich plant diversity and its status as one of the country's hotspots (74). Other major contributions were the Eastern Cape (29.7%), North West and KwaZulu-Natal (13.5%), Mpumalanga (5.4%) while Gauteng and Free State province were the least (2.7%). The most studied ethnic groups were Xhosa (28.9%), baPedi (15.7%), Zulu, VhaVenda, and Batswana (13.2%) and Tsonga (10.5%) while the least responses were from the Basotho and Afrikaner (2.6%). A diverse range of participants involved in the studies were farmers, herbalists, traditional healers, community members (households), knowledge holder (elders), extension officers/animal health technicians, herders, and local authorities (**Table 1**).

There are numerous methods and approaches used for studying ethnoveterinary knowledge used to treat cattle diseases. Depending on the nature of the knowledge and the degree of certainty researchers had a variety of options. As a result, classification of such a range is critical in order to detect potential systematic patterns in the research literature. Twelve research methodologies were used to collect data, 5 sampling techniques, and 2 analysis methods used in South African EVM

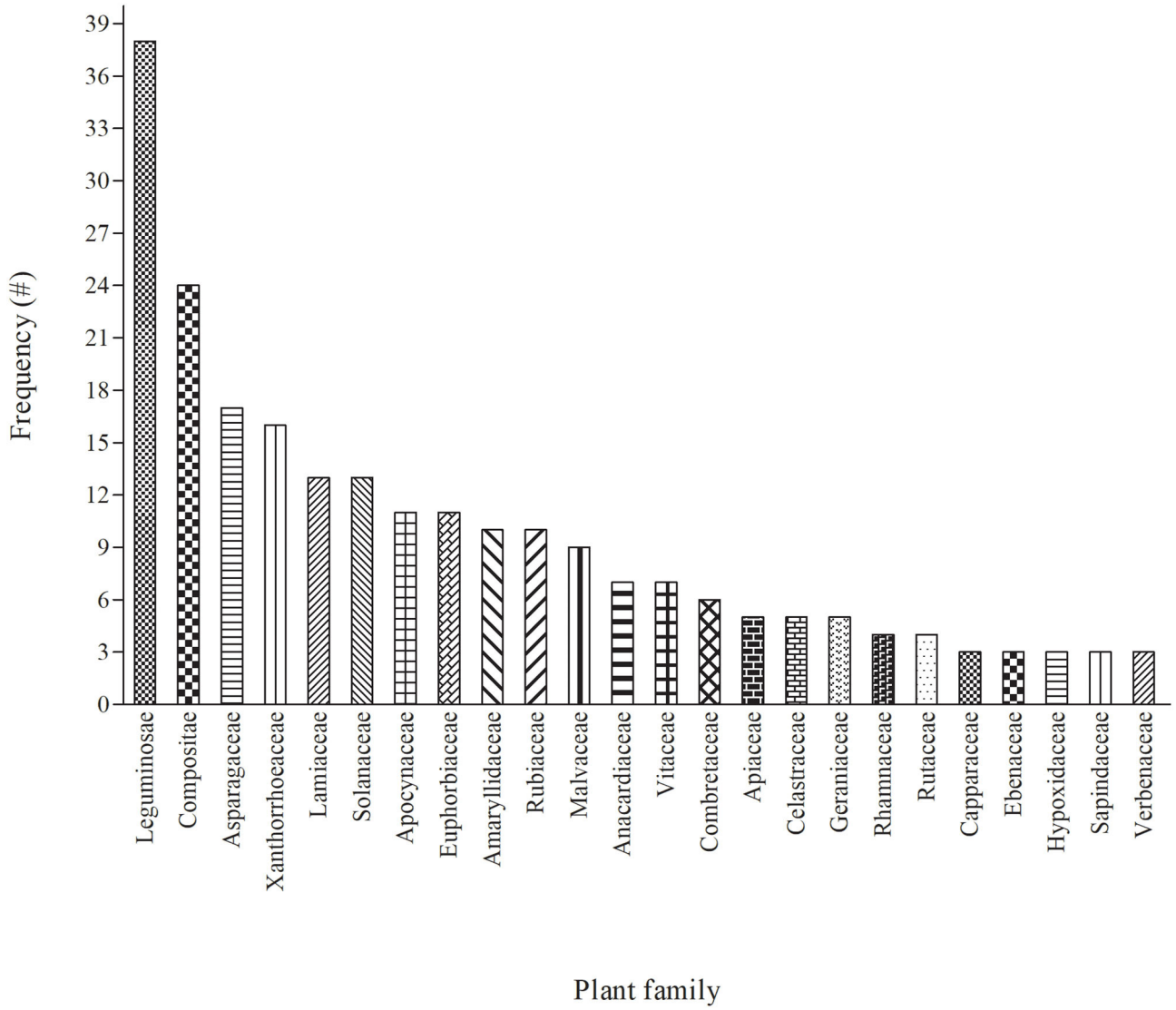

FIGURE 2 | The 24 major (with ≥ 3 mentions) plant families used for treating cattle diseases in South Africa. Each of the remaining 57 plant families were mentioned once or twice each (see **Supplementary Tables 1**, **2** for details).

studies (**Table 1**). Semi-structured interview guides were the most commonly used data collection tool as demonstrated in 40% of the reviewed literature while Rapid Rural Appraisal was used in 16% of the articles. It is worth noting that some of the researchers used a variety of methodologies to conduct their research. The majority of studies did not demonstrate the use of approaches and theories to underpin the use of EVM in the treatment of cattle diseases (**Table 1**). The development of theories and approaches are necessary requirement for the proper development of any field (81). However, the process of developing theories is contentious. Some researchers believe that existing theories should be expanded upon (82) while others believe that new innovative theories should be encouraged in the spirit of plurality (83). Furthermore, none of the articles that took theoretical perspectives proposed a novel EVM theory but were all based on pre-existing theories (76).

Overview of Medicinal Plants and Families Used in Treating Cattle Diseases

An inventory of plants used against cattle diseases across seven (7) provinces of South Africa was generated (**Supplementary Table 1**). The plants are arranged in alphabetical order based on the botanical name (with synonyms in the brackets), as well as their families, local names (were available in Setswana/Tswana, Venda, English, Afrikaans, Tsonga, Zulu, and Xhosa), plant parts used, preparation and administration process, and diseases treated are provided. A total of 310 plant species (from 81 families) were used

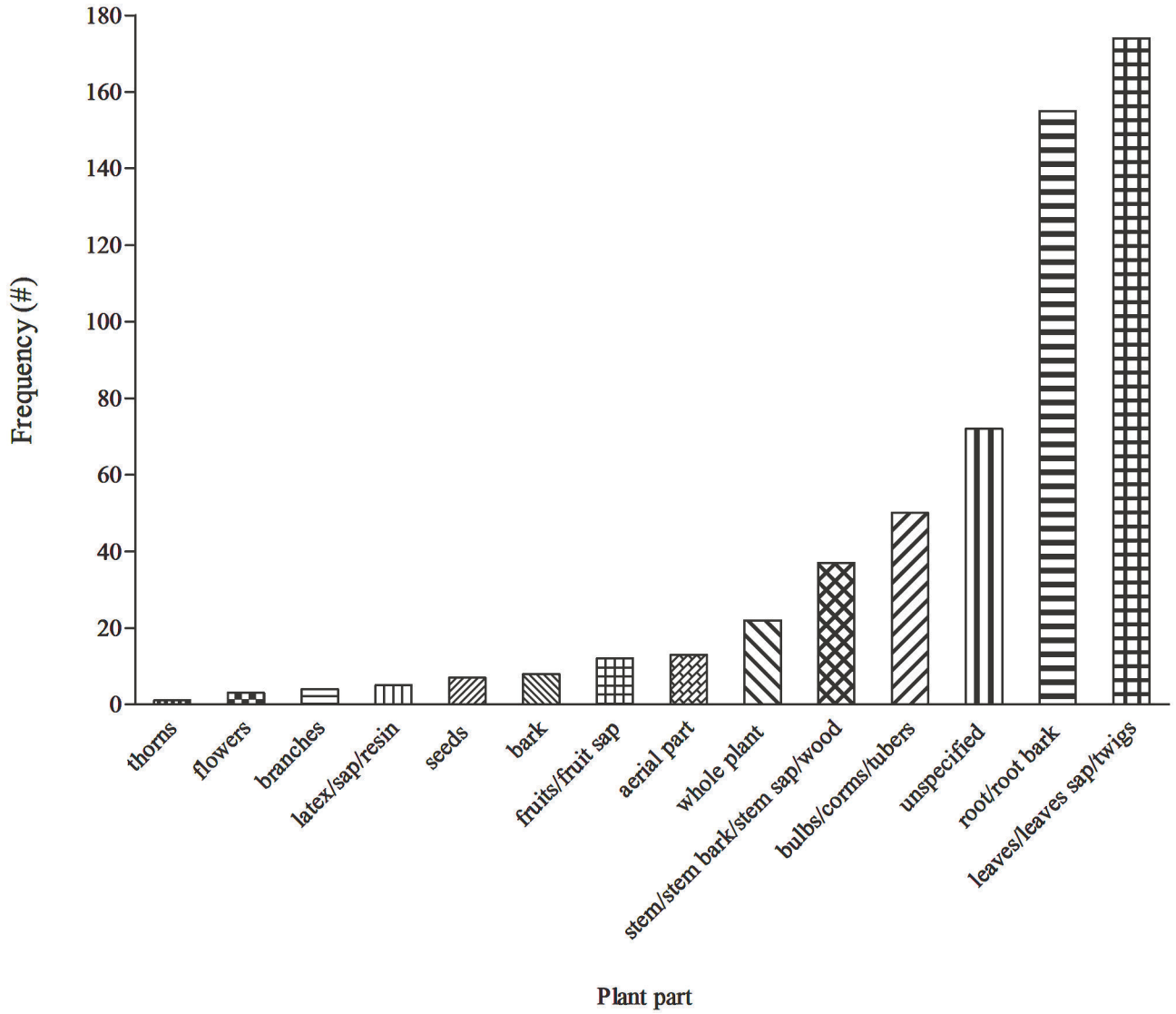

FIGURE 3 | Distribution of medicinal plant parts used to treat cattle diseases in South Africa.

against different cattle diseases. The current review provides a strong indication that South Africa has rich diversity of EVM plants and associated indigenous knowledge. The most frequently mentioned plant which represents 5.5% of the inventory were *Elephantorrhiza elephantina* (Burch.) Skeels, *Aloe ferox* Mill., *Dicerocaryum eriocarpum* (Decne.) Abels, *Senna italica* Mill., *Aloe marlothii* A.Berger, *Boophone disticha* (L.f.) Herb., *Solanum panduriforme* E. Mey, *Spirostachys africana* Sond., *Drimia sanguinea* (Schinz) Jessop, *Pappea capensis* Eckl. & Zeyh, *Calpurnia aurea* (Aiton) Benth., *Gunnera perpensa* L., *Carissa bispinosa* (L.) Desf. ex Brenan, *Clutia pulchella* L., *Gymnanthemum corymbosum* (Thunb.) H.Rob., *Volkameria glabra* (E.Mey.) Mabb. & Y.W.Yuan, and *Ximenia americana* L. Their frequent use and higher number of mentions (3–4 times) in South Africa for diseases in cattle was established in the current review. The relatively high frequency of mentions for these plants is an indication of their effectiveness against diverse diseases in cattle.

In terms of diversity, 81 families were used as herbal medicine to treat and manage cattle diseases in South Africa (**Figure 2** and **Supplementary Tables 1**, **2**). Leguminosae/Fabaceae was the most dominant family and contributed 38 plants, followed by Compositae (24), Asparagaceae (17), Xanthorrhoeaceae (16), Lamiaceae and Solanaceae (13), Apocynaceae and Euphorbiaceae (11), Rubiaceae (10), Malvaceae (9) and Vitaceae (7). Leguminosae/Fabaceae had the highest number of plants used to treat cattle diseases which may be attributed to their higher abundance in the study area or due to high bioactivity (84). Similar studies have also been reported from other

TABLE 2 | Examples of plants used in combination therapy for treating cattle diseases in South Africa.

No of plants	Combined plants	Plant parts used	Preparation and administration methods	Disease/condition	Reference
2	*Asparagus setaceus* + *Rhus incisa*	Roots	Infusion	Shock	(59)
2	*Curtisia dentata* + *Rapanea melanophloeos*	Bark	Decoction	Unspecified	(59)
2	*Cussonia spicata* + *Olea europaea*	Bark + leaves	Decoction	Endometritis/ vaginitis	(59)
2	*Dicoma galpinii* + *Senna italica*	Roots	Infusion, oral	Gala	(76)
2	*Grewia flava* + *Ziziphus zeyheriana*	Roots	Decoction, oral	Diarrhea	(76)
2	*Helichrysum caespititium* + *Artemisia afra*	Roots + leaves	Decoction, oral	Coughs	(76)
2	*Hippobromus pauciflorus* + *Protorhus longifolia*	Bark	Decoction	Heartwater, diarrhea	(59)
2	*Pelargonium reniforme* + *Plumbago auriculata*	Roots	Decoction	Diarrhea	(59)
2	*Pelargonium sidoides* + *Ziziphus zeyheriana*	Unspecified	Decoction	Anthelmintics	(15)
2	*Phoenix reclinata* + *Arctotis arctotoides*	Roots + leaves	Decoction, topical	Foot rot	(59)
2	*Ziziphus zeyheriana* + *Helichrysum caespititium*	Roots	Decoction, oral	Pains	(76)
3	*Bulbine abyssinica* + *Solanum lichtensteinii* + *Withania somnifera*	Roots	Infusion, oral	Internal sores	(76)
3	*Drimia sanguinea* + *Senna italica* + *Elephantorrhiza elephantina*	Bulb + roots + bulb	Maceration, oral	Intestinal parasites	(12)
3	*Drimia sanguinea* + *Ziziphus oxyphylla* + *Ziziphus mucronata*	Bulb + roots+ roots	Poultice, topical	Cleaning the kidney	(12)
3	*Hypoxis hemerocallidea* + *Aloe vera* + *Pouzolzia mixta*	Bulb + leaves + roots	Maceration, oral	Heart problems	(12)
3	*Leucas capensis* + *Brachylaena ilicifolia* + *Aloe ferox*	Leaves + sap	Decoction	Unspecified	(59)
3	*Peltophorum africanum* + *Elephantorrhiza elephantina* + *Jatropha zeyheri*	Bulb + roots + bulb	Maceration, oral	Constipation	(12)
3	*Plectranthus laxiflorus* + *Eucomis punctata* + *Kedrostis africana*	Unspecified	Decoction	Gallsickness	(59)
3	*Senna italica* + *Ziziphus zeyheriana* + *Cadaba aphylla*	Roots	Decoction, oral	Pains	(76)
3	*Solanum campylacanthum* + *Helichrysum caespititium* + *Withania somnifera*	Roots	Decoction, oral	Pain	(76)
3	*Solanum lichtensteinii* + *Bulbine abyssinica* + *Withania somnifera*	Roots	Infusion, oral	Internal sores	(76)
3	*Withania somnifera* + *Helichrysum caespititium* + *Solanum campylacanthum*	Tuber + roots	Decoction, oral	Pain	(76)
3	*Withania somnifera* + *Solanum lichtensteinii* + *Bulbine abyssinica*	Tuber + roots	Infusion, oral	Internal sores	(76)
4	*Dicoma galpinii* + *Ziziphus zeyheriana* + *Senna italica* + *Cadaba aphylla*	Roots	Decoction. Oral	Pains	(76)
4	*Grewia occidentalis* + *Olea europaea* + *Zanthoxylum capense* + *Aloe ferox*	Leaves + sap	Infusion	Gallsickness	(59)
5	*Drimia sanguinea* + *Terminalia sericea* + *Senna italica* + *Elephantorrhiza elephantina* + *Jatropha zeyheri*	Bulb + roots + roots+ bulb + bulb	Poultice, topical	Anemia	(12)
6	*Dicerocaryum senecioides* + *Drimia sanguinea* + *Pouzolzia mixta* + *Peltophorum africanum* + *Senna italica* + *Hypoxis hemerocallidea*	Whole plant + bulb + roots + leaves + bulb + bulb	Poultice, topical	Flea eradication	(12)

parts of world where participants mostly use the members of Leguminosae/Fabaceae for the preparation of EVM for the treatment of different livestock diseases (10, 85–87). However, the findings differ from those of other EVM studies in which the other families such as Apiaceae (88), Poaceae (30), Aloaceae (22), Asteraceae (89, 90) and Solanaceae (91) were ranked as the highest. The difference among these studies may be related to the dominant vegetation of the areas or cultural significance (30).

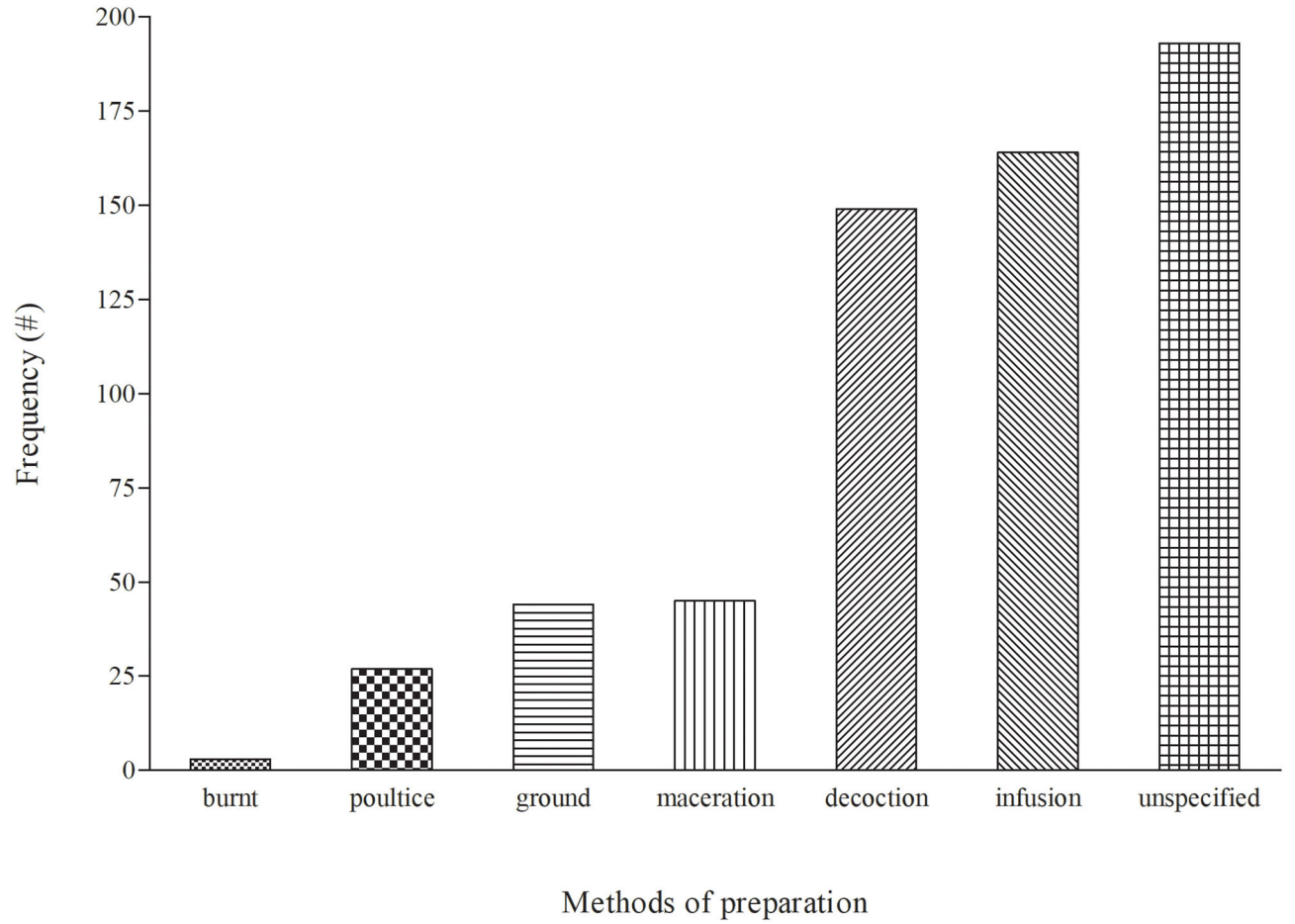

FIGURE 4 | Distribution of the modes of preparation for medicinal plants used to treat cattle diseases in South Africa.

Plant Parts Used to Treat Cattle Diseases

In total, 14 plant parts/components were used for treating cattle diseases in South Africa (**Figure 3**). Leaves (30.7%) were the most widely used in EVM for treating cattle diseases. The popularity of leaves as one of the most preferred plant part has been a common pattern in South African EVM (46). Preference of leaves over other parts of plants remain common for various reasons including the relatively ease of access when compared to other plant parts. Furthermore, leaves are synthesizing organ for some important plant secondary metabolites that may exert medicinal properties (92–94). From a conservation perspective, individual plants are often not threatened by leaf harvesting for medicinal purpose. Roots constituted 27% and were the second most widely used plant parts, which may be due to rich pool of active compounds, especially terpenes (94). However, the selection of underground parts of the plant including the roots is not viable as it affects plant life and is considered to be highly detrimental to the survival of the whole plant if not done in a sustainable manner (95). As a result, proper harvesting strategies and conservation measures are required to ensure the long-term utilization of medicinal plant resources (95, 96).

Mono vs. Multi-Plants Application for the Treatment of Cattle Diseases

Even though monotherapy was the most common, the combination of two or more plants were evident in some instances as remedies for treating cattle diseases in South Africa (**Table 2**). In some instance, a combination of six (6) plants was indicated as treatment remedy for eradicating flea in cattle. Based on the findings by Moichwanetse et al. (12), these type of mixtures are often formulated with more than one plant in order to achieve synergistic or potentiating effects in cattle. Based on the study by Sarswat and Purohit (97), the use of plant mixtures is common for mitigating bovine infertility. Furthermore, the combination of various parts of a plant is commonly used to manufacture medicines for different health conditions in traditional medicine (98–100).

Method of Preparing Medicinal Plants for the Treatment of Cattle Diseases

Before administration of medicinal plants to treat cattle diseases, diverse methods of preparation are utilized, which may differ depending on the location and culture. Six (6) preparation

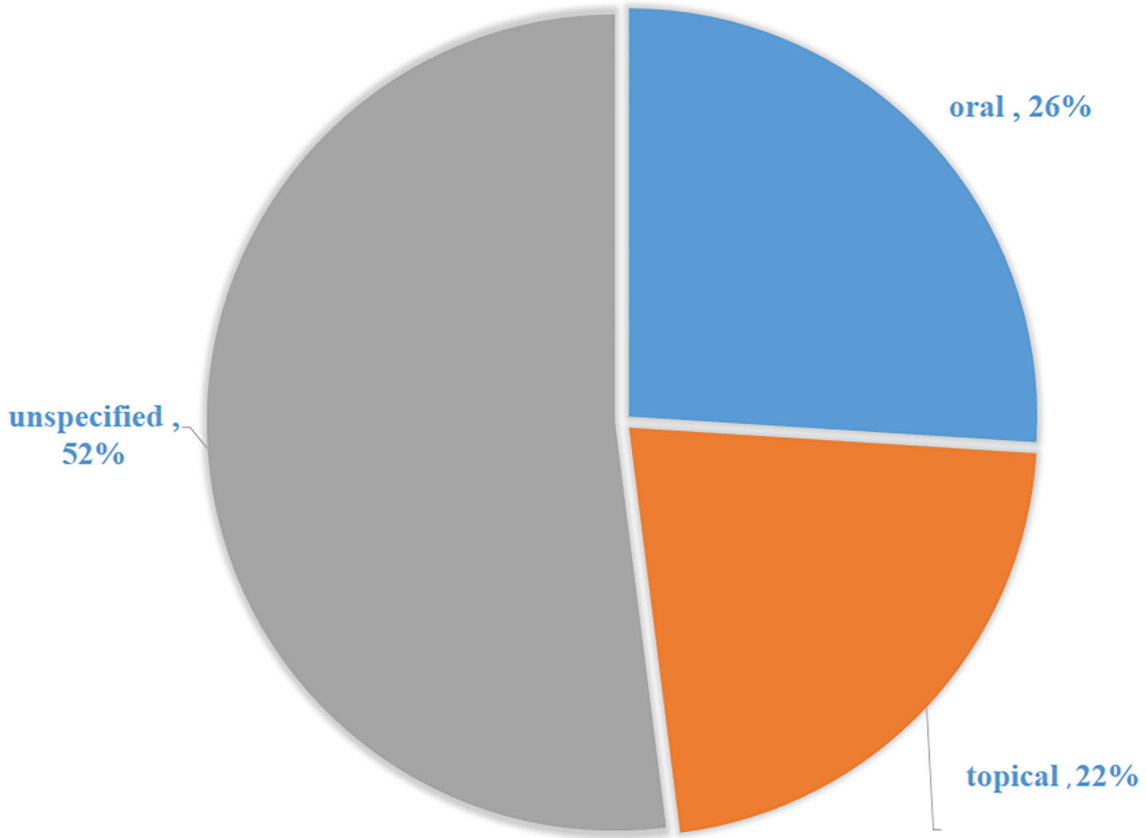

FIGURE 5 | Distribution of the mode of administration for medicinal plants used to treat cattle diseases in South Africa.

methods (burnt, decoction, ground, infusion, maceration, and poultice) were used for treating diseases in cattle (**Figure 4**). Infusion (166 = 27.25%) was a popular method and it involves pouring cold/hot/warm water onto the plant material and allowing the mixture to cool. This was followed by decoction (149 = 24.46%), which involved boiling plant materials in a specific amount of water and allowing the mixture to cool before administration. However, the current observation differs from other countries whereby crushing and pounding were the most common used preparation methods for livestock diseases (101–103). Other methods of preparation such as maceration, grinding and poultice had low frequencies in the range of 4–7%. The methods of preparation differ depending on the type of disease being treated and the site of the ailment. The majority of the preparations were made using water.

Mode of Administration/Application of Medicinal Plants for the Treatment of Cattle Diseases

The local communities use a variety of methods to administer EVM plants when treating diseases in cattle (**Figure 5**). The major route of administration for EVM plants was oral-based (157 = 26.5%). Oral administration is a simple and non-invasive form of systemic treatment. The route allows for the rapid absorption and distribution of the prepared medicines and allowing for sufficient curative power to be delivered (104). Topical which contributed 21.8% (105) was the second widely mode of application while 51.9% (308) of cases did not specify how herbal remedies should be administered. Across many African cultures, oral administration of medicinal plants is the most common route used to treat disease in cattle, as this ensures fast and direct interaction with different plant compounds at the site of action (101, 106, 107). The majority of the research documented in the current review omitted the dosage and vehicle usage. The dosage is important because it indicates how much should be used to treat the cattle and the units of measurement. However, EVM are generally known to have a significant flaw in terms of accuracy and standardization (102, 108).

Common Diseases in Cattle Treated With Plants and Associated Indigenous Knowledge

A total of 310 medicinal plants were used to treat several diseases in cattle which were categorized into 10 major groups

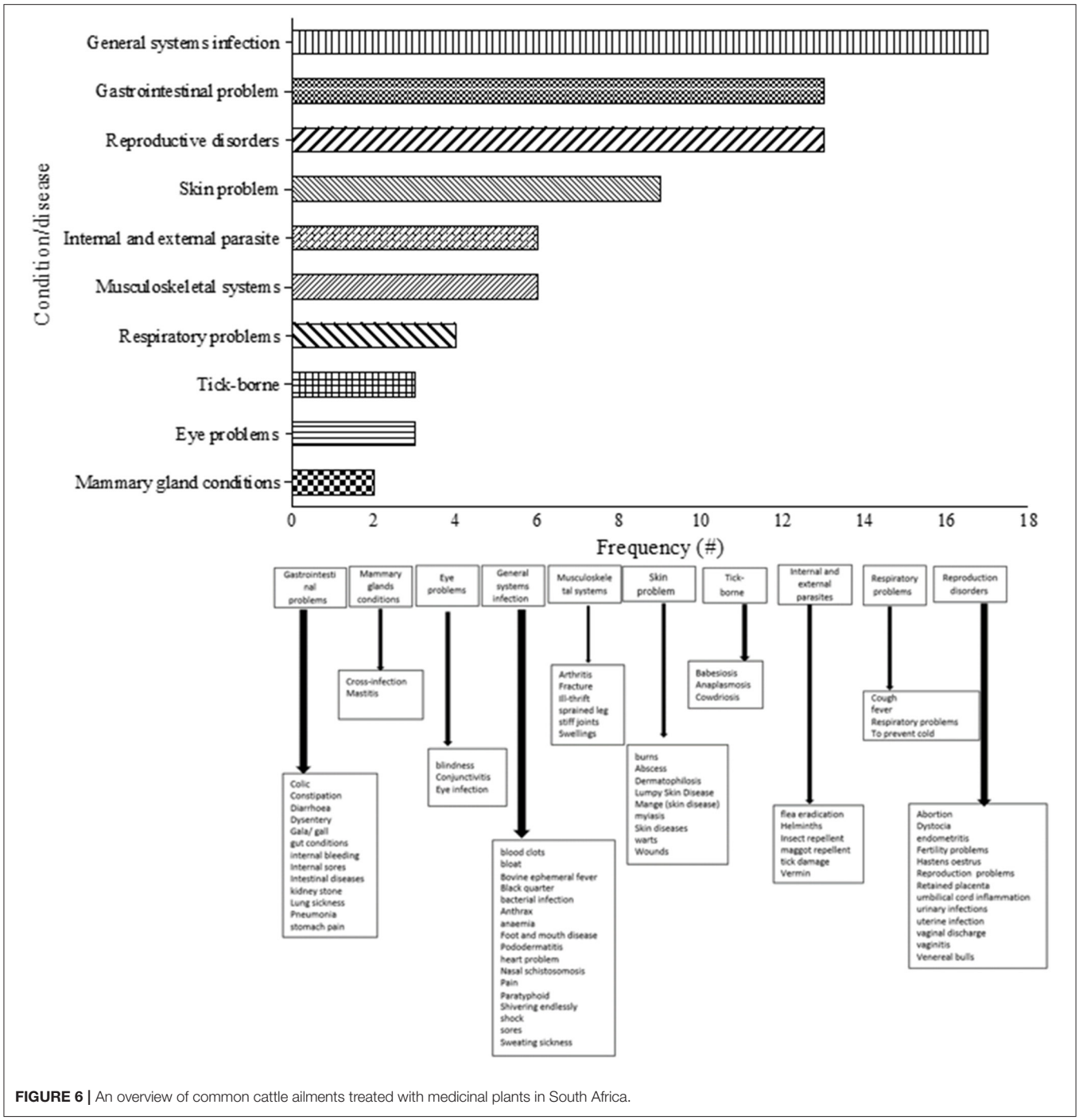

FIGURE 6 | An overview of common cattle ailments treated with medicinal plants in South Africa.

(**Figure 6**). The classification of the different diseases was based on the study by Ndou (76), with slight modification. Some of the dominant categories included general systems infection, reproduction disorders, gastrointestinal problems, skin problem, internal/external parasites, musculoskeletal systems, and respiratory problems. On the other hand, treatment of conditions such as eye problems, tick-borne and mammary glands problem were relatively lower in terms of mentions in the reviewed literature. General systems infection was regarded as the most common disease category in cattle (**Supplementary Table 3**). The majority of these health challenges including digestive problems were easily diagnosed by participants through observation which may explain their high degree of mentions (30, 33–37). The current review identified that the 9 common conditions were anaplasmosis (treated with 69 plants), retained placenta and wounds (treated with 59 plants),

TABLE 3 | Overview of the biological evaluation of plants used to manage cattle diseases in South Africa.

Scientific name	Screened activity (Reference)	Number of Assays conducted
Aloe marlothii A.Berger	Antibacterial, Antifungal, Antimycobacterial, and Cytotoxicity (78) Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anti-rickettsial, Anti-babesial, and Antioxidant (116) Anti-ticks and Toxicity (117)	11
Aloe arborescens Mill.	Antibacterial and cytotoxicity (118)	2
Aloe ferox Mill.	Anti-parasitic (73) Anti-ticks and Toxicity (117) *In vitro* and *in vivo* acaricidal (65) Anthelminthic (119) Anthelminthic (120) Anti-ticks (121)	5
Apodytes dimidiata E.Mey. ex Arn.	Antiparasitic, Antibacterial, Antioxidant, Cytotoxicity, and Antifungal (122)	5
Balanites maughamii Sprague	Antibacterial (64)	1
Bauhinia thonningii Schum. (Sny: *Piliostigma thonningii* (Schum.) Milne-Redh.)	Antibacterial (64)	1
Bolusanthus speciosus (Bolus) Harms	Antibacterial and Cytotoxicity (123)	2
Breonadia salicina (Vahl) Hepper & J.R.I.Wood	Antibacterial (64)	1
Calpurnia aurea (Aiton) Benth.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Antibacterial and Cytotoxicity (123) Acaricidal (127)	3
Cassia abbreviata Oliv.	Anthelmintic (69)	1
Cissus quadrangularis L.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anthelmintic and toxicity (128) Acaricidal (127)	7
Clausena anisata (Willd.) Hook.f. ex Benth.	Antiparasitic, antibacterial, antioxidant, cytotoxicity, and antifungal (122)	5
Coddia rudis (E.Mey. ex Harv.) Verdc.	Antibacterial (68)	1
Combretum caffrum (Eckl. & Zeyh.) Kuntze	Antibacterial and Antifungal (129)	2
Curtisia dentata (Burm.f.) C.A.Sm.	Anthelmintic (130)	1
Cussonia spicata Thunb.	Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anthelmintic and toxicity (128)	5
Cynanchum viminale (L.) L. (Syn: *SarcoStemma viminale* (L.) R.Br.)	Antibacterial, Anti-inflammatory, and Mutagenicity (115)	3
Dicerocaryum eriocarpum (Decne.) Abels	Anti-parasitic (73) Anthelmintic (120) Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anthelmintic and toxicity (128)	7
Dombeya rotundifolia (Hochst.) Planch.	Antibacterial (64) Antibacterial, Anthelmintic and toxicity (128)	3
Drimia sanguinea (Schinz) Jessop Syn: *Urginea sanguinea* Schinz	Antibacterial, Anti-rickettsial, Anti-babesial, and Antioxidant (116)	4
Elephantorrhiza elephantina (Burch.) Skeels (Syn: *Acacia Elephantorrhiza*)	Antibacterial, Anti-rickettsial, Anti-babesial, and Antioxidant (116) Anthelminthic (119)	5
Elephantorrhiza obliqua Burtt Davy	Antibacterial, Antifungal, Antimycobacterial, and Cytotoxicity (78)	4
Gardenia volkensii K.Schum.	Anti-parasitic (73) Anthelmintic (120)	2
Gnidia capitata L.f.	Antibacterial, Anthelmintic and toxicity (128)	3
Harpephyllum caffrum Bernh.	Antibacterial (68)	1
Helichrysum caespititium (DC.) Sond. ex Harv.	Anthelmintic (120)	1
Helichrysum kraussii Sch.Bip.	Anti-parasitic (73)	1
Heteromorpha arborescens (Spreng.) Cham. & Schltdl.	Antibacterial and cytotoxicity (123)	2
Hippobromus pauciflorus Radlk.	Antibacterial, Anthelmintic and toxicity (128)	3

(Continued)

TABLE 3 | Continued

Scientific name	Screened activity (Reference)	Number of Assays conducted
Hyperacanthus amoenus (Sims) Bridson	Antibacterial (64)	1
Hypoxis rigidula Baker	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 126)	3
Jatropha curcas L.	Anti-ticks and toxicity (117)	2
Jatropha zeyheri Sond.	Antibacterial, Anti-inflammatory, and Mutagenicity (115)	3
Lantana camara L.	*In vitro* and *in vivo* acaricidal (65)	1
Leonotis leonurus (L.) R.Br.	Anthelminthic (119) Anti-ticks (121)	2
Maerua angolensis DC.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 126)	3
Melia azedarach L.	Antiparasitic, antibacterial, antioxidant, cytotoxicity, and antifungal (122)	4
Pappea capensis Eckl. & Zeyh.	Anthelmintic (120, 131)	1
Pelargonium luridum (Andrews) Sweet	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 126) Acaricidal (127)	3
Peltophorum africanum Sond.	Antioxidant, antibacterial, anthelmintic and toxicity (132) Anthelmintic (69)	4
Pittosporum viridiflorum Sims	Antibacterial and cytotoxicity (123)	2
Plumbago zeylanica L.	Antiviral and cytotoxicity (133)	2
Pouzolzia mixta Solms	Antibacterial, Anthelmintic and toxicity (128)	3
Ptaeroxylon obliquum (Thunb.) Radlk.	*In vitro* and *in vivo* acaricidal (65)	1
Pterocarpus angolensis DC.	Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anthelmintic, and toxicity (128)	5
Rhoicissus tridentata (L.f.) Wild & R.B.Drumm.	Antibacterial, Anti-rickettsial, Anti-babesial, and Antioxidant (116)	4
Ricinus communis L.	Anti-ticks and toxicity (117) Antibacterial, Anti-inflammatory, and Mutagenicity (115) Antibacterial, Anthelmintic and toxicity (128)	6
Salix capensis Thunb.2	Antibacterial and Antifungal (129)	
Schkuhria pinnata (Lam.) Kuntze ex Thell.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Antibacterial, Anti-inflammatory, and Mutagenicity (115) Acaricidal (127)	7
Schotia brachypetala Sond.	Antibacterial, antifungal, Antimycobacterial, Cytotoxicity (78) Antibacterial, Anthelmintic and toxicity (128) Anthelmintic (69)	6
Schotia latifolia Jacq.	Antibacterial and Antifungal (129)	2
Sclerocarya birrea (A.Rich.) Hochst.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Antibacterial, Anthelmintic and toxicity (128) Acaricidal (127)	5
Searsia lancea (L.f.) F.A. Barkley (Syn: *Rhus lancea* L.f.)	Antibacterial, Anthelmintic and toxicity (128)	3
Secamone filiformis J.H. Ross	Antibacterial, Anthelmintic and toxicity (128)	3
Senecio barbertonicus Klatt	Anthelmintic (120)	1
Senna italica Mill.	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Anti-tick (134) Acaricidal (127) Anthelmintic (69)	4
Synadenium cupulare L.C. Wheeler	Antibacterial, Anthelmintic and toxicity (128)	3
Tabernaemontana elegans Stapf	Anthelmintic and cytotoxicity (124) Acaricidal and cytotoxicity (105, 125, 126) Acaricidal (127)	3
Tagetes minuta L.	*In vitro* and *in vivo* acaricidal (65) Anti-ticks (135)	2

(Continued)

TABLE 3 | Continued

Scientific name	Screened activity (Reference)	Number of Assays conducted
Tephroseris palustris (L.) Rchb. (Syn: *Senecio congestus* (R.Br.) DC.)	Anthelmintic (120)	1
Tetradenia riparia (Hochst.) Codd	Antibacterial and cytotoxicity (118)	2
Trema orientalis (L.) Blume	Antibacterial and cytotoxicity (118)	2
Vachellia nilotica (L.) P.J.H. Hurter & Mabb. (Syn: *Acacia nilotica* (L.) Delile)	Antibacterial and cytotoxicity (118)	2
Volkameria glabra (E.Mey.) Mabb. & Y.W. Yuan (Syn: *Clerodendrum glabrum* E.Mey.)	Anti-ticks and toxicity (117) Antiparasitic, antibacterial, antioxidant, cytotoxicity, and antifungal (122)	7
Zanthoxylum capense (Thunb.) Harv.	Antiparasitic, antibacterial, antioxidant, cytotoxicity, and antifungal (122)	5
Ziziphus mucronata Wild.	Antibacterial, anti-inflammatory and Mutagenicity (122) Antibacterial, anthelmintic and toxicity (128)	5

diarrhea (treated with 50 plants), babesiosis (treated with 47 plants), helminths (treated with 46 plant) and constipation (treated with 25 plants). Plants such as *Drimia sanguinea* (Schinz) Jessop, *Elephantorrhiza elephantina* (Burch.) Skeels, *Senna italica* Mill., *Boophone disticha* (L.f.) Herb., *Dicerocaryum eriocarpum* (Decne.) Abels, *Aloe ferox* Mill., *Cassia abbreviata* Oliv., *Cussonia spicata* Thunb., and *Cissus quadrangularis* L. were recorded as the most frequently mentioned ones for treating cattle diseases (**Supplementary Table 3**).

Given that the incidence and severity of various cattle diseases are widespread in rural areas (109–112), the detrimental effect on meat and milk production are often enormous on small-holder livestock farmers (11, 30, 42, 46). As a result, indigenous communities extensively depend on the use of EVM and associated indigenous knowledge to understand the cause, clinical signs and transmission mode of disease occurrence (39, 113). The ability of the community members to understand the diseases is achieved through experiences. They use techniques such as observing the breathing and vocalization, urine and dung, tasting milk, behavioral change, knowledge of vectors and social interaction (76, 114).

Overview of Biological Evaluation of Plants Used to Manage Cattle Diseases

Out of the 310 plants, ∼21% (66 plants) have been screened for biological activity in targeted assays relating to EVM used against cattle diseases (**Table 3**). Plants were tested for biological activities including antibacterial, antifungal, anti-ticks, antioxidant, antimycobacterial, anti-inflammatory and cytotoxicity. An estimated 70% of the plants (46 of the 66) were screened for antibacterial activity which make it the most studied biological activity. In addition, 51% of the plants (34 of the 66) were evaluated for anthelmintic property while 38% (25 of the 66 plants) have been tested for safety based on cytotoxicity effect. The most frequently screened plant was *Aloe marlothii* that have been screened in 11 bioassays. Other plants that have been subjected to multiple bioassays were *Cissus quadrangularis, Dicerocaryum eriocarpum, Schkuhria pinnata,* and *Volkameria glabra* (7 bioassays), *Ricinus communis* and *Schotia brachypetala* (6 bioassays), *Aloe ferox, Apodytes dimidiata, Clausena anisata, Cussonia spicata, Elephantorrhiza elephantina*, *Pterocarpus angolensis, Sclerocarya birrea, Zanthoxylum capense,* and *Ziziphus mucronata* (5 bioassays). Plants used for therapeutic purposes are normally assumed to be safe. This is mainly due to the long-term use of medicinal plants for the treatment of diseases based on basic knowledge accumulated and shared from generation to generation over many centuries (136).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Based on this extensive review, South Africa has a diverse range of plants used for mitigating diseases affecting cattle. The distribution and utilization pattern of EVM reveals a significant variation across a range of geographical settings for 7 out of the 9 provinces in South Africa. Despite the gradual socio-cultural transformation over the years, the inhabitants have retained remarkable knowledge of the plants and their uses up to present days. This suggests that the use of plants for the management of cattle diseases remain culturally rooted among South Africans. The leaves were the most commonly used plant part while the most common methods of preparation were infusions and decoctions. Even though we successfully generated an inventory of 310 medicinal plants used to treat cattle diseases, significant knowledge gaps such as the absence of diagnostic methods for the diseases, preparation methods, administration route and plant parts existed for a number of the plants. This fragmented information emphasizes the need for a well-planned and holistic approach when conducting EVM surveys. The need to adhere to good practices and guidelines particularly "The recommended standards for conducting and reporting ethnopharmacological field studies" (137) and "The need for accurate scientific nomenclature for plants" cannot be overemphasized (138). Furthermore, documenting the use of plants in EVM among South African ethnic groups should

embrace indigenous research methodologies in order to gain more cultural insight from the participants. South Africa's unique heritage, both in terms of its rich plant diversity and its cultural traditions, need to be studied, and developed for the benefit of all its people and animals. Furthermore, pharmacological properties studies of EVM plants are a worthwhile endeavor that can contribute to the discovery of new entity to existing drug pools. Establishment of the mechanisms of action remain pertinent to mitigate the drug resistance issues that is increasingly encountered among disease-causing organisms. Toxicology studies must also be strongly incorporated so that potential toxic effects of plants can be identified at early stage of bio-prospecting. In addition, the study of the synergistic effects of plants used in combination would also be beneficial in the development of potent extracts or herbal mixture for resource-poor livestock farmers.

AUTHOR CONTRIBUTIONS

The project was conceptualized by MVC with guidance from AOA and MM. MVC prepared the draft manuscript under the supervision of AOA and MM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.710884/full#supplementary-material

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The Next Step to Further Decrease Veterinary Antibiotic Applications: Phytogenic Alternatives and Effective Monitoring; the Dutch Approach

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Antibiotics are used to control infectious diseases in both animals and humans. They can be life-saving compounds but excessive use in animal husbandry leads to the development of antibiotic resistance which can impact the public health. Since similar antibiotics are used in both animal and human healthcare, it is important to reduce the use of antibiotics in production animals. In the Netherlands policies have been developed aiming for a decrease of antibiotic usage in animals, and alternatives to antibiotics are investigated. Currently, a one-on-one relationship between farmer and veterinarian is successfully implemented and (national) registration of antibiotic usage is mandatory. Unfortunately, after a 70% decrease in antibiotic usage since 2009, this decrease is now stagnating in most sectors. Innovative strategies are required to facilitate a further reduction. One promising option is a focus on farm management and natural alternatives to antibiotics. The Dutch government has invested in the spread of knowledge of natural remedies and good animal management to support animal health via so called Barnbooks for farmers and veterinarians. Another option is the analysis of on-farm antibiotic use to prevent unregistered applications. New (bio)analytical strategies to monitor the correct and complete registration of antibiotic usage have been developed and trial-tested in the Netherlands. Such strategies support a risk-based monitoring and allow effective selection of high-risk (high antibiotic use or illegal antibiotic) users. Both effective monitoring and the availability and knowledge of alternatives is a prerequisite to achieve a further significant decrease in antibiotic veterinary usage.

Keywords: phytogenics, antibiotics, monitoring, Netherlands, strategy

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INTRODUCTION

In the Netherlands, strategies have been developed aiming to decrease use of antibiotics in animals. In response to several outbreaks of multi-resistant bacteria and the resulting societal and political pressure, several compulsory and voluntary measures were initiated to reduce antibiotic usage by farmers and prescription by veterinarians (1, 2). These measures have led to a decrease of antibiotic use of almost 70% in the past 10 years (3). Further decrease might be achieved by optimizing farm management and housing, the application of natural products to improve animals health, and

effective monitoring of antibiotic use and registration. In this overview we discuss the use of plant based products which have become part of farm health plans in the Netherlands and monitoring of antibiotic use to detect non-registered applications.

DUTCH REDUCTION OF ANTIMICROBIALS BY ALMOST 70% NOW STAGNANT IN MOST SECTORS

Antibiotics are used to control infectious diseases in both animals and humans. They can be life-saving compounds but excessive use in animal husbandry contributes to the development of antibiotic resistance which impacts public health. Since similar antibiotics are used in both animal and human healthcare, it is important to reduce the use of antibiotics in production animals (4). In recent decades, these risks to public health have been seen with the identification of livestock associated methicillin-resistant *Staphylococcus aureus* and extended spectrum beta-lactamase-producing bacteria (5, 6). "Therapeutic antimicrobial use in farm animals in the Netherlands doubled between 1990 and 2007" (1). Next to the development of antibiotic resistance, excessive use of antibiotics in livestock may lead to residues in animal products impairing human health. These and other events have contributed to more pressure from the public to increase the monitoring and control of the use of antimicrobials in Dutch farms.

"The Netherlands Veterinary Medicines Authority" (SDa), an independent body, was established in 2010 with the main aim of reducing antimicrobial use in the farm industry (3). The SDa annually reports the use of antimicrobials in farm animals in the Netherlands. The SDa is a partnership between the government, the Royal Dutch Veterinary Association (KNMvD) and livestock industries, with the aim to monitor antibiotic usage on farms and set reduction targets. To achieve this, multiple goals were set comprising of monitoring current antimicrobial use on individual farms and at veterinarian level and creating benchmark indicators to identify high risk users and prescribers. The body was granted the authority to apply disciplinary sanctions and extra monitoring of high users and prescribers by the Dutch Food and Consumer Product Safety Authority (NVWA).

Additional legislation was set in 2014 which prescribed that only veterinarians were allowed to administer antimicrobials. Exceptions are possible under strictly regulated and monitored conditions. The goal was to create closer interaction between veterinarian and farmer, compulsory herd health checks and the presence of a farm health plan and farm therapeutic plan. Currently, a one-on-one relationship between farmer and veterinarian has been successfully implemented and (national) registration of antibiotic usage is mandatory.

The current figures (**Figure 1**) show that the Dutch approach to antimicrobial use reduction set up by the government, farmers and veterinarians has resulted in a reduction of antimicrobials by almost 70% in 2019 compared to 2009. While in some sectors (veal calves) this decrease is still occurring, in other sectors (dairy cattle, broilers, and swine) this decrease is now slowing down or stagnating (3). Further reduction in antimicrobial use will require an animal sector-specific approach as there are different trends in the various sectors. Some sectors (like broiler and pig farms) show many farmers in the low usage category and a small trail of high users. Other sectors (like veal farms) show a generalized high use of antimicrobials, which would therefore justify a more general approach for that whole sector. Strategies to further reduce use of antimicrobials will therefore depend on the sector and will require a broader approach to farm animal health. This will include looking at alternatives to antimicrobials but also housing and ventilation methods, focusing on preventative strategies to yield a further decrease. Also, non-registered use should be prevented and therefore an effective monitoring system is indispensable.

USE OF PHYTOGENICS TO KEEP ANIMALS HEALTHY

Phytotherapeutics are plant-based medicine that were traditionally used to prevent and cure disease in humans and livestock (8–10). Nowadays in animal husbandry phytotherapeutics are mostly not registered as veterinary medicine but used as complementary feed or feed additives. Since the word phytotherapeutics may lead to the suggestion that these products are veterinary medicine, phytogenics (plant derived) products is more appropriate.

Phytogenics can be a huge resource for improving animal health since bioactive plant components can work on gut flora, modulate the immune system, affect digestion and many other systems, exerting a multi-target action (11–13). The control of multi-drug resistance in human and animal pathogens might profit from the multi-target action of plant-based products (14).

The Dutch government invested in providing information for farmers and veterinarians concerning alternatives in management and use of health supporting products. Medicinal plants have been used worldwide for prevention and treatment of diseases in animals and humans for ages. The broad spectrum of plant metabolites represents a huge potential for medication of herbivore and omnivore livestock. While there is a large amount of evidence-based knowledge about medicinal plants published in literature in both English and German language (8), this is hard to access for Dutch farmers.

To make the information on alternatives and their possible application accessible for a wider public, so-called "Barn books" per animal category were published by WFSR in a project funded by the Dutch Ministry of Agriculture, Nature and Food Quality. First books were aimed at organic farmers and focussed on dairy cattle, poultry and pigs. These books are also translated into English as Natural Dairy Cow Health, Natural Swine health, Natural Poultry Health, Guide to keeping your animals healthy with herbs, and other natural products (15–17). These books are freely available via the internet. Since conventional farmers also needed to reduce the use of antibiotics, the series was extended in a governmental project and consists at the moment of books for dairy cows, swine, poultry, turkeys, rabbits, veal calves, dairy

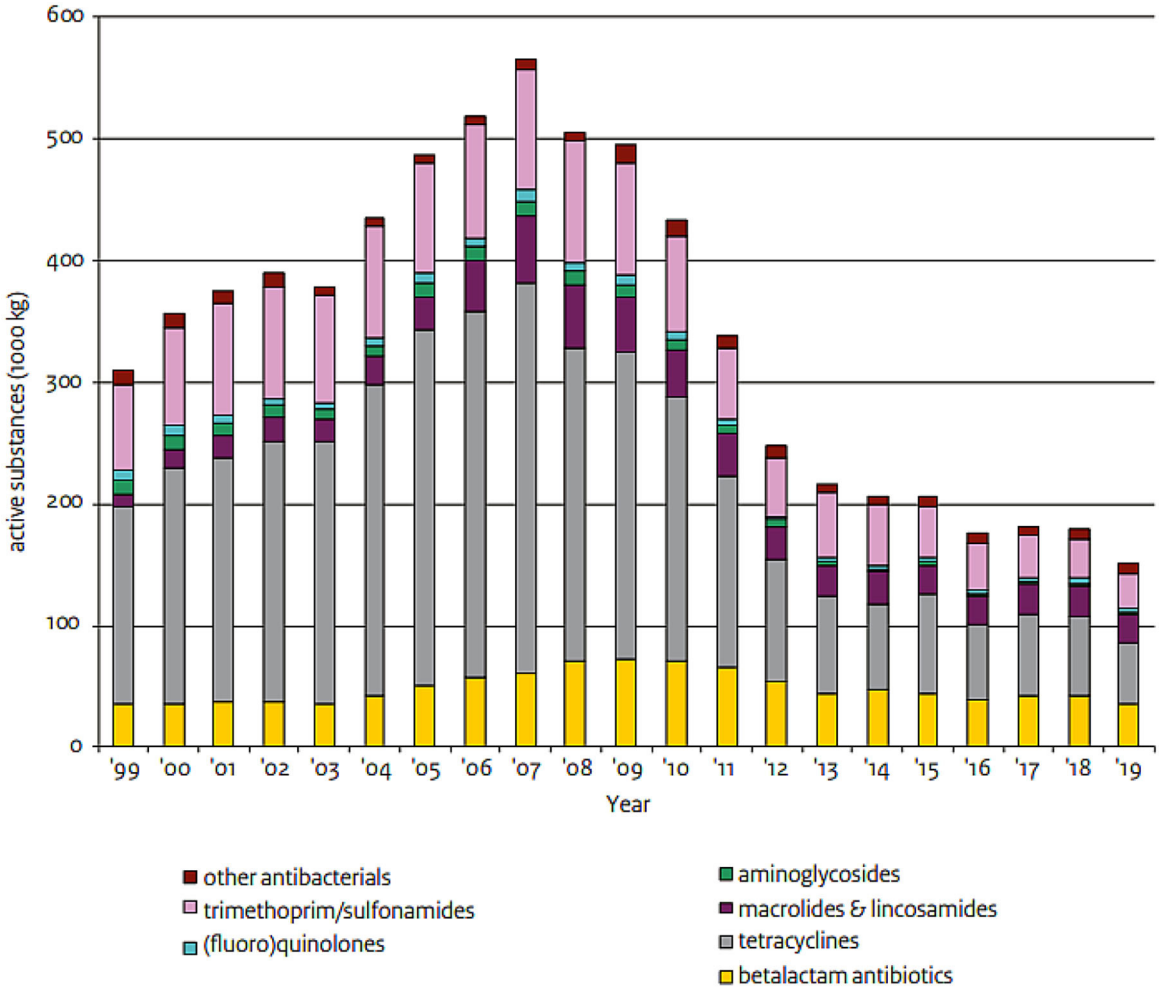

FIGURE 1 | Antimicrobial veterinary medicinal product (AVMP) sales 1999–2019 in kg (thousands). Trends in antibiotic sales from 1999 to 2019 in The Netherlands. Reproduced from NethMap (7).

goats and sheep (18–25). The aim was to provide both farmers and their veterinarians with objective information on products and what to expect from their effects.

The books consist of chapters per life stage of the animal with general management advice and natural products that can support the animals' health or prevent the animals from becoming sick. The products described are complementary feeds or feed additives. In the annexes there is a list of herbs with their main constituents and use, and per product general information and information from research with references is provided. The barn books give information on products for which the supplier provided the full composition of the product and results from field trials and literature. Products with no full disclosure of the composition or without underlying substantiation are not included. The aim was to provide both farmers and their veterinarians with objective information on products and their expected effects.

Natural products used as feed additives have shown to improve zootechnical parameters such as growth performance, feed conversion and gut health in livestock, and so reduce the need for antibiotics (26–31). The use of natural products such as phytogenics and other natural products alone cannot provide the solution for the use of antibiotics in animal husbandry. In animal production most antibiotics are used in young animals (broilers, pigs, turkeys, and veal calves) for disorders of the intestinal or respiratory tract. The immune system of young animals is not yet fully developed in the first weeks of live and in combination with insufficient intake of colostrum, contact to pathogens often gives rise to high morbidity and mortality. Inadequate management, such as long distance transports, fasting, mingling animals from different sources, high stock density, changes in climate, inappropriate diets, and suboptimal hygiene play a role in the susceptibility to disease of these animals. Therefore, other principles that can be used to reduce the need for antibiotics are breeding (slower growing breeds, more resilient animals, optimal instead of maximum production, dual purpose breeds), management (hygiene, feeding, housing, climate, stock density), quality control and chain management

and a quality based payment system (low residue animals get a bonus), and development of antibiotic free production chains. In some aspects the abovementioned principles are already used in organic farming, where due to European regulations the use of antibiotics is limited, production is in most cases lower than in conventional farms, stock density is lower and organic products get a better price. Because the use of antibiotics in organic production is limited by European regulations on organic management, natural healing methods like phytotherapeutics, vitamins, and minerals are used to keep the animals healthy or treat when sick.

ANALYTICAL STRATEGIES THAT SUPPORT AND STIMULATE PRUDENT USE OF ANTIBIOTICS

Next to management and phytogenics, (on-site) analysis of antibiotic residues to check for correct registration of antibiotic usage will prevent unregistered application and thus contribute to the prudent use of antibiotics.

Compulsory registration of antibiotic use is an important step toward monitoring antibiotic use on farm level (1). However, such a measure can only achieve its full potential and effectiveness if registration of antibiotic use is enforced. An important tool that can be applied is the analysis of antibiotic residues in non-invasive matrices (matrices that can be taken from live animals) such as hair and feathers (32, 33). We expect that this tool will become even more effective if analysis is done on-site so that appropriate actions can be taken immediately. This measure will also have a preventive effect and contribute to the prevention of unregistered use of antibiotics.

Routine monitoring of veterinary drug residues is most commonly carried out in the slaughter phase. Animal tissue is sampled, transported to the laboratory and analyzed for violation of the maximum residue limit. Sampling is also done at the farm. Usually urine, milk or eggs depending on animal type and production use are collected. Taking urine samples can be quite time-consuming. Excretion studies have shown that many antibiotics have a relative high excretion rate in urine and that residues can only be detected shortly after administration. Therefore, the analysis of urine samples to check legally correct antibiotic use, is not an economically feasible and practical strategy.

Studies have demonstrated that antibiotic residues are present in hairs and feathers in relatively high concentrations (34). As residue excretion from feathers is slow (32, 34–37) and predominantly occurs during molting, feathers make an ideal matrix for long term monitoring. Studies in broilers demonstrated that a decrease in the concentration of residues in feathers after dispositioning (after administration of antibiotics) is mainly the result of the growth of the animal and the increase of feather weight, rather than excretion of the drugs from the feathers. We previously demonstrated that in practice, feather analysis is an effective strategy to determine the antibiotics that broilers were exposed to throughout their (short) lives (33). This strategy also applies to hairs of calves and swine. Due to these animals having a much longer life compared to broilers, most likely not all antibiotics administered throughout the whole lifetime of the animal can be detected at the slaughter age. Note, that for some antibiotics, residues were even detectable up to 100 days after administration in hair.

As hair and feather samples are rather easy to collect on site and contain as well as retain information about the history of antibiotic use in the animal (33), these samples are ideal for antibiotic use detection to check for correct use and registration. In a pilot study that included 20 broiler farms, two cases of antibiotics residues were detected in feathers for which no recent applications were registered. This pilot demonstrated the potential of using feathers for antibiotic residue detection.

Additional research (not published) demonstrated that, for screening of antibiotics, cotton pads could also be used to obtain samples from calves. Instead of shaving hair (which is an elaborate procedure), these cotton pads were wiped over the animal's neck or back and stored in a tube. The (qualitative) analytical results of the cotton pad samples proved to be comparable to the results of the hair samples taken from the same animals. This demonstrated that also a quick and easy sample taking procedure can be applied for cattle and swine.

Even though the above presented approach has been proven successful in finding non-registered use of antibiotics, a main disadvantage of this approach is that the whole procedure (sampling, transport, and analysis) is time consuming and relatively expensive. We are therefore, currently investigating the application of on-site lateral flow devices (LFD) for easy and quick analysis of antibiotics in hair, feathers and preferably cotton pad swipes.

LFDs for the detection of antibiotics are commercially available (e.g., CHARM sciences inc (Lawrence, MA, USA) and PerkinElmer (Waltham, MA, USA), and can also be easily prepared and adapted in-house, as long as residue specific binding molecules (e.g., antibodies) are available. Samples can easily be extracted on-site with a buffer and a small volume of the extract is then transferred onto a LFD. After several minutes, a qualitative result is obtained. LFDs can detect a single specific compound or compound class, but multiplex devices are available. This development allows inspectors (after a short training) to carry out a quick assessment of antibiotic exposure on site. After screening multiple animals, results of the LFDs are compared with the farms antibiotic use registration system and within 15 min the inspector can obtain a good indication of the status of the farm with regard to antibiotic use and registration thereof. In case no discrepancies are observed, the inspector can quickly move on to the next farm. If differences are observed, the inspector can discuss this with the farmer and, if deemed necessary take additional samples for laboratory analysis. This approach is very promising and, as it is a very quick and easy approach, is expected to have a preventive effect of misuse of antibiotics in animal husbandry. The large scale implementation to LFD application on hairs and feathers at farm level will probably present new challenges. These especially regard the application of the test in a potentially contaminated environment and the legal status of the result. The latter includes the validation of the LFD under field conditions. Those hurdles are expected

to be overcome in the coming years. Nonetheless, the currently proposed strategy can easily be applied at laboratory level already.

CONCLUSION

In conclusion, in the Netherlands an important decrease of antibiotic use in animal husbandry has been achieved since 2009. However, it is currently plateauing even though a further reduction is urgent. New approaches are suggested to further decrease antibiotic usage. The differences between antimicrobial use reduction achieved per sector suggest that approaches should probably be sector and sometimes farm specific. These could include optimized management and raising awareness for the risk of antimicrobial resistance. Additionally, we advocate the public availability of knowledge on natural products as alternatives and an effective strategy to monitor and enforce correct use and registration of antibiotic applications. These are promising tools to achieve lower antibiotic inputs and additional monitoring of antibiotic use data must demonstrate their effectiveness.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: data available via the references.

AUTHOR CONTRIBUTIONS

MG is the responsible author and wrote the part: use of phytogenics to keep animals healthy. NC wrote the part about the Dutch policy: Dutch reduction of antimicrobials by almost 70% now stagnant in most sectors. BB wrote the part: analytical strategies that support and stimulate prudent use of antibiotics. All authors have contributed the whole article.

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Anticoccidial Activity of Qinghao Powder Against *Eimeria tenella* in Broiler Chickens

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Artemisia annua (AAH) is traditionally used as an anti-malarial, expectorant and antipyretic Chinese medicine. The aim of this study was to explore the therapeutic effect of Qinghao Powder (QHP) on chicken coccidiosis, evaluate the safe dosage of QHP, and provide test basis for clinical medication. High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) were used to detect artemisinin in Qinghao Powder (QHP) for quality control. The level of artemisinin in QHP was 81.03 mg/g. A total of 210 chicks (14 days of age) were divided randomly into seven groups: three QHP treatments (0.15, 0.30, and 0.60 g/kg), a toltrazuril control (1.00 mL/L), a sulfachloropyrazine sodium control (SSC, 0.30 g/L), an *E. tenella*-infected control, and a healthy control group. All the groups were inoculated orally with 7×10^4 *E. tenella* oocysts except for the healthy control group. After seven days of administration, compared with the infected control group, chicks which were administered QHP, SS, and toltrazuril showed less bloody feces, oocyst output, and cecal lesions, and the protection rates were improved. The maximum rBWG and ACI were found in the SS-medicated group, followed by the groups medicated with 0.60 and 0.30 g/kg QHP. Therefore, a 0.30 g/kg dose level of QHP in the feed was selected as the recommend dose (RD) in the target animal safety test, in which 80 broiler chicks (14 days of age) were randomly divided into four major groups (I-healthy control group; II-1× RD; III-3× RD; IV-6× RD), with each group subdivided into two subgroups (A and B) consisting of 10 chicks each. After 7-day (for sub-group A) or 14-day (for sub-group B) administration, compared with the healthy control, treatment-related changes in BWG, feed conversion ratio (FCR), relative organ weight (ROW) of the liver, WBC counts, and levels of RBC, HGB, ALT, AST, and TBIL were detected in the 3× and 6× RD groups. No differences were noted in necropsy for all doses, and histopathological examinations exhibited no QHP-associated signs of toxicity or abnormalities in the liver or kidney. The findings suggest that QHP at a dose of 0.30 g/kg feed would be appropriate for therapy and intermittent treatment of *E. tenella*-infected chicks, the dosage in clinical applications should be set according to the recommended dose to ensure animal safety.

Keywords: *Eimeria tenella*, Qinghao Powder (QHP), recommended dose (RD), safety test, target animal

INTRODUCTION

Chicken coccidiosis, a disease caused by apicomplexan protozoa of the genus *Eimeria*, is a significant problem in the poultry industry. There are seven *Eimeria* species affecting chickens, including *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, and *Eimeria tenella*. The parasites multiply in the intestinal epithelia, destroying the cells and reducing digestive capacity and nutrient absorption in the bird, with *Eimeria tenella* being highly pathogenic and causing caecal coccidiosis (1–5). Coccidiosis is responsible for 6–10% of all broiler mortalities, and the annual loss caused by *Eimeria* infection to the poultry industry is estimated at more than $3 billion (6, 7). At present, chicken coccidiosis has largely been controlled through the use of chemoprophylaxis and anticoccidial drugs added to feed, but there are complications due to the emergence of drug resistance and the toxic side effects of such additives on animal health (8–10). Increasing the development of drug-resistant coccidial species has stimulated the search for alternative control methods or new drugs, and this has become a top priority for the poultry industry (11–13). One alternative is the use of live virulent or attenuated vaccines or recombinant vaccines (14–16). However, live vaccines, particularly virulent ones, may have short-term adverse effects on chicken growth rate. Recombinant vaccines are still in the early stages of development. Therefore, until vaccines become more sophisticated, the use of anticoccidial drugs will continue.

Artemisiae annuae herba (AAH) is the dried aerial part of *Artemisia annua* L. [Asteraceae], a plant that has been traditionally utilized as an antimalarial, expectorant, and antifebrile compound in Chinese medicine. Moreover, AAH has anticoccidial properties when used alone or as the main herb in a complex formulation during treatment; however, as an animal coccidiostat, the content of the artemisinin within the crude extract of AAH or AAH complex has always been low, and the clinical use of artimisinin has been restricted due to the complexity of the synthetic route and the high synthetic cost. The findings from previous studies indicated that high doses of artemisinin can have adverse side effects such as neurotoxicity, renal toxicity, and cardiotoxicity in animals; the n-hexane extract of *Artemisiae annuae herba* at 0.50 g/kg of dose in feed reduced food intake and weight gain in chicks, and artemisinin administered continuously for 16 days at a high dose significantly inhibited the body weight gain of chickens (17–19). Qinghao Powder (QHP) prepared from the petroleum ether extract of the traditional Chinese medicine *Artemisiae annuae herba* has been shown to be effective against chicken coccidiosis (20, 21). As a plant-derived medicine, there are no scientific reports available concerning the content of active ingredients, anticoccidial activity, or safe dosage range. In the present study, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) were used to detect artemisinin in QHP for quality control; furthermore, a pathological model of chick coccidiosis was produced after *E. tenella* (Guangdong strain) was inoculated into chicks to determine the therapeutic effects and optimal recommended dose (RD) of QHP. The safe RD in target animals was further assessed for consequent clinical drug security according to the CVDE Guidelines (22). These experiments will provide a basis for the subsequent application of QHP and may assist in the approval of new animal drugs and preparations against chicken coccidiosis.

Abbreviations: QHP, Qinghao Powder; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PI, post infection; HC, healthy control group; IC, infected control group; TC, toltrazuril control; SS, sulfachloropyrazine sodium; SSC, sulfachloropyrazine sodium control; BWG, body weight gain; rBWG, relative body weight gain; ACI, anticoccidial index; RBC, red blood cells; HGB, hemoglobin concentration; HCT, hematocrit; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell; Lym, lymphocyte; ML, monocytes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; TBIL, total bilirubin; BUN, blood urea nitrogen; CER, creatinine.

MATERIALS AND METHODS

Drugs and Reagents

Petroleum ether, acetic ether, acetic acid, and n-hexane were purchased from Tianjin Chemical Reagent Company, China. Ethanol and xylene were purchased from Shanghai Chemical Reagent Company, China. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (England); artemisinin was purchased from the National Institutes for Food and Drug Control (Purity of all $\geq$ 98%, China).

QHP (batch number: 20190520) was purchased from Heima Animal Pharmaceutical Co., Ltd., Henan Province, China. The positive control drug was toltrazuril (2.5%, w/v, batch number: 20190329, Bayer (Sichuan) Animal Health Co., Ltd., China). Sulfachloropyrazine sodium (SSC, 30%, w/w, batch number: 20190620) was purchased from Chongqing Yongjian Biotechnology Co., Ltd., China. Artemisinin from the petroleum ether extract of *Artemisiae annuae herba* was chosen to be a biomarker in TLC and HPLC evaluation for quality control.

Thin Layer Chromatography Analysis (TLC)

As the active ingredient of *Artemisiae annuae herba*, the artemisinin in QHP was assayed by TLC according to the standardized experimental protocols of the Veterinary Pharmacopoeia of P. R. China (23). Petroleum ether (60–90°C)–acetic ether (4:5) was used as the developing solvent for artemisinin in silica gel–GF254 plates (Qingdao Haiyang Chemical Reagent Factory, China); 10% sulfuric acid ethanol solution containing 2% vanillin was used as the color developing reagent, and artemisinin was used as the standard preparation.

High Performance Liquid Chromatography Analysis(HPLC)

The contents of artemisinin in QHP were determined by HPLC. Quantitative analysis of artemisinin in QHP was performed on the Agilent 1290 Infinity apparatus comprising two solvent delivery systems and a photodiode array detector (Agilent, USA). The column was an Agilent ZORBAX SB-C18 chromatographic column (4.6 mm × 250 mm, 5.0 μm). The mobile phase consisted of acetonitrile and H_2O (60: 40), and the pH value was 6.8–7.2. Reagents were filtered through a Millipore 0.45 mm filter and degassed prior to use. The entire run was carried out by

TABLE 1 | Effects of Qinghao Powder on bloody feces, oocyst output, and protection rate of chicks inoculated with *Eimeria tenella*.

Groups/Drug concentration	No.	Treatments	Bloody feces[A]				Total blood feces	Oocyst output ($\times 10^6$)[B]	Protection rate (%)[C]
		Day 15 ~ Day 21	PI day 4	PI day 5	PI day 6	PI day 7			
1-HC Healthy control	30	Feed without QHP and anticoccidial	0	0	0	0	0	0.00 ± 0.00^{b}	100.00
2-IC Infected control	30	Feed without QHP and anticoccidial	3.6	4.0	4.0	4.0	15.6	17.62 ± 2.56^{a}	0.00
3-TC 1.00 mL/L toltrazuril	30	Feed with 1.00 mL/L of toltrazuril for 4 d	3.0	3.6	4.0	4.0	14.6	8.63 ± 1.95^{bc}	51.02
4-SSC 0.30 g/L of SS	30	Feed with 0.30 g/L of SS for 4 d	2.4	3.0	3.0	3.0	11.4	0.90 ± 0.32^{b}	94.89
5-Low dose 0.15 g/kg of QHP	30	Feed with 0.15 g/kg of QHP for 7 d	3.0	3.2	3.6	3.6	12.6	9.76 ± 2.38^{bc}	44.60
6-Middle dose 0.30 g/kg of QHP	30	Feed with 0.30 g/kg of QHP for 7 d	2.8	3.2	3.2	3.2	12.4	4.43 ± 1.52^{bc}	74.85
7-High dose 0.60 g/kg of QHP	30	Feed with 0.60 g/kg of QHP for 7 day	2.4	2.8	3.2	3.0	11.4	3.90 ± 1.37^{bc}	77.86

QHP, Qinghao powder; PI, post infection; HC, healthy control group (non-treated and non-infected); IC, infected control group (non-treated and E. tenella infected); TC, toltrazuril control; SS, sulfachloropyrazine sodium; SSC, sulfachloropyrazine sodium control.

[A]Bloody diarrhea score (median, IQR) of each group on PI day 4–7 after challenge with E. tenella.

[B]Means of three pens, data were presented as means ± SD. [a,b,c]Columns with different superscripts present significant differences ($P < 0.05$).

[C]Protection rate of each group on PI day 8. Protection rate (%) = (oocyst output of infected/unmedicated control group – oocyst output of medicated groups) ÷ (oocyst output of infected/unmedicated control group) × 100.

gradient elution at a flow rate of 1.0 mL/min; the detection wavelength was set at 210 nm; the column was maintained at 35°C, and the injection volume was 10 μL. Data collection and quantification were performed with Agilent Open LAB A.02.02 CDS ChemStation (Agilent, USA). The peak of artemisinin was identified by comparison with chemical standards.

Ethics Statement

All of the experimental procedures were performed according to the principles of the Center for Veterinary Drug Evaluation (CVDE), Ministry of Agriculture, China (22). All of the animal experiments were conducted in strict accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (24). All of the applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (Approval No. LZMY 2020-016).

STUDY DESIGN

Eimeria tenella Oocysts (Guangdong Strain)

The oocysts were isolated from chicks that had died from *E. tenella* infection in 1996 in Huadu, Guangdong Province, China, as confirmed by microscopic examination and sequence analysis of the rRNA gene internal transcribed spacer regions. The strain were maintained in the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, CAAS. The oocysts were propagated in the broiler chicks without *E. tenella* infection by oral infection, and the feces were collected on post-infection (PI) days 6, 7, 8, and 9. The unsporulated oocysts were sporulated by placing in 2.5% $K_2Cr_2O_7$ solution at suitable humidity and temperature (28°C). Sporulated oocysts were cleaned with water and counted by the McMaster technique described by Foreyt (25). The required concentration of the sporulated oocysts (70,000/mL) was maintained with phosphate buffered saline.

Birds

A total of 290 one-day-old as-hatched Lingnan yellow-feathered broiler chicks (Lanzhou Hualong Commercial Hatchery) of both sexes were used. Chicks were reared under coccidia-free conditions and fed commercial food without coccidiostat additives for 14 days during the study (*ad libitum*). Chicks were reared under the following conditions: temperature (23 ± 2°C), relative humidity (55 ± 15%), and ventilation (air exchange rate of 18 cycles/h) (26).

Anticoccidial Test

At 14 days of age (the day of challenge), the broiler chicks ($n = 210$) free from coccidian infection were weighed individually and randomly divided into seven treatments (1–7) with three pens containing 10 chicks each. Each pen was allocated to a large cage with a single tray per pen to catch the fecal material. All of the groups were inoculated orally with 7.0×10^4 sporulated oocysts except for the healthy control group (1-HC). At 15 days of age, all of the chicks except for those in the healthy control (1-HC) and infected control group (2-IC) began drug treatment for 7 or 4 days (**Table 1**). At

22 days of age (post-inoculation day 8, PI day 8), after weighing the surviving chicks individually, all of the chicks were euthanized for the grading of cecal lesions, and the survival rate was calculated for each group. The clinical observations of bloody diarrhea and mortality for all the chicks were recorded daily throughout the experimental period. Treatments were as follows: 1-HC (healthy/negative control group; non-treated and non-infected); 2-IC (infected/positive control group; non-treated and *E. tenella*-infected); 3-TC (toltrazuril control, 1.00 mL/L water); 4-SSC (sulfachloropyrazine sodium control, 0.30 g/L water); 5-LG (low-dose group, 0.15 g/kg feed); 6-MG (middle-dose group, 0.30 g/kg feed); 7-HG (high-dose group, 0.60 g/kg feed).

Body weight gain (BWG) and survival rate (%): chicks from each treatment were weighed individually on day 14 (the day of inoculation) and day 22 (PI day 8). The individual and mean body weight gains were calculated for the period of days 14–22. The relative body weight gain (rBWG) and survival rate were calculated as follows:

BWG rate (%) = (final body weight – initial body weight) ÷ initial body weight × 100.

rBWG (%) = (BWG rate of the infected/unmedicated control or drug-treated group ÷ BWG rate of healthy control group) × 100.

Survival rate (%) = (number of surviving chicks in each group ÷ number of initial chicks in each group) × 100.

Fecal score and oocyst output in the feces: fecal droppings were examined visually for bloody diarrhea during 4–7 days PI and scored on a scale of 0–4 based on the evaluation standard of Suo and Li (27). Furthermore, on days 6, 7, and 8 after inoculation, the total daily fecal output of each pen of chicks was collected, and the daily oocyst production was determined using the McMaster technique (25, 28).

Cecal lesion score and oocyst value: on PI day 8, all the surviving chicks were euthanized, and the ceca were removed and opened. The infected ceca were examined and scored (from 0 to 4) according to the method described by Johnson and Reid (29). Lesion score = the average lesion score in each group ×10.

The cecal contents were ground, and the complete cecum contents of each chick were pooled. The total number of oocysts was determined from duplicate counts of diluted samples of homogenates using a hemocytometer counting technique (30). The results were expressed as OPG based on the method of JIAO (31).

Oocyst ratio = (OPG in healthy control or drug-treated group) ÷ (OPG in infected/unmedicated control group) × 100.

The anticoccidial index (ACI) established by Merk and Dohome (32) was calculated as ACI = (rBWG + survival rate) × 100 – (lesion score + oocyst value).

The total oocyst output per bird from feces and cecum was used to calculate the protection rate by the formula Protection rate (%) = (oocyst output of infected/unmedicated control group–oocyst output of healthy control or medicated groups) ÷ (oocyst output of infected/unmedicated control group) × 100 (14, 33, 34).

Safety Test

This trial was conducted in line with the Guidelines on the Target Animal Safety Tests of Veterinary Traditional Chinese Medicines and Natural Medicines (22). Based on RD from the above-mentioned anticoccidial test, broiler chicks (n = 80, 14 days old) free from coccidian infection were selected as the target animals and were randomly divided into four major groups (I–IV) after weighing individually; each group was divided into two subgroups (A and B) consisting of 10 chicks each. At 14 days of age, chicks were fed with QHP at 1×, 3×, and 6× the recommended dose for 7 days (for the A sub-group) or 14 days (for the B sub-group). The groups were classified as I-A, I-B (healthy/negative control group, *ad libitum*); II-A, II-B (1× recommend dose, 1× RD); III-A, III-B (3× recommend dose, 3× RD); IV-A, IV-B (6× recommend dose, 6× RD). The behavior, feed intake, and deaths of the chicks were observed and recorded every day after administration. The weight on day 14 was considered as the initial weight. The feed conversion ratio (FCR) was calculated as grams of feed consumed to produce one gram of live weight. FCR was determined from the 3rd to 4th week of age.

On the 7th or 14th days after administration, the surviving chicks from subgroup A (only on the 7th day) or subgroup B (only on the 14th day) were individually weighted and euthanized. Blood was collected via cardiac puncture into EDTA-containing and non-heparinized tubes for hematological (automatic hematology analyzer, Sysme XT-1800i, China) and biochemical parameters assays (automatic blood biochemical detector, Olympus AU640, Japan). White blood cells (WBC), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT, %), mean corpuscular hemoglobin concentration (MCHC), lymphocytes (Lym), monocytes (ML), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), total bilirubin (TBIL), blood urea nitrogen (BUN), and creatinine (CRE) were measured.

After blood collection, all of the organs were examined and observed carefully, and macroscopic pathological changes were recorded. The selected organs (liver, kidney, spleen, heart, lung) were removed and weighed individually. The selected tissue samples (liver and kidney) were fixed with 10% buffered formalin solution and underwent routine histological processes for paraffin embedding and light microscopic examination. The relative organ weight (ROW) was calculated as organ weight (OW) as a percentage of body weight (BW).

Statistical Analysis

The data were analyzed with the SPSS software program version 19.0 (IBM-SPSS Inc., Chicago, IL, USA). The bloody diarrhea score and lesion score of each group were compared by the non-parametric Kruskal-Wallis H test, and the results were presented as median (IQR, inter-quartile range). The parameters of oocyst output, body weight for each time point, body weight gains, relative organ weights (ROW), and biochemical and hematological indexes were analyzed by one-way ANOVA, followed by least significant difference (LSD) and Student's two-tailed t-test for the comparison between the test and control

group, and Dunnett's test when the data involved three or more groups. Data are expressed as the mean ± standard deviations, *P*-values <0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

The Chemical Component Analysis of Qinghao Powder (QHP)

According to TLC analysis, artemisinin was present in QHP (**Figure 1**). As the active compound of *Artemisiae annuae herba* (AAH), artemisinin was present in QHP at 81.03 mg/g as per HPLC analysis (**Figure 2**).

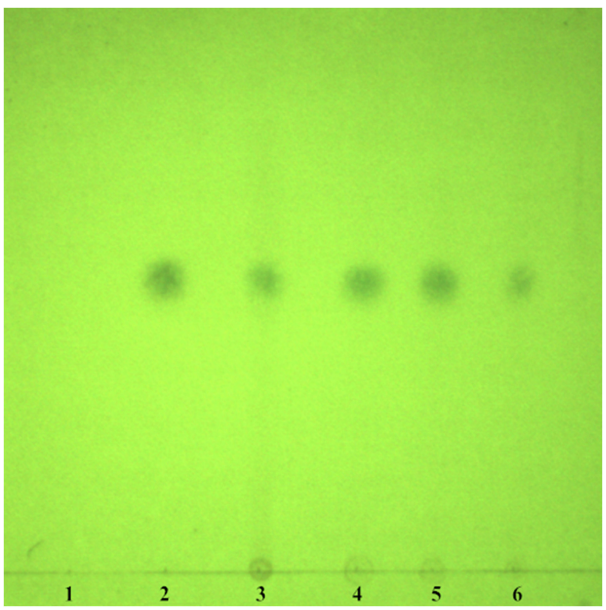

FIGURE 1 | TLC chromatogram for detecting artemisinin in QHP. (1) Negative control. (2) Artemisinin. (3) Standard Chinese herbal medicine. (4-6) Samples of QHP.

Anticoccidial Test

As shown in **Tables 1**, **2**, bloody feces were observed in all infected groups after challenge, and 21 dead chicks were found during PI days 4–7. The oocyst output of groups administered with QHP, sulfachloropyrazine sodium, and toltrazuril were significantly less than that of infected control group ($P < 0.05$, $P < 0.01$). Furthermore, chicks treated with 0.30 g/L sulfachloropyrazine sodium and 0.60 g/kg QHP, which excreted less bloody feces and got higher protective rate among 5 infected groups. Among the drug medicated groups, the maximum rBWG was observed in the group administered with sulfachloropyrazine sodium (0.30 g/L water), followed by the groups administered with 0.30 g/kg feed QHP and 0.60 g/kg feed QHP. The lowest rBWG was observed in the group medicated with 1.00 mL/L toltrazuril.

Chicks in the infected/non-treated group (2-IC) displayed the most severe swelling in the cecum. Cecal lesions were also found in all chicks in other infected groups, and the degree of damage was lower than that of the infected control group. No obvious lesions in other organs were found in all groups. Chicks in the five drug-treated groups showed a reduction in oocyst production; the maximum reduction was found in the sulfachloropyrazine sodium medicated group followed by the groups medicated with 0.60 g/kg QHP and 0.30 g/kg QHP. The anticoccidial activity in the sulfachloropyrazine sodium and QHP (at doses of 0.60 g/kg feed and 0.30 g/kg feed) treatments were superior to toltrazuril treatment in terms of oocyst output, protection rate, rBWG, cecal damage, and ACI values.

Safety Test

No deaths or abnormal changes in behavior, clinical condition, or feed intake occurred in chicks during the experimental period. There were no visible pathological changes in the heart, liver, lung, spleen, kidney, or other organs 7 or 14 days after administration. Histopathological examination of the liver and kidney revealed no abnormal pathological lesions in

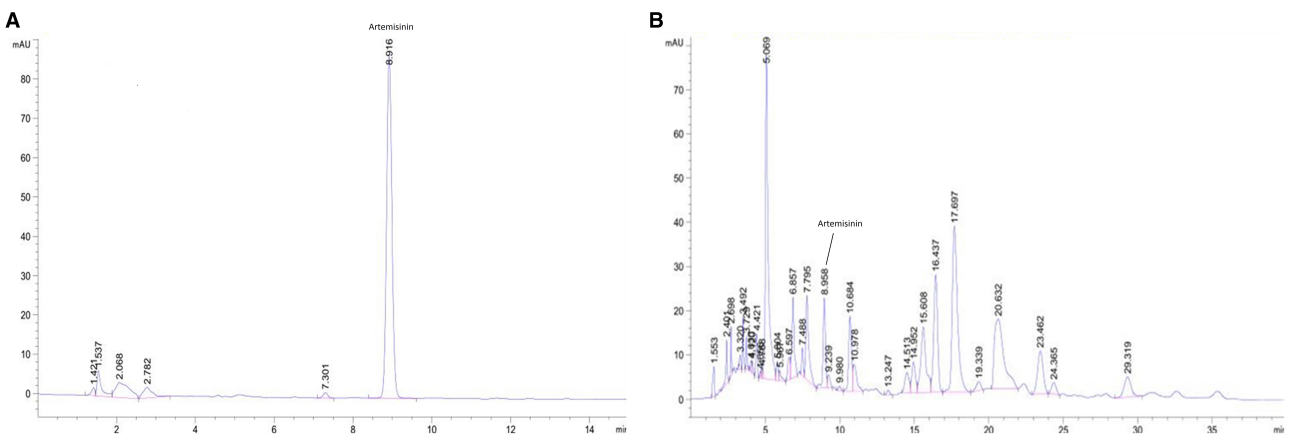

FIGURE 2 | HPLC chromatogram for detecting artemisinin in QHP. **(A)** Artemisinin control. **(B)** Sample of QHP.

TABLE 2 | Effects of Qinghao Powder on the rBWG, lesion score, and ACI in chicks against *Eimeria tenella* ($n = 30$).

Groups/Drug concentration	No.	Initial body weight (g)[A]	Final body weight (g)[B]	rBWG (%)[C]	Survival rate (%)[D]	Lesion score[E]	Oocyst value[F]	Anticoccidial index (ACI)[G]
1-HC Healthy control	30	302.53 ± 29.61[a]	453.76 ± 20.85[a]	100	100	0	0	200
2-IC Infected control	30	303.68 ± 28.56[a]	404.65 ± 26.07[bc]	66.82	80.00	37.50	40	69.32
3-TC 1.00 mL/L toltrazuril	30	297.53 ± 27.45[a]	382.33 ± 29.55[bc]	56.74	83.33	28.75	10	101.32
4-SSC 0.30 g/L of SS	30	303.78 ± 27.88[a]	429.95 ± 34.01[a]	84.29	100.00	28.25	0	156.04
5-Low dose 0.15 g/kg of QHP	30	302.92 ± 27.54[a]	406.13 ± 32.64[b]	68.87	80.00	29.50	20	99.37
6-Middle dose 0.30 g/kg of QHP	30	306.59 ± 29.15[a]	420.58 ± 35.22[b]	75.75	86.66	28.25	5	129.16
7-High dose 0.60 g/kg of QHP	30	298.55 ± 28.87[a]	417.96 ± 40.28[b]	75.30	100.00	26.25	5	144.05

HC, healthy control group (non-treated and non-infected); IC, infected control group (non-treated and E. tenella infected); TC, toltrazuril control; SS, sulfachloropyrazine sodium; SSC, sulfachloropyrazine sodium control; BWG, body weight gain; rBWG, relative body weight gain.

[A,B]Data were presented as means ± SD. [a,b,c]Values with different superscripts in the same column differ significantly ($P < 0.05$).

[C]rBWG (%) = (BWG of the infected/unmedicated control or drug-treated group ÷ BWG of healthy control) × 100.

[D]Survival rate (%) = (number of surviving chicks in each group ÷ number of initial chicks in each group) × 100.

[E]Lesion scores (median, IQR) of cecum examined on the PI day 8.

[F]Oocyst value of each group on PI day 8. Oocyst value = 0 (an oocyst ratio of 0–1%); oocyst value = 5 (an oocyst ratio of 1–25%); oocyst value = 10 (an oocyst ratio of 26–50%); oocyst value = 20 (an oocyst ratio of 51–75%); and oocyst value = 40 (an oocyst ratio of 76–100%); Oocyst ratio = (OPG in healthy control or drug-treated group) ÷ (OPG in infected/unmedicated control group) × 100%; OPG, oocyst per gram.

[G]Anticoccidial index (ACI) of each group. ACI = (rBWG + survival rate) × 100 − (lesion score + oocyst value).

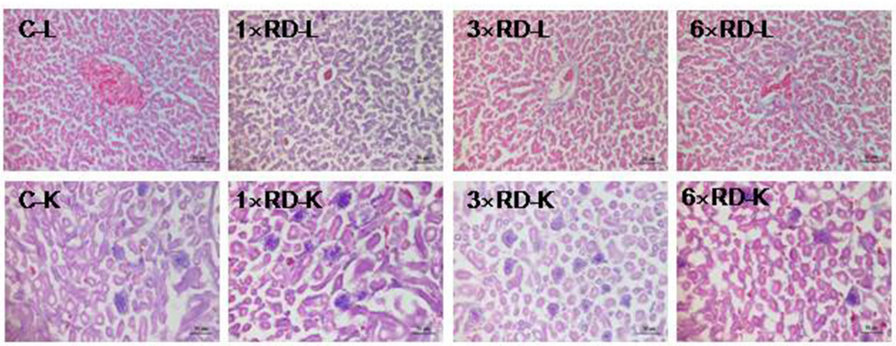

FIGURE 3 | Histopathological analysis of organs in the control (C) and three QHP-treated groups (1× recommend dose, 1× RD; 3× recommend dose, 3× RD; 6× recommend dose, 6× RD) after 7-day administration (H&E stained); livers (L, 100×); kidney (K, 100×). Scale bar = 50 μm.

the QHP-treated chicks compared with the controls, as shown in **Figures 3**, **4**.

Compared with the control, after 7- or 14-day administration, there were significant differences in the body weight gain (BWG) of chicks in 3× or 6× RD groups ($P < 0.05$, $P < 0.01$); moreover, lower rBWG and higher feed conversion ratios (FCR) were exhibited in these two groups at 14th day after administration, but similar BWG and lower FCR were observed in the 1× RD group (**Table 3**). The results of ROWs showed that there were no significant differences between the chicks in three dose groups of QHP and the control group after 7 or 14 days of administration (except for ROW of liver in the 6× RD group at 14th day after administration, $P < 0.05$) (**Table 4**). Furthermore, no significant differences were observed in any of the hematological/biochemical indexes of the two QHP-treated groups (3× RD and 1× RD) after 7 days of administration; while the total counts of RBC and WBC, as well as the levels of ALT, AST, and TBIL in the 6× RD group, which was significantly different to the control at 7th or 14th day after administration ($P < 0.05$) (**Table 5**).

DISCUSSION

Artemisiae annuae herba (AAH) exhibits good clinical efficacy in treatments as an antimalarial, expectorant, or antifebrile agent in Chinese traditional medicine. In addition, artemisinin exerts control effects against toxoplasmosis, chicken coccidia,

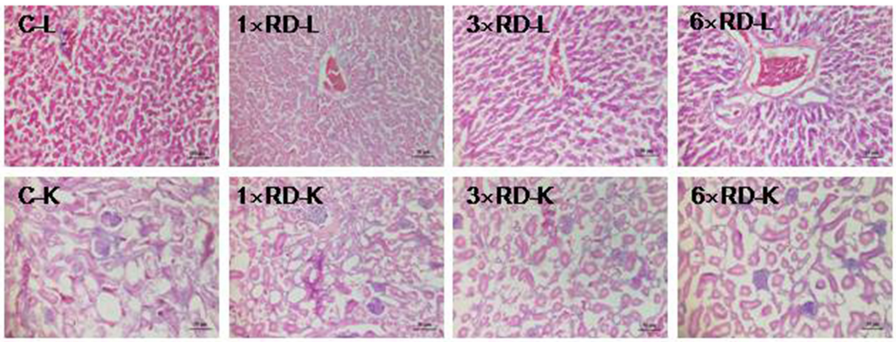

FIGURE 4 | Histopathological analysis of organs in the control (C) and three QHP-treated groups (1× recommend dose, 1× RD; 3× recommend dose, 3× RD; 6× recommend dose, 6× RD) after 14-day administration (H&E stained); livers (L, 100×); kidney (K, 100×). Scale bar = 50 μm.

TABLE 3 | Effect of Qinghao Powder on growth performance of chicks in the safety test ($n = 10$).

Treatments	Initial BW (g)[A]	Final BW (g)[B]	BWG (g)[C]	rBWG (%)[D]	Feed intake (g)[E]	FCR[F]
7th day after administration						
Control	238.87 ± 12.83[a]	387.59 ± 13.03[a]	142.75 ± 20.37[a]	100	245.30	1.718
1× RD (0.30 g/kg)	241.26 ± 12.28[a]	389.57 ± 12.54[a]	143.34 ± 19.79[a]	100.41	257.69	1.798
3× RD (0.90 g/kg)	242.45 ± 14.79[a]	402.54 ± 15.06[a]	157.17 ± 13.28[b]	110.10	279.56	1.779
6× RD (1.80 g/kg)	241.60 ± 12.53[a]	399.08 ± 16.99[a]	151.42 ± 18.95[b]	106.07	288.07	1.902
14th day after administration						
Control	238.87 ± 12.83[a]	547.35 ± 22.84[a]	301.58 ± 21.36[a]	100	545.26	1.808
1× RD (0.30 g/kg)	241.26 ± 12.28[a]	546.77 ± 21.67[a]	300.60 ± 20.36[a]	99.68	537.60	1.788
3× RD (0.90 g/kg)	242.45 ± 14.79[a]	485.30 ± 20.70[bc]	237.95 ± 24.38[bc]	78.90	537.45	2.259
6× RD (1.80 g/kg)	241.60 ± 12.53[a]	464.79 ± 21.08[bc]	216.11 ± 22.25[bc]	71.66	529.17	2.449

RD, recommend dose; BWG, body weight gain; rBWG, relative body weight gain; FCR, feed conversion ratio.
[A–C]Data were presented as means ± SD, [a,b,c]Values with different superscripts in the same column differ significantly ($P < 0.05$).
[D]rBWG (%) = (BWG of the drug-treated group ÷ BWG of control group) × 100.
[E]The feed intake of each group was measured by subtracting the residual feed weight from the offered feed weight during the trial.
[F]FCR was calculated as grams of feed consumed to produce one gram of live weight. FCR = feed intake (g)/BWG (g).

TABLE 4 | Effect of Qinghao Powder on the relative organ weight (ROW) of chicks in the safety test ($n = 10$).

Indexes	Groups and treatments (g/kg)			
	Control	1× RD (0.30 g/kg)	3× RD (0.60 g/kg)	6× RD (1.80 g/kg)
7th day after administration				
Heart[A]	0.75 ± 0.08[a]	0.72 ± 0.05[a]	0.74 ± 0.09[a]	0.75 ± 0.10[a]
Liver[B]	2.52 ± 0.06[a]	2.48 ± 0.07[a]	2.44 ± 0.08[a]	2.46 ± 0.09[a]
Spleen[C]	0.13 ± 0.05[a]	0.12 ± 0.06[a]	0.14 ± 0.08[a]	0.11 ± 0.03[a]
Lung[D]	0.67 ± 0.09[a]	0.67 ± 0.10[a]	0.69 ± 0.04[a]	0.71 ± 0.05[a]
Kidney[E]	0.83 ± 0.07[a]	0.82 ± 0.06[a]	0.82 ± 0.07[a]	0.84 ± 0.10[a]
14th day after administration				
Heart[A]	0.64 ± 0.13[a]	0.63 ± 0.10[a]	0.65 ± 0.11[a]	0.66 ± 0.09[a]
Liver[B]	2.65 ± 0.12[a]	2.63 ± 0.08[a]	2.63 ± 0.13[a]	2.52 ± 0.10[b]
Spleen[C]	0.11 ± 0.05[a]	0.13 ± 0.04[a]	0.13 ± 0.06[a]	0.12 ± 0.07[a]
Lung[D]	0.71 ± 0.16[a]	0.68 ± 0.21[a]	0.71 ± 0.18[a]	0.69 ± 0.19[a]
Kidney[E]	0.82 ± 0.20[a]	0.83 ± 0.17[a]	0.82 ± 0.15[a]	0.81 ± 0.21[a]

[A–E]Data were presented as means ± SD. [a,b]Values with different superscripts in the same row differ significantly ($P < 0.05$).

TABLE 5 | Effects of Qinghao Powder on hematological and serum biochemical indexes of chicks in the safety test ($n = 10$).

Indexes	Groups and treatments (g/kg)			
	Control	1× RD (0.30 g/kg)	3× RD (0.60 g/kg)	6× RD (1.80 g/kg)
7th day after administration				
RBC ($\times 10^{12}$/L)^{A}	2.53 ± 0.10^{a}	2.47 ± 0.26^{a}	2.53 ± 0.25^{a}	2.59 ± 0.30^{a}
HGB (g/L)^{B}	94.60 ± 5.82^{a}	95.60 ± 7.88^{a}	97.00 ± 6.86^{a}	98.60 ± 7.74^{a}
HCT^{C}	32.06 ± 2.15^{a}	31.04 ± 2.25^{a}	31.12 ± 2.91^{a}	32.45 ± 2.37^{a}
MCHC (g/L)^{D}	270.00 ± 20.51^{a}	272.00 ± 27.38^{a}	275.80 ± 28.01^{a}	280.00 ± 27.43^{a}
WBC ($\times 10^{9}$/L)^{E}	253.10 ± 18.24^{a}	256.26 ± 15.32^{a}	259.14 ± 23.50^{a}	254.50 ± 20.33^{a}
Lym ($\times 10^{9}$/L)^{F}	10.50 ± 1.22^{a}	10.96 ± 1.18^{a}	11.22 ± 1.23^{a}	10.80 ± 1.25^{a}
ML^{G}	1.13 ± 0.20^{a}	1.16 ± 0.22^{a}	1.28 ± 0.25^{a}	1.23 ± 0.28^{a}
ALT (U/L)^{H}	2.05 ± 0.23^{a}	2.12 ± 0.25^{a}	2.30 ± 0.36^{a}	2.60 ± 0.88^{b}
AST (U/L)^{I}	230.40 ± 15.24^{a}	237.07 ± 19.60^{a}	238.40 ± 17.15^{a}	250.60 ± 14.80^{b}
TP (g/L)^{J}	32.70 ± 2.12^{a}	33.54 ± 2.36^{a}	36.80 ± 7.24^{a}	37.20 ± 2.58^{a}
TBIL (μmol/L)^{K}	9.68 ± 1.20^{a}	9.62 ± 1.10^{a}	9.70 ± 1.36^{a}	10.80 ± 1.06^{b}
BUN (mmol/L)^{L}	0.77 ± 0.12^{a}	0.70 ± 0.15^{a}	0.70 ± 0.18^{a}	0.61 ± 0.16^{a}
CRE (μmol/L)^{M}	10.54 ± 0.70^{a}	10.58 ± 0.97^{a}	10.66 ± 0.90^{a}	10.70 ± 1.06^{a}
14th day after administration				
RBC ($\times 10^{12}$/L)^{A}	2.44 ± 0.20^{a}	2.47 ± 0.18^{a}	2.51 ± 0.22^{a}	2.25 ± 0.17^{b}
HGB (g/L)^{B}	98.43 ± 7.45^{a}	99.4 ± 9.60^{a}	97.80 ± 8.96^{a}	88.40 ± 10.25^{b}
HCT^{C}	31.62 ± 2.20^{a}	30.23 ± 2.30^{a}	32.05 ± 2.18^{a}	31.28 ± 2.16^{a}
MCHC (g/L)^{D}	278.40 ± 25.38^{a}	272.60 ± 23.59^{a}	268.95 ± 25.67^{a}	270.40 ± 26.55^{a}
WBC ($\times 10^{9}$/L)^{E}	264.22 ± 24.35^{a}	260.34 ± 26.50^{a}	259.52 ± 24.38^{a}	276.04 ± 27.10^{b}
Lym ($\times 10^{9}$/L)^{F}	10.20 ± 1.26^{a}	10.48 ± 1.25^{a}	10.36 ± 1.17^{a}	10.51 ± 0.93^{a}
ML^{G}	1.14 ± 0.41^{a}	1.25 ± 0.32^{a}	1.14 ± 0.27^{a}	1.26 ± 0.86^{a}
ALT (U/L)^{H}	2.10 ± 0.22^{a}	2.11 ± 0.24^{a}	2.18 ± 0.23^{a}	2.20 ± 0.28^{a}
AST (U/L)^{I}	232.80 ± 12.45^{a}	233.40 ± 12.69^{a}	235.60 ± 14.23^{a}	240.15 ± 17.62^{a}
TP (g/L)^{J}	30.14 ± 2.59^{a}	33.21 ± 2.48^{a}	32.48 ± 2.82^{a}	32.67 ± 3.50^{a}
TBIL (μmol/L)^{K}	9.58 ± 1.20^{a}	9.60 ± 1.08^{a}	9.95 ± 1.36^{b}	10.50 ± 1.98^{b}
BUN (mmol/L)^{L}	0.75 ± 0.12^{a}	0.78 ± 0.15^{a}	0.79 ± 0.10^{a}	0.80 ± 0.15^{a}
CRE (μmol/L)^{M}	10.64 ± 1.10^{a}	10.69 ± 1.02^{a}	10.72 ± 1.03^{a}	10.85 ± 1.26^{a}

RBC, red blood cells; HGB, hemoglobin concentration; HCT, hematocrit; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell; Lym, lymphocyte; ML, monocytes; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; TBIL, total bilirubin; BUN, blood urea nitrogen; CER, creatinine.
$^{A–M}$Data were presented as means ± SD. ^{a,b}Values with different superscripts in the same row differ significantly ($P < 0.05$).

schistosomiasis, eperythrozoonosis, and *Pneumocystis carinii* infection (17, 18, 35). However, preclinical studies such as animal acute/chronic toxicity experiments revealed that high doses or long-term exposure to artemisinin can have toxic effects in multiple systems and organs (17–19). Therefore, QHP prepared from the petroleum ether extract of *Artemisiae annuae herba* required a deeper evaluation of its efficacy and safety concerning its anticoccidial properties prior to clinical applications. Control of the quality of medicinal materials and preparations with modern analytical tools is important to ensure their efficacy. In this study, artemisinin in QHP was identified and assayed using HPLC and TLC. The results showed that artemisinin was present in QHP, and the content of artemisinin in QHP was 81.03 mg/g. We can make a preliminary conclusion that the quality of QHP in terms of artemisinin content remains acceptable.

In this study, supplementation of QHP, sulfaclopyrazine sodium (SSC), and toltrazuril in feed alleviated the signs of infection. After seven days of administration, the number of oocysts significantly decreased ($P < 0.05$) after treatment of the infected chicks with QHP, SS, and toltrazuril. The protection rate for groups 4-SSC (0.30 g/L of SS), 6-middle dose (0.30 g/kg of QHP), and 7-high dose (0.60 g/kg of QHP) was 94.89, 74.85, and 77.86%, respectively. QHP at different concentrations had a therapeutic effect on chicken coccidiosis, as the degree of severity of cecal lesions was significantly improved and the presence of bloody feces was reduced in groups medicated with 0.30 and 0.60 g/kg QHP, and both oocyst value and oocyst output were significantly reduced. In addition, the results for rBWG revealed a pattern relatively similar to that of lesion scores, oocyst output and oocyst values among different groups. However, according to the ACI values, the anticoccidial effects of 0.30 g/kg (ACI = 129.16) and 0.60 g/kg (ACI = 144.05) QHP were moderate, and 0.15 g/kg QHP (ACI = 99.37) was insufficient. Research by Del Cacho et al. (36) showed that adding 10 or 17 ppm of artemisinin to the feed significantly affected the formation of oocysts, inhibited the sporulation of oocysts, and reduced

cecal damage, but did not affect the formation and development of gametes. Findings from Loredana et al. (19) confirmed that artemisinin supplementation at doses of 5 ppm, 50 ppm, and 500 ppm had prevention and treatment effects on single or mixed infections with *Eimeria acervulina*, *Eimeria tenella*, and *Eimeria maxima*. The treatments significantly lessened cecum lesions and reduced oocyst output. In this study, the content of artemisinin in QHP was 81.03 mg/g, and the recommended dose was 0.30 g/kg, equivalent to 24-ppm artemisinin. The anticoccidial effect was similar to that of the above-mentioned study.

The lesion score, oocyst value, and ACI value are commonly applied parameters used to evaluate the anticoccidial efficacy of animal drugs. In this study, the ACI values from the drug-treated groups were all <160, which may be related to the virulence of the *E. tenella* strain (Guangdong strain) selected or the concentration of the sporulated oocysts (7×10^4) in the test. Considering that the mortality rate in the infection control group reached 20%, a value that was beyond the optimal range (5–10%) in the experimental design, we can infer that the chicks in the five drug-treated groups were inoculated with a relatively high dose of sporulated *E. tenella* oocysts (37). In addition, the lower ACI value expressed in the toltrazuril control revealed that the *E. tenella* strain used in this study might have high resistance to toltrazuril while having greater sensitivity to SSC. Although the 0.60 g/kg dose of QHP in this experiment had a stronger anticoccidial effect in terms of the lesion score, oocyst value, and survival rate, the cost of choosing 0.60 g/kg is higher, and the research by Yin et al. (17) showed that high-dose artemisinin can have side effects such as neurotoxicity, renal toxicity, and cardiotoxicity. Engberg et al. (38) indicated that the n-hexane extract of *Artemisiae annuae herba* at 0.50 g/kg of dose in feed reduced the food intake and weight gain in chicks. Loredana et al. (19) also confirmed that artemisinin administered continuously for 16 days at a high dose significantly inhibited the body weight gain of chickens. From the above research results, high doses of QHP would be expected to have certain side effects on chicks. Considering that the decrease in body weight gain would also have an economic impact on the poultry breeding industry, a drug dose of QHP at 0.30 g/kg was determined as the recommended dosage and was used for the subsequent target animal safety test to evaluate the long-term effects after 7-day or 14-day continuous administration of QHP. From the results of reduced average weight gain and increased feed conversion rate in chicks in the two QHP-treated groups (0.90 g/kg, 3× RD; 1.80 g/kg, 6× RD) (**Table 3**) as well as the significant difference in the ROW of liver in the 6× RD group after 14-day administration (**Table 4**), we conclude that long-term high doses of QHP are likely to be toxic, and that such doses would have an inhibitory effect on the BWG and cause weight loss in chicks.

In this study, except for WBC counts, levels of HGB and MCHC in the 6× RD group, all of the other tested hematological parameters were within the normal range at all stages of the study, and no significant differences were observed between the control and the three treatment groups. The increase in WBC counts may be related to the inflammation induced after hepatocyte injury and to the changes in ALT and TBIL levels (17). RBCs or reticulocytes are sensitive indicators of the toxic effects of artemisinin drugs. *In vivo* animal experiments have found that artemisinins have an inhibitory effect on erythropoiesis. For example, after intravenous administration of artesunate at a dose of 240 mg/kg/d for 3 days, the peripheral blood reticulocytes, RBC counts, and HGB levels in rats were reversibly reduced according to the report by Xie et al. (39). Furthermore, the toxicity of dihydroartemisinin to RBCs is selective or phased, and it mainly affects primitive and young red blood cells. This may affect the cell cycle to inhibit RBC differentiation, as reported by Finaurini et al. (40). The findings of this study indicated the production of circulating white/red blood cells in chicks was not significantly affected by QHP at 3× and 1× the recommend dose levels. In this study, no significant differences in the levels of BUN or CRE in any of the drug groups were noted compared to the control groups; since BUN and CRE serve as confirmatory markers for renal dysfunction and failure (41–43), the above results suggest that 14-day continuous administration of QHP had little negative impact on the kidney function of chicks. As for the biochemical parameters, ALT and AST are well-known as markers of cell damage, especially hepatocyte necrosis (44–46). Moreover, TBIL (the product of hemoglobin degradation) is an important indicator and sign of liver damage and cholestasis and is also related to increased hemolysis (47–49). In our assay, the levels of ALT, AST, and TBIL in the 6× RD and 3× RD groups increased significantly after seven days of administration ($P < 0.05$); however, at the 14th day after administration, the concentrations of ALT and AST returned to normal levels, indicating that the increase in the levels of these two enzymes may be related to the reversible damage of liver cells stimulated by high artemisinin concentrations. Moreover, this signified that QHP has moderate toxic effects on chicks after 14 days of administration at a daily dose above the 0.60 g/kg recommended dose. However, the macroscopic examinations of the organs of chicks in the three QHP-treated groups produced no apparent changes compared with the control groups, and the necropsy results were not in agreement with the hematological and serum biochemical analyses. Moreover, these findings were not confirmed or supported by the histopathological analysis of the livers and kidneys, where QHP did not show toxic effects on the vital organs, and there was no abnormal tissue damage in the three QHP-treated groups. In conclusion, the present study recommends a dose of 0.30 g/kg feed of QHP, as this is likely to be non-toxic.

CONCLUSION

In this study, the content of artemisinin in QHP was 81.03 mg/g, and the addition of QHP (0.30 and 0.60 g/kg feed) could increase the rBWG and survival rate of broiler chicks infected with *E. tenella* (Guangdong strain) while reducing bloody diarrhea, oocyst output, and lesion scores in the cecal region. The ACI values in 0.30 and 0.60 g/kg QHP-treated groups were between those of toltrazuril and SSC treatments, indicating that QHP had prevention and treatment effects in chicks. From the safety test, a dose of 0.30 g/kg feed of this plant-derived anticoccidial was recommended as it presented no QHP-related signs of toxicity

or abnormalities in target animal safety tests. Findings from this study provided information for designing new plant-based drug against coccidiosis infection. Therefore, the dosage in clinical applications should be set according to the recommended dose to ensure animal safety, and QHP at a dose of 0.30 g/kg feed would be appropriate for the therapy and intermittent treatment of *E. tenella*-infected chicks.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

LW: development of methodology, analysis and interpretation of data, and writing of the manuscript. WZG: performed the experiments and coordinated and supervised the study. SUH: acquisition of data and writing of the manuscript. ZTG: conception and design and provided background information. DAC: acquisition and analysis of data and development of methodology. FY: analyzed and interpreted the references and material support. FC: development of methodology and analysis and interpretation of data. XJW: preparation of serum samples and material support. JWL: provided background information and references amended. All authors contributed to the article and approved the submitted version.

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Multifarious Trajectories in Plant-Based Ethnoveterinary Knowledge in Northern and Southern Eastern Europe

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Over the last century in the European context, animal production has been transformed by the dynamics of centralization and decentralization due to political and economic factors. These processes have influenced knowledge related to healing and ensuring the welfare of domestic animals. Therefore, our study aimed to document and compare current and past ethnoveterinary practices, and to identify trajectories in ethnoveterinary knowledge in study regions from both northern and southern Eastern Europe. In the summers of 2018 and 2019, we conducted 476 interviews, recording the use of 94 plant taxa, 67 of which were wild and 24 were cultivated. We documented 452 use reports, 24 of which were related to the improvement of the quality or quantity of meat and milk, while the other 428 involved ethnoveterinary practices for treating 10 domestic animal taxa. Cattle were the most mentioned target of ethnoveterinary treatments across all the study areas, representing about 70% of all use reports. Only four plant species were reported in five or more countries (*Artemisia absinthium*, *Hypericum* spp., *Linum usitatissimum*, *Quercus robur*). The four study regions located in Northern and Southern Eastern Europe did not present similar ethnoveterinary knowledge trajectories. Bukovinian mountain areas appeared to hold a living reservoir of ethnoveterinary knowledge, unlike the other regions. Setomaa (especially Estonian Setomaa) and Dzukija showed an erosion of ethnoveterinary knowledge with many uses reported in the past but no longer in use. The current richness of ethnoveterinary knowledge reported in Bukovina could have been developed and maintained through its peculiar geographical location in the Carpathian Mountains and fostered by the intrinsic relationship between the mountains and local pastoralists and by its unbroken continuity of management even during the Soviet era. Finally, our results show some patterns common to several countries and to the veterinary medicine promoted during the time of the Soviet Union. However, the Soviet Union

and its centralized animal breeding system, resulted in a decline of ethnoveterinary knowledge as highly specialized veterinary doctors worked in almost every village. Future research should examine the complex networks of sources from where farmers derive their ethnoveterinary knowledge.

Keywords: alternative and complementary veterinary medicine, animal husbandry, livestock, local ecological knowledge, plant-based remedies

INTRODUCTION

In many societies, livestock significantly contribute to human food security by providing several important food products, other valuable goods (e.g., wool, leather, and fat), agricultural inputs (e.g., manure), and services (e.g., transport, plowing). However, over the last century in the European context, animal production has been transformed by dynamics of centralization and decentralization (1, 2). These phenomena have also modified the associated veterinary knowledge and practices. While industrialized areas of Western Europe have increasingly shifted to highly technological animal breeding, in several rural communities of Europe, especially in mountainous regions, circum-Mediterranean areas, and post-Soviet contexts, livestock maintain their historical role in the livelihoods of peasants and they have been considered truly part of the family realm (3).

Animal breeding involves maintaining animal health and welfare. Scholars have found that the knowledge related to healing and ensuring the welfare of domestic animals has been largely abandoned in industrialized areas of Europe (3), while it appears to be relatively alive in rural contexts of the Mediterranean (4) and in some areas of Eastern Europe, e.g., Belarus (5–7). In the Mediterranean region, folk veterinary knowledge has been partially preserved due to geographical isolation and distance from veterinary services, yet it is rapidly declining and being replaced by modern livestock farming technologies and administrative veterinary controls (4). In several areas of Eastern Europe, a similar trend may have its roots in the political events that occurred during the time of the Soviet Union and after its collapse. Indeed, during most of the Soviet period, kolkhozes (collective farms) for animal (and crop) production were implemented, and each household could own only a small number of livestock (e.g., chickens, pigs, cows, sheep, goats) for subsistence (8). At the beginning of the 1990s, when the Soviet Union collapsed, political changes resulted in a profound transformation of agricultural production in Eastern Europe, with a concurrent decentralization and relocalization of veterinary knowledge production and implementation (9).

The use of plants for veterinary practices is well-studied in some regions of Eastern Europe. For instance, in Ukraine several scholars have investigated this topic [e.g., (10–13)], focusing on specific regions of the country (14, 15) or specific diseases (16, 17). However, most of the studies focusing on Eastern European ethnoveterinary medicine have been published in local languages. Indeed, in the international literature written in English, ethnoveterinary medicine is an underexplored field in Eastern Europe, but its preservation and implementation are increasingly considered a promising alternative for improving animal health and welfare. For instance, veterinary phytotherapy can find new applications in agroecological or organic agricultural practices, a fast-developing sector in Western Europe (18). In addition, ethnoveterinary knowledge can contribute to local biodiversity conservation (19, 20). Indeed, the health of an ecosystem and the livestock and people who inhabit it are strictly interdependent and need to be considered holistically (21).

Within this framework, our study aimed to document and compare current and past ethnoveterinary practices, and to identify trajectories in ethnoveterinary knowledge in rural borderland areas of eight countries from northern and southern Eastern Europe, namely Finland, Russia, Estonia, Lithuania, Poland, Belarus, Ukraine, and Romania. We further discuss what factors may have contributed to the persistence/erosion of ethnoveterinary knowledge in Eastern Europe.

MATERIALS AND METHODS

Study Area

In the summers of 2018 and 2019, we conducted semi-structured interviews in four regions in eight countries that are home to nine main ethnolinguistic groups (**Table 1**).

Bukovina is a historical region of the Austro-Hungarian Empire that has been split between Romania and Ukraine since 1940. It is inhabited by several ethnic groups including Jews, Ukrainians, Poles, Romanians, and Hutsuls. Bukovina is partially occupied by the North-Eastern Carpathians which reach an altitude of 1,651 m a.s.l. We conducted our research among Hutsuls living in the Carpathian villages of the upper Suceava Valley in Romania and Putyla Rayon in Ukraine and among Romanians living in the pre-Carpathian hills of Straja (Romania) and Storozhenets and Glybotskyi district in Ukraine. Both in Ukraine and in Romania, most of the interviewees rely on family farming.

Dzukija is a historical and cultural region located in the borderlands of Poland, Lithuania, and Belarus. This border area has long been a crossroads for trading routes and has been subject to a series of changes in national status. It is now mainly inhabited by Lithuanians and Poles. It is characterized by plain and hilly rural areas and is currently experiencing a remarkable rural emigration. Soils throughout the studied region are sandy and of little agricultural value. We conducted interviews among Lithuanians living in several villages of Augustów and Sejny counties of Podlaskie Voivodeship (Poland), Šalčininkai district of Vilnius County (Lithuania), and Hrodna, Voranava, and Ašmiany districts of Hrodna Region (Belarus).

TABLE 1 | Main characteristics of the study area.

Region	Country	Main groups	Languages	Number of SSI	Dominant landscape
Karelia	Finland	Finns, Karelians	Finnish, Karelian	71	Plains covered by forests and lakes
	Russia	Russians, Karelians	Russian, Karelian	61	Plains covered by forests and lakes
Setomaa	Estonia	Estonians, Setos	Estonian, Seto	78	Mixed forests, fields, and marshy areas
	Russia	Russians, Setos	Russian, Seto	46	Plains covered by pastures and forests
Dzukija	Belarus	Lithuanians	Lithuanian, Belarusian	33	Plains covered by pastures
	Lithuania	Lithuanians	Lithuanian	30	Plains covered by pastures
	Poland	Lithuanians	Lithuanian	32	Mild hills covered by pastures
Bukovina	Ukraine	Hutsuls, Romanians	Ukrainian, Romanian, Russian	65	Mountain and hilly areas covered by forest and pastures
	Romania	Hutsuls, Romanians	Romanian	60	Mountain/Forest and pastures

Karelia is a historical region currently divided between Finland and the Russia. Agricultural lands occupy only a small percentage of its territory, which is mainly covered by forests, a crucial resource for the local economy (22, 23). In Russian Karelia, during the last century, significant areas of meadow have appeared in place of abandoned arable land, but due to the cessation of grazing and haymaking, field degradation occurs which results in tree overgrowth and the spreading of shrub vegetation (24). In Finnish Karelia, forestland has been maintained over the last few decades through forestry intensification (25). On the Russian side, fieldwork was carried out in Petrozavodsk, Zaozer'e, Lekhnavolok, Novaya Vilga, Priazha, Essoila, Korza, Rubchoila, Siamozero, and Kalevala. On the Finnish side, the interviews were conducted for the most part in North Karelia (Joensuu, Lieksa, Nurmes, Valtimo, Ylä-Valtimo, Puukari, Rasimäki, Varpasenkylä, Viensuu, Viinijärvi, Sotkuma), while a few were conducted in Helsinki.

Setomaa is a region located at the Estonian-Russian border inhabited by speakers of the Seto and Russian languages. After WWII, the largest part of the formerly united historical Setomaa was incorporated into the Russian Soviet Federative Socialist Republic and remained there even after Estonia regained independence. Since the twentieth century, rapid alterations to the environment (like climate change and changes in the landscape due to the abandonment of agricultural activities) have led to the disappearance of some native plants (26) and the primary sector is continuously declining (27). Interviews were conducted in the villages of Pechorsky District of Pskov Oblast in the Russia and in Setomaa, Võrumaa-, and Tartumaa in Estonia. On the Russian side, most of the interviewees were retirees. During their lives, they worked in various positions in sovkhozes (state farms)–dairymaid, crop specialist, accountant, and head of the land plot. We also spoke to two zootechnicians–one has changed jobs since then while the other is still practicing.

Field Study

The research was part of a wider study, namely the ERC-funded DiGe project, aiming to understand the mechanisms of change in ethnobotanical knowledge that occur among cross-border minorities when a dominant group tries to modify this knowledge. The four regions were selected in order to give an overall comparative picture of the current and past uses of plant-based ethnoveterinary remedies in Eastern Europe (**Figures 1**, **2**). We conducted 476 semi-structured interviews among rural people conveniently selected while they were walking down the street, sitting on public benches (in the regions in which this was common), or working in their gardens. Our aim was to understand the existence and dynamics of ethnoveterinary knowledge among the non-specialist population living in the studied rural regions. We interviewed people mainly in one-to-one interviews, although on some occasions interviews involved other members of the household as well.

In Finnish Karelia, interviewees were also identified in advance through various social networks as it was not always possible to approach them directly. We consider this sample robust given the fact that saturation was reached after about 10–15 interviews per country.

Before each interview, prior informed consent was obtained following the Code of Ethics of the International Society of Ethnobiology (28). Upon consent, questions on ethnoveterinary medicine were asked in the context of documenting the whole plant use-system using the same methodology in all the countries. The questions were directed toward the healing of the livestock and other animals in the households using plant-based remedies. The general question on the presence of animals in the household was followed by questions regarding the healing of every animal mentioned by the interviewee and on the ways in which these plant-based products improved the quality of animal products (e.g., milk, meat) both in the past and in the present. We consider quality that which, in different ways and based on interviewees' perceptions, improves, enhances, and intensifies the characteristics (e.g., in terms of taste, smell, nutritional properties, etc.) of milk and meat. We allowed interviewees to freely talk about the topic, asking follow-up questions when needed. When a wild plant was mentioned, we asked the interviewee to show it to us if possible. Collected voucher specimens are stored in the herbarium of Ca' Foscari University of Venice (UVV) for countries of the European Union (Finland [bearing numbers KAR02–KAR012; KARDR10 and KARDR24], Estonia [bearing numbers SE001–SE135], Lithuania [bearing numbers DZULT01–DZULT126, and DDZULT01–DDZULT45], Poland [bearing numbers DZUPL001–DZUPL107 and DDZUPL01–DDZUPL39], Romania [bearing numbers

FIGURE 1 | Research sites.

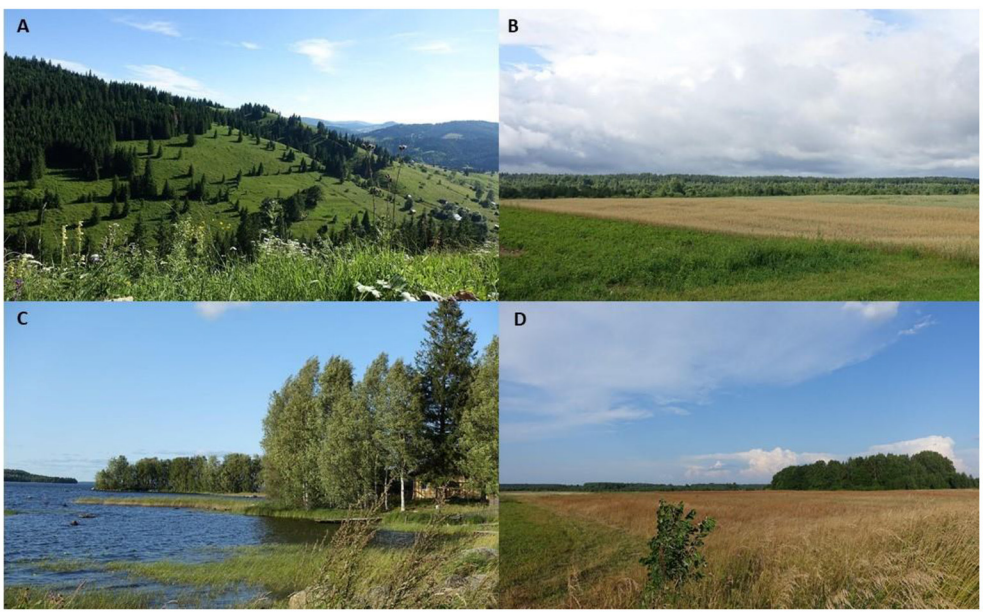

FIGURE 2 | Landscapes of **(A)** Bukovina, Izvoarele Sucevei (Romania), summer 2019 (credit: Nataliya Stryamets); **(B)** Dzukija, Tabariškes (Lithuania), summer 2018 (credit: Julia Prakofjewa); **(C)** Karelia, Essoila (Russia), summer 2018 (credit: Valeria Kolosova); **(D)** Setomaa Panikovichi (Russia), summer 2018 (credit: Olga Belichenko).

SB003–SB096]), while the specimens collected outside of the EU are deposited in Roztochya Nature Reserve for Ukraine [bearing numbers NB001–NB085] and in the Komarov Botanical Institute, Russian Academy of Sciences, Saint Petersburg for the Russia [bearing numbers LE 01063392–LE 01063946 (http://en.herbariumle.ru/)].

When possible, interviews were recorded upon the interviewee's approval and transcribed in the local language; in the few cases when recording was refused, we took notes. Later, we entered this information in English into an Excel spreadsheets organized as detailed use reports (DUR) of plant-based remedies, where each row contained the country and ethnic community of the interview, its code, the scientific name of the plant, its local name, the part used, when it was used, the mode of preparation, and its use. Botanical taxa were classified using World Flora Online (2021). The botanical families were classified according to the Angiosperm Phylogeny Website (29).

The research protocol was approved by the Ethics Committee of Ca' Foscari University of Venice.

RESULTS

In the four regions where this study was conducted, we recorded the use of 94 plant taxa, 67 of which were wild and 24 were cultivated, from 189 interviewees. We documented 452 use reports, 19 of which were related to the improvement of the quality or quantity of meat and milk, while the other 428 involved ethnoveterinary practices for treating 10 domestic animal taxa. Out of the 476 interviews we conducted in the four study regions, 189 reported ethnoveterinary uses. Below, we focus first on ethnoveterinary knowledge related to cattle as about 70% of the use reports concerned cattle illnesses (**Figure 3**).

The Importance of Cattle

Cattle were the most mentioned target of ethnoveterinary treatments across all the study areas. We recorded the use of 55 plants belonging to 25 families (**Supplementary Tables 1**, **2**) for this purpose, 35% of them were cultivated and 65% were wild.

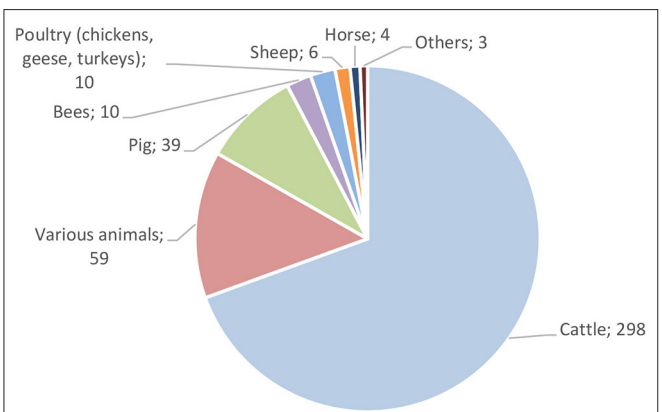

FIGURE 3 | Distribution of ethnoveterinary use reports by animal category.

Only four species were reported in five or more countries (*Artemisia absinthium*, *Hypericum* spp., *Linum usitatissimum*, *Quercus robur*).

The majority (61%) of the DUR were currently in use, while 39% referred to past uses. However, excluding the Bukovinian Carpathian area (our Ukrainian and Romanian case studies), the proportion of currently used DUR drops to 12%, while the remaining 88% refers to past ethnoveterinary uses. Indeed, as illustrated in **Figure 4**, current uses were widely reported in Romania and Ukraine and to a lesser extent in Russian Setomaa, Poland, and Belarus. Karelians, Estonian Setos, and Lithuanians referred only or mainly to past uses.

The digestive and reproductive systems were the most common targets of ethnoveterinary remedies (**Figure 5**). For the digestive system, diarrhea was the most reported ailment followed by stomach illnesses and other issues such as intestinal gas, rumination problems, and abdominal pain. For treating diarrhea, the most commonly used plants were *Rumex* spp. (reported in four countries across three regions), *Hypericum* spp. (reported in five countries across all four regions), and *Quercus robur* (reported in five countries across all four regions). In regard to the reproductive system, the most common issues involved calving and the use of postpartum supplements. The most utilized plant for treating the reproductive system was *Linum usitatissimum*, mentioned in four countries and three regions. Among the listed plants some (e.g., *Atropa belladonna*, *Cannabis sativa*, *Hypericum* spp.) could potentially have negative effects on animals. While no interviewee explicitly mentioned possible adverse side-effects, they were not assessed, being out of the scope of this article.

Ethnoveterinary Remedies Related to Other Livestock

Excluding cattle, we documented 130 DURs related to 44 plant taxa belonging to 28 botanical families that were used for treating nine animal taxa, including pigs, honeybees, sheep, turkeys, chickens, geese, horses, dogs, and cats. Only three species were found to be used in four countries: *Picea abies* (three regions), *Quercus robur* (three regions), and *Urtica dioica* (two regions). The livestock most commonly treated with ethnoveterinary remedies were pigs (39 DURs), which were mainly mentioned in Russian Karelia and Russian Setomaa. As in cattle, the most widely treated illness was diarrhea, primarily in pigs. Also, five plant taxa (above all *Urtica dioica*) were used, especially in Dzukija, as feed supplements (13 DURs).

Plants to Improve Animal Product Quality

In addition to ethnoveterinary remedies, we also recorded the use of 19 DURs referring to ten plant taxa for improving the quality of milk, meat, and in one case pig bristles (**Table 2**). More than half of the uses (11) were reported in Bukovina, the rest in Lithuania and Russian Setomaa. None were reported in Karelia. The most common plant was *Urtica dioica*, which was reported

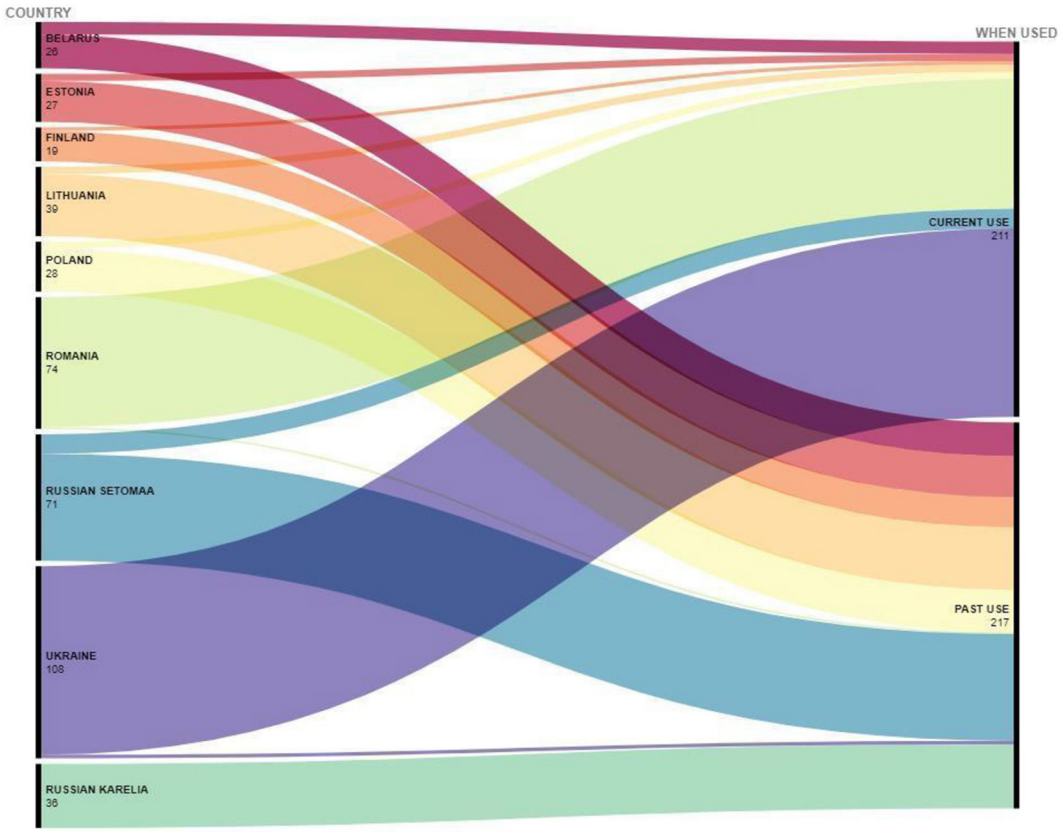

FIGURE 4 | Alluvial graph of the number of ethnoveterinary remedies (colored stripes), country (on the left), and time of use (on the right).

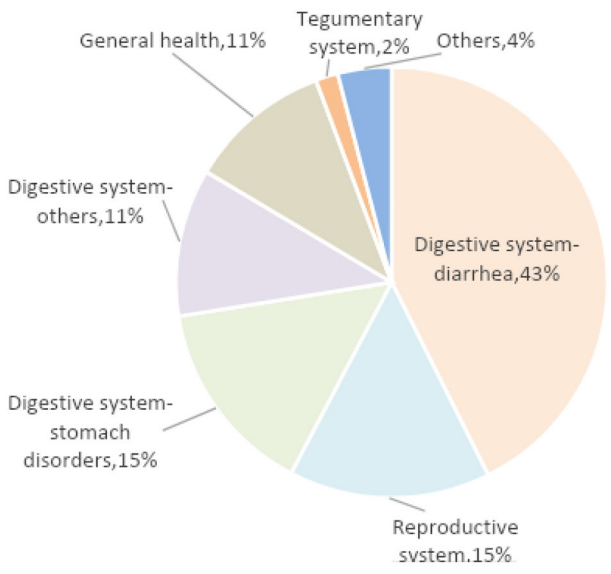

FIGURE 5 | Distribution of detailed use reports (DUR) related to cattle ethnoveterinary remedies.

by six people in Bukovina for improving milk quality and by one interviewee in Lithuania for improving pork meat quality.

Ethnoveterinary Knowledge Dynamics

Out of the 476 interviews we conducted in the four study regions, 189 interviewees reported ethnoveterinary uses (**Table 3**). Specifically, Bukovina had the highest percentage of people who mentioned ethnoveterinary remedies (58%), followed by Dzukija (44%) and Setomaa (41%). Karelia was the region where less ethnoveterinary knowledge was reported (only 21% of respondents could mention at least one remedy). Bukovina was also the region with the highest rate of DUR per person (2.5), followed by Dzukija (2.2). When examining the data from a cross-border perspective, we can observe that bordering countries in Bukovina and Karelia reported similar percentages of interviewees mentioning ethnoveterinary remedies and DUR referring to the past. However, in Dzukija, especially in Poland and Lithuania, a higher percentage (86 and 90%, respectively), of past uses were mentioned compared to Belarus (73%). Similarly, in Setomaa the percentage of interviewees who could mention at least one ethnoveterinary remedy was 23% in Estonia, while it was more than double that (59%) in Russian Setomaa.

TABLE 2 | Plant taxa used for improving the technological quality of some animal products.

Latin name and family	Local name	Part used	Preparation	Use	Use reports (*past)
Arctium tomentosum Mill. (*Asteraceae*) [W]	Лопух (RS)	Leaves	Fresh	Improving cow milk quality	RS
Chelidonium majus L. (Papaveraceae) [W]	Czystaciel (LT); чистотіл (UA)	Aerial parts	Fresh; Infusion	Improving cow milk quality	2 UA; LT*
Chenopodium album L. (*Amaranthaceae*) [W]	Balanda (BL)	Aerial part	Fresh	Improving pork meat quality	LT*
Linum catharticum L. (*Linaceae*) [C]	Linučiai, linuciai (LT)	Seeds	Infusion	Improving cow milk quality (increase fat in the milk)	LT*
Linum usitatissimum L. (*Linaceae*) [C]	Лён (RS)	Seeds	Fresh	Improving pig bristle quality	RS*
Rumex confertus Willd. (*Polygonaceae*) [W]	Щіва (UA)	Leaves	Fresh	Improving sheep milk and wool quality	UA
Salix spp. (*Salicaceae*) [W]	Paju (FI)	twigs and leaves	Fresh or dried	Improving cow milk quality	FI*
Stellaria media (L.) Vill. (*Caryophillaceae*) [W]	Žliuge (LT)	Aerial parts	Fresh	Improving pork meat quality	LT*
Trifolium spp. (*Leguminosae*) [W]	Trifoi (UA)	Aerial parts	Fresh	Improving cow milk quality	UA
Urtica dioica L. (*Urticaceae*) [W]	Dilgele (LT); Urzica (RO); кропива (UA)	Aerial parts	Infusion	Improving cow milk quality	4 RO; 2 UA
			Fresh	Improving pork meat quality	LT*

*W, Wild species; C, Cultivated species; LT, Lithuania; RO, Romania; RS, Russian Setomaa; UA, Ukraine. *No longer in use, number of use reports (UR) is indicated if more than 1.*

TABLE 3 | Ethnoveterinary knowledge by region.

	DUR (*n*)	DUR referring to past uses	EV respondents (*n*)	Total respondents (*n*)	% of interviewees who mentioned EV remedies	% of DUR referring to past uses	DUR per interviewee (*n*)
Romania	74	1	34	60	57	1	2.2
Ukraine	108	2	38	65	58	2	2.8
Total Bukovina	**182**	**3**	**72**	**125**	**58**	**2**	**2.5**
Belarus	26	19	15	33	45	73	1.7
Lithuania	39	35	15	30	50	90	2.6
Poland	28	24	12	32	38	86	2.3
Total Dzukija	**93**	**78**	**42**	**95**	**44**	**83**	**2.2**
Finland	20	18	15	71	21	90	1.3
Russian Karelia	36	36	15	61	25	10	2.4
Total Karelia	**56**	**54**	**30**	**132**	**23**	**95**	**1.9**
Estonia	27	23	18	78	23	85	1.5
Russian Setomaa	71	60	27	46	59	85	2.6
Total Setomaa	**98**	**83**	**45**	**124**	**41**	**85**	**2.0**

"I Call the Doctor!" and Other Attitudes Toward Ethnoveterinary Practices

We recorded different attitudes regarding the use of plants for treating animals (**Table 4**). Interviewees often expressed a dichotomy between official veterinary services and plant-based solutions. For instance, when asked about their actions in the case of animal illness, several interviewees said, "Normally we call the veterinary doctor" (Romanian Hutsul man born in 1951) and "I call the vet and he gives me something but I do not know what" (Romanian Hutsul man born in 1934). In Ukraine, some interviewees specified that "Now we go to the doctor" (Ukrainian Romanian woman born in 1939) or "For this [mastitis], now we need to call the doctor" (Ukrainian Romanian woman born in 1983), underlining that this was not the case in the past.

TABLE 4 | Prevalent attitudes toward ethnoveterinary remedies.

Prevalent attitudes toward ethnoveterinary remedies	Countries in which the concept was mentioned
(Now) we call the veterinary doctor	Romania, Ukraine, Belarus, Russian Karelia, Russian Setomaa, Estonia, Lithuania, Poland, Finland
Better to use drugs, they are more reliable	Belarus, Poland, Lithuania
Better to use drugs, they are faster	Russian Setomaa, Russian Karelia
Better to use herbs, they are more effective	Romania
In the old days, animal healers knew incantations	Russian Karelia, Russian Setomaa, Belarus, Lithuania
And if we can, we cure the cattle ourselves	Russian Karelia, Romania

In the Dzukija region, older respondents reported the loss of ethnoveterinary knowledge as a reason for turning to official veterinary medicine: "There are no more people who know the right herbs and remedies. Now there are veterinarians. If someone needs something, they turn to veterinarians" (Belarusian Lithuanian woman born in 1946). Informants from both the Belarusian and Lithuanian sides of Dzukija noted that state-sanctioned veterinary medicine appeared with the advent of the Soviet state and the formation of kolkhozes [∼1950]. On the Polish side, older Lithuanian interviewees mentioned that they mainly healed animals with drugs (Polish Lithuanian woman born in 1929).

At the same time, although the older generation of interviewees living on the Belarusian and Lithuanian sides of the Dzukija region is characterized by a lack of trust in official medicine, the middle and younger generations prefer medicines over medicinal herbs. For example, a middle-aged female respondent noted that it is better to treat cows with drugs, not herbs. For her, using chemicals is a more reliable option (Lithuanian woman living in Belarus woman born in 1954).

Among the respondents who worked on kolkhoz between the 1950s and 1990s, some noted that they used home remedies for the kolkhoz animals: "I worked on the farm, raised calves, and none of them died. I made bottles for them at home. There were pałyn (*Artemisia absinthium*), čystacieł (*Chelidonium majus*). And I carried these bottles to the kolkhoz" (Lithuanian woman living in Belarus woman born in 1941).

In Russian Karelia and Setomaa, the decision to call a veterinary service (instead of treating livestock using plant-based remedies) was sometimes perceived as a matter of time, as one veterinary doctor mentioned, "You know, when I started working, there were already antibiotics [. . .]. But even at the beginning of my work [in the late 1970s], in fact, they did not pay much attention to folk remedies. Well, maybe because this is a longer path to recovery, whereas one needs the result immediately. After all, they use more modern ones here" (Russian Seto woman born in 1939). This was also confirmed by a retired kindergarten nanny: "And as with pills, you call a doctor, because it is fast for calves, yeah, for calves. Quicker with them, because herbs are too slow" (Russian woman, born in 1962, living in Karelia). Conversely, in Romania, some older interviewees were skeptical about official veterinary medicine: "Teas of *Hypericum*, *Equisetum*, *Carum carvi*, dried bread, and the grains of *Coriandrum sativum* are better than furazolidonul [a vet medicament]" (Romanian woman, born in 1948, living in Romania).

In the Soviet Union, it was not difficult to receive veterinary consultations ("The vet lived right here. They [vets] came on foot, came [by bus or truck], some on horseback," said a Russian Seto woman born in 1956). However, some interviewees also reported that "there were specialists, but at home, you were mostly on our own" (Russian Karelian man born in 1950). Contrastingly, another interviewee stated, "Cattle were not treated at home, only by a veterinarian. In the old days, there were very good veterinarians. Only the veterinarian treated animals" (Russian Karelian man born in 1929). Indeed, during Soviet times there was a veterinary doctor working in every large village with kolkhoz who helped locals to treat cattle. An Estonian Seto woman born in 1938 narrated: "Animals had flax seeds after calving, but otherwise, if something was wrong, we had to call the vet."

In Finnish Karelia, an interviewee claimed that in the 1950s, "Veterinarians were not used as the nearest veterinarian was 35 km away. There was someone among the people, who was familiar with these problems, and he/she was called only if there was some illness and you were unsure of what you should do" (Finnish woman, born in 1978, living in Finland).

Human-Livestock Connectedness in Eastern Europe

Our interviewees from the four regions often mentioned the deep connection they have or had with their livestock. For instance, in Russian Karelia, an Ingrian-Russian woman (born in 1954) mentioned, "We had goats, sheep, yes... well, at that time it was kind of necessary, because living was not very... how to say?–easy. Therefore, we kept them," stressing the role livestock played in guaranteeing food security.

Also, a Russian woman, born in 1966, living in Karelia reported using human food for feeding animals: "They just subsidized bread very, very much. [. . .] My Karelian grandmother fed all her cattle with bread. It was cheaper than growing potatoes and buying grain from a sovkhoz. And my dad, having a car, every day brought her two loaves, four loaves, of bread. It was a strange idea of our household, but bread was subsidized, and the rest was not."

In Romania, a Hutsul interviewee (Romanian Hutsul woman born in 1961) clearly explained the nexus between "eating in a healthy way and livestock breeding. When asked about any possible product she could give animals to produce more milk she exclaimed, "Ah, no, I do not give them anything. Well, to be precise, sometimes a bit of bran, when animals come in at night and in the morning when milking, but otherwise, grass. Here we eat healthily." It is worth noting how "we" includes both humans and animals, as a whole, and therefore if animals eat healthily humans will as well.

In the Dzukija region, however, among the villagers, there is often a reasonably practical approach: "We did not contact anyone. If an animal lives, it lives. Nothing was treated. As the old then died" (Lithuanian woman living in Belarus woman born in 1939). We recorded a profound respect for livestock. Many respondents said that, for example, a cow is a very clever animal that knows what to eat and what not to eat. At the same time, the respondents remembered many instances of using herbal remedies in various rituals related to the prevention of diseases in livestock. In particular, the tradition of blessing animals with a palm bouquet has survived to this day: "And also animals were smoked with that Easter Palm. Yes, if they got sick then they did it" (Lithuanian woman living in Lithuania born in 1938). Setos used *Salix* sp. twigs consecrated on Palm Sunday. They placed these twigs on the barn door to protect animals. Both Seto and Russian people recalled drawing a black cross with charcoal above the barn door, on baptism day, to protect animals.

It should also be noted that a large number of our respondents' recollections concerned the use of various plants for the treatment and prevention of culture-bound illnesses in domestic animals, such as fright and the evil eye: "For fright, there is uročnikas [*Gymnocarpium dryopteris*] [. . .] which is good for the evil eye as well. [. . .] Especially for animals, we used to collect it, if someone casts the evil eye, you smoke it, and it was a medicine" (Lithuanian woman living in Lithuania born in 1939).

Perceived Changes in Livestock Keeping

Among the changes in livestock keeping mentioned by interviewees who lived during Soviet times, the crucial role of cows was reported: "When collectivization took place, only one cow was left for the family. Whether it was one person, or seven people, still only one cow was left for the family. And they also handed over meat, milk, and eggs [to the procurement system]. It was a difficult period then" (Russian woman, born in 1940, living in Setomaa). This was also confirmed by an elder Finnish Karelian woman who remembers that whipped cream from the only cow remaining was an important resource: "I remember, in the 1970s, when it was decided that we would be left with one cow. At that time, cream was skimmed from milk and whipped as whipped cream." Another participant commented, "It was not considered a farm if there was no cow" (Russian Karelian man born in 1928). In addition, a retired Karelian seamstress (born in 1954) living in the Russia mentioned that cattle disappeared because "the mowing was very bad, and it was necessary first to give hay to the sovkhoz, and therefore my parents got rid of the cows." This issue of the lack of hay was also mentioned by a Karelian forestry specialist: "It was torture to keep a cow. When I was in elementary school, my father had a MAZ lorry. Mowing was not allowed then, and they drove in the direction of Interposiolok to steal hay; they mowed on the side of the road and returned with it at night–either to the neighbors or to our own farm. They helped each other, dried... they dried hay, we dried hay, and that's all... so... and lived" (Russian Karelian woman born in 1948).

Among other more recent changes, a Seto interviewee claimed, "And now you can list the cow in the Red Book, only a few cows remain. So, there are no animals to treat actually" (Russian Seto man born in 1943). An Estonian Seto woman, born in 1938, commented, "I had three cows, and for a short time also four. When the Estonian state started, it destroyed all the cows, no one needed milk anymore. Milk churn stands were lost." A younger Estonian Seto man (born in 1953) explained his point of view, referring to the current availability of state jobs: "There were those "state" works [e.g., kolkhoz and sovkhoz], and, on top of them, people had their own animals and farmland. But have you seen that now no one raises animals like cows or pigs at all? Pigs can't be kept anymore, because that bastard plague (African swine fever) is here." In Ukraine, an older Romanian woman explained, "I do not have a cow now, but I had one. I have had it for many years, but now I am old, weak and I really cannot keep it."

A couple of Romanian Hutsuls reflected upon our question regarding agricultural changes in the last few decades: "Agriculture has changed since our youth. It has changed a lot [. . .]. Now they make much more hay, but the grass lasts, which in the past did not happen because everyone here had livestock, much more than now since there are no animals, because milk is paid almost nothing, and so why keep a cow if the milk. . . [is paid nothing]" (Romanian Hutsul man and woman born in 1934 and 1939, respectively). However, they also proudly stated, "Milk here is natural, from flowers, cows graze on flowers and also eat hay. The milk is fatty, it is good." Finally, a Romanian teacher living in Romania claimed, "I have had livestock, but my children have left, parents have passed away, and working and caring for animals is hard, so I gave it up," and then she continued counting the number of livestock in the village: "For instance, my grandparents had a lot of livestock, sheep and cows, and in summer they used to go to the mountain pastures, but now I think that in the entire village only five people go to the mountains. Also, the animals are few: we had about 50 animals, the same number as our neighbors, but now, in all the area, there are barely 10."

In the Dzukija region, the respondents noted that cows grazed everywhere in the village in the recent past [1980–1990s]. "And only this year locals killed the last cow" (Lithuanian woman living in Belarus woman born in 1939). Now, mostly older people keep at the most chickens, although, in comparison with the past, they had a rather large farm. Thus, a respondent from the Belarusian side of Dzukija noted that in her youth, she and her husband kept three cows, three pigs, a horse, a lot of chickens, geese, and sheep, and now she only has three chickens (Lithuanian woman living in Belarus woman born in 1946).

The most evident difference in livestock keeping was in Poland, where the Lithuanian minority are larger-scale farmers. They still keep many animals and sell the milk to the state. In Communist times, farming in Poland was nationalized only to a small degree. Just a small proportion of food production was state based, while the majority remained in private hands. This also meant that private farming in Poland was highly fragmented, as the state did not encourage mergers and land acquisition.

DISCUSSION

Our results showed that the four study regions located in Eastern Europe do not present similar ethnoveterinary knowledge trajectories. Bukovinian Romanians and Hutsuls appear to hold a living reservoir of ethnoveterinary knowledge, unlike the other regions as summarized in **Figure 6**. However, despite differences in the richness of ethnoveterinary knowledge, cattle were the livestock most commonly treated with herbal remedies in all four regions. Setomaa (especially Estonian Setomaa) and Dzukija showed an erosion of ethnoveterinary knowledge with many uses reported in the past but no longer in use.

Bukovinian Carpathians as a Reservoir of Ethnoveterinary Knowledge

The current richness of ethnoveterinary knowledge reported in Bukovina could have been developed and maintained through its peculiar geographical location. Unlike the other study areas of this research, our fieldwork in Bukovina was carried out in

FIGURE 6 | Possible drivers of the persistence of ethnoveterinary knowledge in Bukovina (left) compared to possible drivers of ethnoveterinary knowledge erosion in Setomaa, Dzukija, and Karelia (Credits: Johanna Lohrengel).

mountainous and peri-mountainous areas. For centuries, the (Bukovinian) Carpathian Mountains have served pastoralists and specifically transhumant herders (30). In particular Hutsuls, one of the studied ethnic groups, are well-known for their centuries-long expertise in mountain pastoralism (31). We observed that several Hutsuls still use their *polonyna* (summer pastures) or send their animals out with other shepherds. However, long-term (pacific) coexistence of Hutsuls with other ethnic groups, could have facilitated the sharing of their knowledge of animal breeding (especially ethnoveterinary practices) among Romanians living in neighboring pre-Carpathian areas. Therefore, the mountainous nature of the area could be a key element in the resilience of ethnoveterinary knowledge, which has also been confirmed by the intrinsic relationship found between pastoralists and European mountains [e.g., (32, 33)] and possibly, as a result, the related ethnoveterinary knowledge (34). In a geographically remote and rather inaccessible territory, such as the Carpathian (and pre-Carpathian) territory of Bukovina, cattle play a crucial role in terms of food security and economic importance, especially in times of crisis. Still, at the present time, Hutsuls and Romanians rely heavily on animal husbandry based on small-scale family farms (35). While sheep are very important from a cultural perspective, especially among Hutsuls, the small proportion of sheep remedies mentioned may be due to several factors including their limited economic importance (it is cow, and not sheep, milk which provides income), and possibly to the fact that we conducted interviews in the summertime when sheep are not with their owners but in mountain areas with shepherds who might have special treatments for sheep which the owners do not know. Therefore, livestock husbandry, particularly cattle breeding, is a daily activity for both Hutsuls and Romanians living in Bukovina. This is specifically due to the importance of milk, which can provide a regular and reliable source of protein and/or cash income throughout the year, in a context where no other agricultural commodities can do this, as observed by Kitching (36). Indeed, milk, when processed, is a crucial element of the local diet and cuisine (30, 37). Among the dairy products which play an important cultural role, identifying Hutsuls as mountain pastoralists, the most salient is bryndza, a complex cheese produced in summer pastures, which has recently obtained Geographical Indication status when produced with sheep milk in the Rakhiv region [the first in Ukraine, (38)] and has been included in the Ark of Taste promoted by Slow Food (39). Other dairy products, including budz (a smoked cheese produced among Ukrainian Hutsuls) and (v)urda (a soft cheese), also contribute to transfer the centuries-long interaction with the Carpathians into the Hutsul foodscape.

Such everyday practices in animal breeding (milking and cheese-making) are still currently present and so is the veterinary knowledge connected to them. Indeed, as observed by Warchalska-Troll and Troll (40), Carpathian pastoralism was characterized by an unbroken continuity of management even during Soviet times. This could be a key for the resilience of the ethnoveterinary knowledge in the area. While several interviewees reported calling the veterinarian for serious issues, most Bukovinian interviewees reported treating minor ailments related to the digestive system and calf birthing themselves, using ingredients (such as plants) locally available. Often, people mentioned that "this is good for both people and cows," revealing the tight nexus between their own well-being and that of their

cattle. These thoughts may have been fostered by the habit, in several areas of the Soviet Union, to buy bread to feed livestock, a practice which became so popular that a decree banned this "misuse" of bread in 1957 (41). This is also reinforced by the observation of Kitching (36) for whom the smallholder production of livestock (and crops) during Soviet times (and right after) represents a "remarkable history of survival and productivity." Indeed, in the 1990s, home-grown food also played a key role in the survival strategy of communities (42) in several post-Soviet contexts, including Northern and Southern Bukovina. Thus, livestock were somehow needed and cared for as valuable family members. The harsh times and this diffused sense of "extended family realm" reveal a strong connectedness to and a holistic vision of the ecosystem where people and livestock are equally important actors within a larger system.

Possible Erosive Trajectories of Ethnoveterinary Knowledge in Karelia, Setomaa, and Dzukija

In Karelia, agriculture has been marginalized, especially in terms of the area where hay is still cut and the number of natural pastures, due to the declining number of cattle and the intensification of dairy farming (43–45). We can therefore hypothesize that our Karelian interviewees did not mention many ethnoveterinary remedies for three main reasons. First, due also in part to the climatic conditions, agriculture and animal breeding have often played a marginal role throughout Karelian history and therefore few livestock animals were kept, although their numbers have been sharply declining over the last century (46, 47). This was also confirmed by an interviewee who stated, "I ate my first chicken at 18 and a half years old. We always have elk and game. Why do we need artificial chickens?" (Karelian Russian woman born in 1968), underlining that wild food plays a major role in the Karelian diet. Second, Naumov et al. (48) reported that in Russian Karelia cattle breeding is currently highly subsidized and the subsidies may help pay for veterinary services that were well-developed during Soviet times (9), thus abandoning existing ethnoveterinary knowledge.

In Setomaa (especially Estonian Setomaa) and Dzukija, we recorded more uses, but mainly in the past. We can say that, in these territories, livestock were quite important in the recent past, but their importance has declined in the last few decades [e.g., (49) for Belarus and Poland; Kalle and Kass (50) for Estonia]. Our interviewees in Estonia still remember when the "one cow" restriction was lifted in the 1980s and everyone was allowed to keep as many animals as they were able to. The restrictions were lifted as private livestock farmers provided a significant portion of the country's milk. Collective farming system could no longer feed the people and so the economic model had to change. The main reason why cows are no longer kept in Estonia is that dairies stopped buying milk from small rural households during the transition to a market economy. At that time, keeping cows became no longer economically viable because there was nowhere to sell the milk.

The process of knowledge erosion may be occurring as a result of different socio-economic changes. First, in both regions, the aging of the rural population has contributed to the abandonment of small-scale livestock breeding (51–53). Second, the trend of "industrializing" livestock has reduced the number of farms and increased the number of animals per farm [we also observed this phenomenon in Finnish Karelia, and it has been found in the Mediterranean context as well, e.g., (54)]. This phenomenon gathered livestock farms in specialized districts and makes use of specialized "academic" knowledge, rather than any local veterinary knowledge. Third, in Setomaa, we observed that an increasing number of people are looking for off-farm jobs (e.g., government employment) which provide a monetary wage and are thus more appealing than farming activities. Such a change started in Estonia in the 1990s with the transition to a market economy, when the planned economy was abolished. In this type of shift, animal husbandry, which requires daily attention, is often the first activity to be abandoned, followed by labor-intensive crop production. Fourth, the policies concerning food production that have been implemented by the European Union in the last several decades may have impacted the system of livestock farming (55, 56). Finally, there may be a widespread tendency to downplay local knowledge by preferring "official veterinary" medicine, stressing the sanitary and economic perspective of animal husbandry. This phenomenon may be also fostered by the increased income and availability of official veterinary medicine. Our interviewees in Estonia explained that the main reason why it was necessary to call a veterinarian was that the bacterial content of the milk was measured every time milk was sold. If an animal had inflammation, no milk could be purchased, so the animal had to be cured quickly, with an antibiotic; yet one was not allowed to sell milk during treatment. Thus, people lost financially when they simply poured milk into the sewers, and one of the main reasons why the animals were not treated at home with their own resources was that there were strict hygiene requirements for the sale of milk and meat. In addition, today, sanitary requirements to prevent African swine fever have closed all small pig farms.

In addition, some local drivers of decline of ethnoveterinary knowledge can be identified. For instance, in Russian Setomaa, one possible driver could be related to the drastic political and economic changes in the region. Indeed, during Soviet times, animal husbandry accounted for a relevant proportion of food production by ruble value, while after 1991 livestock herds declined precipitously and the number of livestock raised by households continues to decline (57, 58). Also, according to our observations, in Belarus (and partially in Lithuania) a rapid decline in rural population size is one of the drivers of livestock husbandry abandonment and its associated knowledge. In most cases, during the autumn-winter period, the older generation of respondents goes to live with their children in nearby settlements. When we looked for the most senior residents, most of them were in the city under the care of children and usually returned home only in the spring and summer. Another possible factor that affects the evolution of ethnoveterinary knowledge in present-day Dzukija concerns political decisions related to restrictions on keeping domestic pigs and birds in

the border region. This is linked with the implementation of EU and the Republic of Belarus regulations in 2014–2020 to limit the threat of African Swine Fever. These political decisions endanger traditional livestock practices by undermining the very core of their existence, and, accordingly, the practice of ethnoveterinary medicine.

Possible Sources of Ethnoveterinary Knowledge

Our results show that ethnoveterinary knowledge partially overlaps in the selected regions, especially in countries formerly part of the Soviet Union. For instance, some taxa such as the cultivated *Linum usitatissimum* and the wild *Hypericum* spp., *Quercus robur*, *Alnus* spp, and *Rumex* spp. were reported across several countries. As five out of the eight studied countries were part of the Soviet Union, we compared our field data against the uses recommended in three popular veterinary medicine books published in 1919 (Gurin), 1988 (Rabinovich), and 2007 (Korobov), representing the commonly used remedies in Imperial Russia, as well as during the Soviet and post-Soviet periods. Finally, we traced the possible sources of veterinary knowledge related to those plants listed in Bloshenko et al. (59) which were reportedly used for ethnoveterinary purposes in the Soviet Union.

We found that the seeds of *Linum usitatissimum* were used for their mucolytic and diuretic properties, which is comparable with their recorded use for diarrhea, as flaxseed extract has been proven effective for treating enteric and non-enteric pathogens (60). These uses were earlier confirmed in Gurin (61), Rabinovich (62), and Korobov et al. (63), although the seeds were claimed to have a slight laxative effect. In addition, we recorded the use of *Linum usitatissimum* as a postpartum supplement, which has been found to be effective in rats (64) due to the presence of beneficial fatty acids (e.g., alpha-linolenic acid) as reported by Bloshenko et al. (59). Gurin (61) and Korobov et al. (63) also suggested the use of flax seeds in wet poultices to foster cicatrization. Flaxseeds were commonly sold in pharmacies during Soviet times. Flax used to be an important economic crop in Belarus, Pskov Oblast of Russia, and the Estonian part of Setomaa. During our fieldwork in Pskov Oblast, we often observed remnants of that time period on farms: retting ponds (*mochilo*) that were used in the cycle of fabric production from flax. Older informants could remember bringing flaxseed to the commonly accessible mills to produce flaxseed oil.

Bloshenko et al. (59) also reported the ethnoveterinary use of *Hypericum perforatum*, *Quercus robur*, and *Rumex* spp. for wound healing and cell regeneration, as well as for their antiseptic properties. *Hypericum perforatum* was also mentioned by Duke (65) who reported that in Soviet times it was used for several applications including the treatment of diarrhea, which we recorded in five of the countries we studied. Various uses of *Hypericum* were reported by Rabinovich (62) and Korobov et al. (63), but they were not mentioned by Gurin (61). However, this plant seems to have been known and used in folk medicine even before the Soviet Union [see (66)].

Deryabin and Tomalcheva (67) reported the use of *Quercus robur* for treating mild diarrhea (as we recorded in five countries), but they traced such a use back to the medieval European medicinal tradition. The use of *Quercus robur* was also recorded in Gurin (61), Rabinovich (62), and Korobov et al. (63). The roots of *Rumex* spp., and specifically *Rumex confertus*, were used in Russian traditional medicine as an astringent (68), use that we also recorded in the Russia and Bukovina. Its use has only been confirmed in Soviet and post-Soviet sources (62, 63).

We can therefore see some overlapping patterns in our records and the veterinary medicine promoted during the time of the Soviet Union, which established kolkhozes where livestock breeding was managed by highly specialized actors. Indeed, highly specialized veterinary doctors worked in almost every village. Several sources suggest that Soviet medicine relied largely on plants and products based on them and thus such uses were often transferred from the human sphere to the animal one (following the idea that livestock were considered to be part of the family). Although it was not explicitly stressed by our interviewees, we can see that some of our findings reflect the official medicinal and veterinary recommendations of the Soviet Union, possibly through the formation of veterinary specialists who actually treated the animals. The recommendations themselves relied on veterinary knowledge derived partially from ethnoveterinary knowledge that underwent scientific tests, so the local uses supported by the official veterinary medicine had a greater chance for survival.

Future research should investigate the heterogeneous trajectories that ethnoveterinary knowledge takes under different drivers of change to pastoral systems, and the complex networks of sources from where farmers derive their ethnoveterinary knowledge. Additionally, it would be interesting to evaluate the effects of the use of plant-based ethnoveterinary remedies in accordance with current legislation in Europe.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ca' Foscari University of Venice Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GM, GV, and RS conceived the study. NK, VK, OB, RS, RK, JP, NS, and GM gathered the data. RS, AP, and GV

supervised the study. GM analyzed the data with the help of the co-authors. GM drafted the first version of the manuscript which was then reviewed, edited, and approved by OB, RK, VK, NK, JP, NS, AP, GV, and RS. All authors contributed to the article and approved the submitted version.

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Ferrate (VI) Oxidation Is an Effective and Safe Way to Degrade Residual Colistin - a Last Resort Antibiotic - in Wastewater

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The rise of novel *mcr* mobile resistance genes seriously threatens the use of colistin as a last resort antibiotic for treatment of multidrug-resistant Gram-negative bacterial infections in humans. Large quantities of colistin are released annually into the environment through animal feces. This leads to environmental toxicity and promotes horizontal transmission of the *mcr* gene in aqueous environments. We examined colistin degradation catalyzed by the presence of strong oxidant Fe (VI). We found almost complete colistin degradation (>95%) by Fe (VI) at initial colistin levels of 30 μM at a molar ratio of Fe (VI): colistin of 30 using an initial pH 7.0 at 25°C for 60 min. The presence of humic acid did not alter the degradation rate and had no significant impact on the removal of colistin by Fe (VI). Quantitative microbiological assays of Fe (VI)-treated colistin solutions using *Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis* indicated that the residual antibacterial activity was effectively eliminated by Fe (VI) oxidation. Luminescent bacteria toxicity tests using *Vibrio fischeri* indicated that both colistin and its degradation products in water were of low toxicity and the products showed decreased toxicity compared to the parent drug. Therefore, Fe (VI) oxidation is a highly effective and environment-friendly strategy to degrade colistin in water.

Keywords: degradation, colistin sulfate, humic acid, antibacterial activity, toxicity, water

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INTRODUCTION

Veterinary antibiotics are routinely used globally to control infectious diseases and to improve animal growth and feed efficiency (1). However, a large proportion of administered antibiotics are excreted in urine and feces as parent compounds due to poor gut absorption or incomplete metabolism (2, 3). China produces and consumes the most antibiotics of any country and 58% of the annual accumulation of 84,000 tons is excreted by animals (4, 5). This continued release into the environment combined with incomplete removal during wastewater treatment has resulted in the presence of these veterinary antibiotics in aquatic environments (6, 7). Antibiotic residues in water can alter ecosystems and even lead to the development of antimicrobial resistance and this is one of the most serious global threats to human and animal health (8–10). Therefore, measures to degrade or remove antibiotics during wastewater treatment are essential to reduce their harm to the environment, animals and humans.

Colistin (also known as polymyxin E) belongs to the polymyxin family of polypeptide antibiotics derived from *Bacillus polymyxa* var. *colistinus* and was introduced to clinical practice in the late 1950s (11). The bactericidal activity of colistin is essentially that of a cationic detergent that associates with Gram-negative bacterial cell membrane phospholipids and Lipid A. This association leads to rapid permeability changes in the membrane causing leakage of intracellular components, ultimately resulting in cell death (12). Most Gram-negative bacteria are susceptible to colistin and these include the most prevalent animal and human pathogens such as *Enterobacter aerogenes, Escherichia coli, Haemophilus influenzae, Bordetella pertussis, Salmonella* spp., *Legionella pneumophila, Shigella* spp., *Klebsiella pneumoniae, and Pseudomonas aeruginosa* (12). Colistin is widely used in animal farming and especially poultry and swine production because it is extremely effective for treating Gram-negative intestinal infections caused by *E. coli, Salmonella* spp., and *Pseudomonas* spp. Colistin is also used in Asia, Europe, and North America for animal growth promotion but its neuro- and nephrotoxicity restrict its use in humans (9, 12–14). The emergence of multidrug-resistant Gram-negative bacteria (e.g., *Enterobacteriaceae*, *P. aeruginosa,* and *Acinetobacter baumannii*) over the past decade has caused resurgence of colistin use as a last resort antibiotic for human infections (15).

When administered orally, colistin is very poorly absorbed by the gastrointestinal tract and is eliminated almost unchanged and thereby reaches water bodies or wastewater treatment facilities in its active form (12, 16). Conventional wastewater treatment methods do not adequately remove colistin. For instance, we have determined the colistin levels in swine wastewater collected from pig farms in central China and the results revealed colistin concentrations ranging from 145 to 10,628 ng L^{-1}. A toxicity study on the earthworm *Eisenia fetida* revealed that colistin could up-regulate heat shock protein 70 and inhibit metallothionein gene expression while damaging mitochondria and the endoplasmic reticulum (17). In addition to these direct environmental effects of colistin, the development of resistance is increasing. It was formerly believed that bacterial resistance to polymyxins was very low and primarily the result of mutations in chromosomal genes. However, the discovery of a novel mobile colistin resistance mechanism encoded by *mcr*-1 on a mobilizable plasmid by has altered this notion (9, 18). Nine additional *mcr* genes (*mcr*-2-10) have since been discovered from animals and food isolates as well as human clinical strains (19–24). A wide range of human and animal pathogens have been identified that carry these plasmid-mediated resistance genes and this poses a huge threat to sustaining effectiveness of colistin against clinical infections caused by carbapenemase-producing carbapenem-resistant *Enterobacteriaceae.* This scenario heralds the destruction of the last family of the last resort antibiotics, the polymyxins (9, 18, 25).

Recent assessments of the risk of developing resistance when used as a feed additive has been raised by the European Union in June 2016 and China banned colistin as feed additive in April 2017 (26). Nonetheless, colistin still threatens ecosystems and human health thus efficient treatment strategies are needed to remove colistin from wastewater before environmental discharge.

Ferrate VI (FeO_4^{2-}) [Fe (VI)] is an extremely effective oxidant that is in current use for bioremediation of wastewater (27–29). Under acidic conditions, the redox potential of Fe (VI) (2.2 V) is greater than ozone (2.07 V) and Fe (VI) is considered to be the strongest disinfectant and oxidant that are currently in use for wastewater and water treatment (28). This inorganic compound is environmentally friendly and its by-product is the non-toxic Fe (III) (30–32). Fe (VI) is a multipurpose treatment chemical and has been used to coagulant or precipitate arsenic (33) and heavy metals (34) and is also a powerful disinfectant for bacteria (31, 35) and viruses (36). Pre-treatment of an aqueous solution requires only a very small amount of Fe (VI) to improve the removal rate of natural organics including humic acid (HA) (37). Additionally, Fe (VI) was found to be effective in promoting the oxidative transformation of pharmaceuticals such as fluoroquinolones (38–40), sulfonamides (41), β-lactams (42) and β-blockers (43). Since the removal of colistin has not been explored, we examined whether Fe (VI) could promote colistin removal from wastewater.

This study aimed to (i) assess the influence of Fe (VI) level, solution pH and reaction duration on the removal of colistin to identify optimum conditions; (ii) evaluate whether humic acid in wastewater would interfere with colistin removal by Fe (VI); (iii) measure the antibacterial activity of reaction mixtures against *E. coli*, *S. aureus,* and *B. subtilis* and (iv) determine the toxicity of colistin before and after Fe (VI) oxidation using a Microtox bioassay testing system. As far as we know, this is the first paper to study the removal of colistin from water using Fe (VI) treatment.

MATERIALS AND METHODS

Reagents

Colistin sulfate (>66.7%) was obtained from China Institute of Veterinary Drug Control (Beijing, China). Peptone soy broth (TSB) and Mueller-Hinton broth (MHB) were purchased from Qingdao Hope (Qingdao, China). HPLC grade methanol, acetonitrile and formic acid purchased from Sigma-Aldrich (Munich, Germany). All other reagents were of analytical grade. Potassium ferrate solid [K_2FeO_4, Fe (VI)] of 99% purity was provided by Guangzhou Kexing Chemical (Guangzhou, China). Humic acid (HA) was obtained from Tianjin Berens Biotechnology (Tianjin, China). Ultrapure water was prepared using a Millipore Milli-Q system (Molsheim, France). Stock solutions of colistin (60 μM) were prepared by dissolving colistin sulfate in Milli Q water. Fe (VI) solution was prepared by adding solid Fe (VI) to 1 mM $Na_2B_4O_7$/5 mM Na_2HPO_4 at pH 9.0.

Removal of Colistin by Fe (VI) and Influence of Humic Acid

Batch oxidation tests were conducted in 50 mL conical flasks equipped with magnetic stirrers and contained 10 mL of Fe (VI) and 10 mL colistin solution at the initial concentration of 30 μM followed by pH adjustment. The mixtures were stirred at 500 rpm at 25.0 ± 0.2°C and 0.8 mL samples were removed and immediately passed through a 0.22 mm nylon membrane

filter into a 2.0 mL vial containing 0.2 mL of sodium thiosulfate (100 mM). The sample was then applied to an Oasis HLB solid phase extraction (SPE) cartridge (Waters, Milford, MA, USA) prior to LC-MS/MS analysis.

The amount of Fe (VI) that optimized colistin removal was examined by adding Fe (VI) into a colistin solution to maintain the molar ratios of Fe (VI) and colistin at 5:1, 10:1, 20:1, 30:1, and 40:1. The pH of the mixture was then adjusted to 9.0, stirred for 60 min at ambient temperature and then quenched with sodium thiosulfate followed by being applied to Oasis HLB SPE cartridges as per above.

The effects of pH on Fe (VI)-mediated colistin removal was examined in a Fe (VI): colistin 30:1 solution and the pH of the mixture was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10. The samples were stirred for 60 min and then quenched and applied to SPE cartridges as per above. These initial conditions were also used to determine the optimal reaction durations for the Fe (VI)-mediated colistin removal at pH 7.0. The samples were stirred and samples were taken at 30 s, 1, 2, 5, 10, 15, 30, 45, and 60 min, quenched with sodium thiosulfate and then cleaned up by Oasis HLB SPE as per above. HA interference in the Fe (VI)-mediated colistin removal was examined using 10 mL colistin solutions individually mixed with HA at 0, 1.0, 5.0, 15, 30, and 60 mg L^{-1} followed by the addition of 10 mL Fe (VI) to maintain a molar ratio of Fe (VI): colistin at 30:1. The pH of the mixture was then adjusted to 7.0. The samples were reacted and quenched and SPE cleaned up as per above.

All experiments were performed in triplicate and the mean ± SD values were calculated. Analysis of variance was conducted with SPSS 17.0 software (IBM, Chicago, IL, USA) to elucidate statistical differences between groups.

Analytical Procedures

Colistin levels in solutions were determined using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Chromatographic separation was performed with a TSKgel Amide-80 column (150 × 2.0 mm, 3.0 μm, Tosoh, Tokyo, Japan) in a column oven maintained at 40°C. Solvent A and B were 0.1% formic acid in water and acetonitrile, respectively. The flow rate was 0.6 mL min^{-1}. Colistin was eluted with a linear gradient of 0–0.8 min, 5% A; 0.8–1.5 min, 5–80% A; 1.5–3.0 min, 80% A; 3.0–3.5 min, 80–5% A; 3.6–5 min, 5% A. The injection volume was 10 μL. An Acquity UPLC I-Class system coupled to a Xevo TQ-S mass spectrometer (UPLC-TQ-S/MS) was used for analyte determination (Waters). The conditions of instrumentation were set as follows: the electrospray ion source was used in positive ionization mode with multi-period multiple reaction monitoring (mpMRM) (**Table 1**). The operation conditions were as follows: source temperature, 120°C; capillary voltage, 3.0 kV; desolvation temperature, 400°C; desolvation gas flow rate, 950 L h^{-1}; collision gas flow rate, 0.15 mL min^{-1}, cone gas flow rate, 150 L h^{-1}. The limit of detection (LOD) of colistin A and colistin B were 5 nM and limit of quantification (LOQ) of colistin A and colistin B were 15 nM.

TABLE 1 | Multiple reaction monitoring settings for MS/MS analysis of colistin.

Analytes	Pre-cursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (eV)
CSA[a]	390.7	379.1, 384.9[c]	2	12, 10
CSB[b]	385.9	374.4, 380.1 [c]	4	12, 10

[a] *Colistin A.*
[b] *Colistin B.*
[c] *Quantification ions.*

Antibacterial Activity Assays

Colistin degradation was determined using a solution of 30 μM colistin and Fe (VI) 900 μM under the optimal reaction conditions of pH 7.0, stirring at 500 rpm at 25.0°C for 60 min. Blank reaction solutions were prepared in the same way except that colistin was replaced by water. The bacterial strains *E. coli* ATCC 25922, *E. coli* K88, *S. aureus* and *B. subtilis* were used at 1 × 10^6 CFU mL^{-1} and added to samples from the degradation assay employing broth microdilution assays to measure the antibacterial activities of the reaction solution. Briefly, colistin samples were 2-fold diluted in a 96-well microdilution plate in MHB. Inoculums (100 μL) prepared in MHB were added to each well and the plates were covered before incubated at 37°C for 16–20 h. The absorbance of the solutions at 600 nm was determined as a surrogate of cell density. The antibacterial activity of colistin at concentration of 30 μM was determined using the same method. Growth inhibition percentage was calculated from the corresponding absorbance value from the plate reader using Equation (1) (41), where the A_{max} represents the maximum value of absorbance which indicates no growth inhibition (0%). The inhibition growth I (%), therefore, varies from 0 to 100%.

$$I(\%) = (A_{max} - A)/A_{max} \times 100\% \quad (1)$$

OriginPro 9.1.0 statistical software (Origin Lab, Northampton, MA, USA) was employed to calculate the 50% growth inhibition (EC_{50}) on basis of the dose-response curve prepared by each reaction solution. To assess the antibacterial activity of each sample, a potency equivalent quotient (PEQ) value was calculated from Equation (2) (44) where $Colistin_0$ and $colistin_t$ represents colistin solution before and after Fe (VI) treatment.

$$PEQ = EC_{50}(colistin_0)/EC_{50}(colistin_t) \quad (2)$$

Ecotoxicity Assessment

The ISO standard (45) with a *Vibrio fischeri* luminescence bioassay was employed to examine the toxicity of colistin and its degradation products. In brief, *V. fischeri* were exposed to the samples before and after Fe (VI) treatment under the optimal reaction conditions for 15 min at 15 ± 0.5°C, followed by determination of their bioluminescence intensities by a GloMax Multi Detection System (Promega, Madison, WI, USA). Then we computed the relative inhibitory rate (IR%) according to Equation (3) (46), in which E_0 represents the normalized

bioluminescence intensities of colistin solution before Fe (VI) treatment and E represents that of colistin solution after Fe (VI) treatment.

$$\text{Relative inhibitory rate (IR\%)} = (E_0 - E)/E_0 \times 100\% \quad (3)$$

RESULTS AND DISCUSSION

The emergence of mobile colistin resistance genes has threatened the role of colistin as the last line of defense against multidrug-resistant Gram-negative bacteria. Nonetheless, colistin is still allowed to be used as a feed additive in many countries and it is therefore necessary to find an effective removal procedure before it enters the aquatic environment. In this study we examined colistin degradation by Fe (VI) oxidization.

Optimization of the Conditions for Degradation of Colistin by Fe (VI)

Fe (VI): Colistin Molar Ratio Dependence

The initial dosage of Fe (VI) used is an important factor affecting its self-decomposition since it is proportional to its decomposition rate. In aqueous solution, this rate for Fe (VI) was previously determined to be 11% in solutions of 25 mM and complete decomposition occurred at levels of >30 mM within 60 min (47). We found that the molar ratio significantly affected colistin degradation (**Figure 1**). When the Fe (VI): colistin ration was increased from 5 to 30, colistin degradation rose from 8.3 to 96.9%. Degradation reached the level of statistical significance at molar ratios of 30 and 40 compared with the other ratios ($P < 0.05$; **Table 2**). The most likely reason for this was that at low K_2FeO_4 levels, the amount of Fe (VI) in solution available for reaction with colistin was insufficient and this was overcome at the higher K_2FeO_4 levels. Consistent with these interpretations, when the molar ratio increased from 30 to 40, colistin degradation increased slightly by only 0.8% and no significant difference between 30 and 40 was found ($P > 0.05$; **Table 2**). Results similar to these have been previously reported when Fe (VI) was employed to degrade bisphenol A (48). These reaction conditions provided sufficient Fe (VI) in solution to react with colistin and the further increase of potassium ferrate provided excess Fe (VI). The latter would however, accelerate its self-decomposition because elevated Fe (VI) concentrations are unstable (48) so that the effective concentration of Fe (VI) available for colistin degradation would be decreased. We therefore used a ratio of 30 for our remaining experiments since this level provided a high level of colistin degradation and was economical.

Initial pH Dependence

The stability of ferrate is enhanced at higher pH (47) according to the following formula (49):

$$4\,FeO_4^{2-} + 10\,H_2O \rightarrow 4Fe^{3+} + 3O_2 + 12OH^- \quad (4)$$

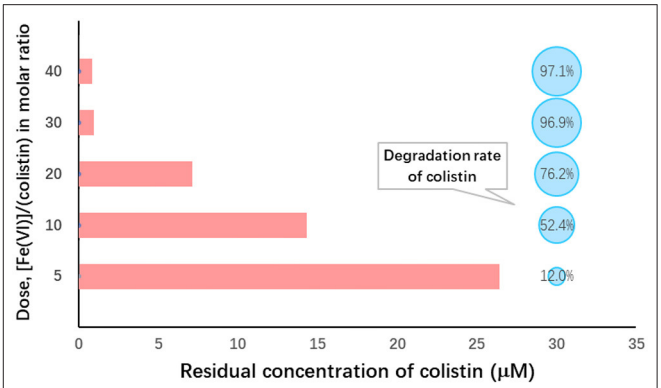

FIGURE 1 | Ferrate (VI): colistin molar ratio dependence on colistin removal from solution. Initial colistin concentration, 30 μM; [Fe(VI)]/(colistin) molar ratio, 5–40; initial pH 9.0; temperature, 25°C; reaction duration, 60 min.

TABLE 2 | Analysis of variance on degradation of colistin by different molar ratio of ferrate (VI) to colistin[a].

Molar ratio of ferrate (VI) to colistin	*N*	Subset of alpha = 0.05 (Degradation rate,%)[b]			
		1	2	3	4
5.00	3	8.3322			
10.00	3		52.3565		
20.00	3			76.2158	
30.00	3				96.9185
40.00	3				97.0968
Significance		1.000	1.000	1.000	0.946

[a] *Experimental conditions: initial colistin concentration = 30 μmol L^{-1}; the molar ratio [Fe(VI)]/(colistin) = 5–40; initial pH = 9.0; temperature = 25°C; reaction duration = 60 min.*
[b] *Group mean values in a subset of the same type are displayed.*

The production of OH^- from the reaction would lead to pH elevation that would also serve to inhibit Fe (VI) self-decomposition, thereby improving the stability of Fe (VI) in solution. In contrast, the presence of elevated H^+ in solution as the pH falls would neutralize the generated OH^- and increase the reaction rate but also accelerate Fe (VI) decomposition. Therefore, Fe (VI) is more stable under alkaline conditions than under neutral and acidic conditions.

In this study, however, we found that the highest level of colistin degradation (96.6%) occurred under neutral conditions (pH 7.0) and the lowest (89.7%) was observed at pH 10 (**Figure 2**). Analysis of variance indicated that colistin degradation at pH 7 was significantly greater than those at all other pH values we examined ($P < 0.05$) and that at pH 10 was significantly lower than those at other pH values ($P < 0.05$; **Table 3**). Similar results were obtained when Fe (VI) was used to oxidize chloramphenicol (50). The initial pH of the solution most likely influences not only Fe (VI) stability which decreases as the pH declines, but also the oxidation capacity of Fe (VI) that decreases at elevated pH. Although Fe

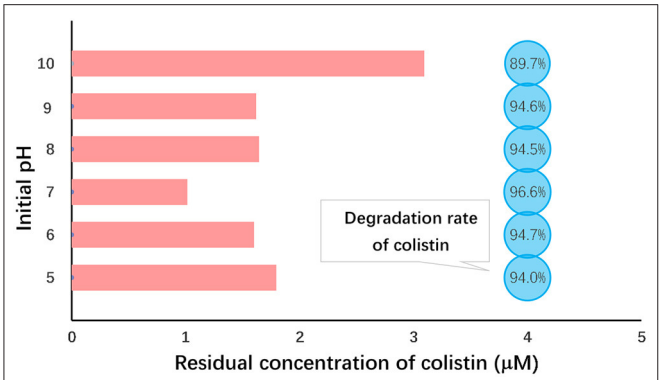

FIGURE 2 | Initial pH dependence of colistin removal. Initial colistin concentration, 30 μM; [Fe (VI)]/(colistin) molar ration, 30; initial pH 5.0–10.0; temperature, 25°C; reaction duration, 60 min.

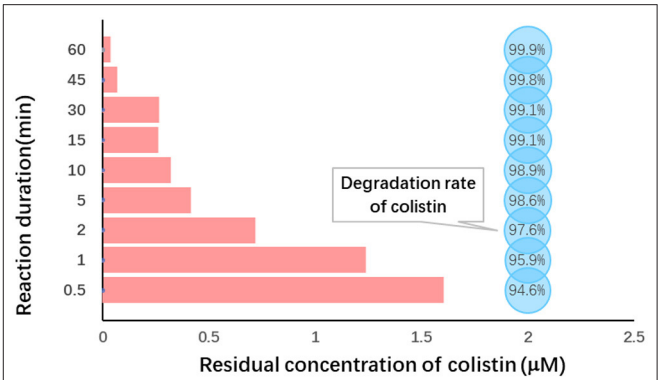

FIGURE 3 | Reaction duration dependence of colistin removal. Initial colistin concentration, 30 μM; [F (VI)]/(colistin) molar ration, 30; initial pH, 7.0; temperature, 25°C, reaction duration, 0.5–60 min.

TABLE 3 | Analysis of variance on degradation of colistin by ferrate (VI) at different pH values[a].

pH	*N*	Subset of alpha = 0.05 (Degradation rate,%)[b]		
		1	2	3
10.0	2	89.6829		
5.00	3		94.0103	
8.00	3		94.5154	
9.00	3		94.5998	
6.00	3		94.6629	
7.00	3			96.6167
Significance		1.000	0.293	1.000

[a]*Experimental conditions: initial colistin concentration = 30 μmol L^{-1}; the molar ratio [Fe(VI)]/(colistin) = 30; initial pH = 5.0–10.0; temperature = 25°C; reaction duration = 60 min.*

[b]*Group mean values in a subset of the same type are displayed.*

(VI) is stable under alkaline conditions, its low redox potential (0.7 V) restricts the reaction and colistin removal. Under acidic conditions, although Fe (VI) shows a high redox potential (2.2 V), its self-decomposition hindered the colistin reaction and Fe (VI) self-decay occurred rapidly within minutes at pH < 6.0 (50, 51). Therefore, extremes of pH were not conducive to colistin removal and we selected pH 7.0 as the optimal initial pH for further study.

Reaction Duration Dependence

In order to achieve better degradation of colistin by Fe (VI), the effect of reaction duration was studied using the optimized level of Fe (VI) at pH 7.0. Colistin was degraded rapidly in the first 10 min and then the rate slowed (**Figure 3**). The degradation rate was significantly increased from 94.6 to 98.9% from 30 s to 10 min ($P < 0.05$) and remained at a plateau until 45 min and then increased slightly (**Table 4**). Due to the strong oxidizing power of Fe (VI), the majority of colistin (98.9%) was removed in the first 10 min. Following this, Fe (VI) consumption in the oxidation reaction as well as self-decomposition slowed the degradation rate. A time limit for the reaction was therefore set at 60 min to maximize colistin removal and was used in the remainder of this study.

Colistin removal at 60 min was 99.9% and exceeded the level in the molar ratio and pH experiments where the maximum values were each 96.9 and 96.6%, respectively. When we examined the influence of Fe (VI) dosage on degradation, we set the initial pH at 9.0. In those experiments, the highest degradation rate was obtained at an Fe (VI): colistin molar ratio of 30 and pH 7.0 for 60 min. These slight discrepancies can be accounted for by differences in reaction batches of Fe (VI) due to (1) moisture, gas and atmospheric reducing substances that would interact with solid K_2FeO_4 and reduce the stability of Fe (VI) and (2) the rapid degradation of Fe (VI) under suboptimal conditions such that occur during pH adjustments that could alter the amount of active Fe (VI) present in the K_2FeO_4. However, since excess Fe (VI) was used in each reaction, trace amounts of colistin in the final reaction mixture were at the level of experimental error and not reaction condition. Therefore, Fe (VI) could readily and effectively remove colistin under the optimal conditions used in this study.

Interference of HA

Dissolved organic matter such as HA are common in wastewaters and natural water (52). We therefore examined whether HA at levels of 0–60 mg L^{-1} inhibited colistin removal by Fe (VI) under optimal conditions. We found that compared to the control, the presence of HA had no apparent impact on the degradation efficiency of colistin ($P > 0.05$; **Table 5**). Similar results were obtained in removal of polychlorinated diphenyl sulfides by Fe (VI) (52). The rapid reaction times for Fe (VI) and colistin most likely allowed this reaction to outcompete reactions with HA.

Antibacterial Activity of Degradation Products

In order to find out if the colistin degradation products disturb the microecological balance in water and if they still pose selective pressure on generation and spread of *mcr*-1 in the environment, the antibacterial activities of colistin and its degradation products

TABLE 4 | Analysis of variance on degradation of colistin by ferrate (VI) at different reaction duration[a].

Reaction duration (min)	*N*	Subset of alpha = 0.05 (Degradation rate,%)[b]					
		1	2	3	4	5	6
0.500	3	94.6452					
1.00	3		95.8749				
2.00	3			97.6093			
5.00	3				98.6102		
10.0	3					98.9248	
30.0	3					99.1104	
15.0	3					99.1210	
45.0	3						99.7714
60.0	3						99.8769
Significance		1.000	1.000	1.000	1.000	0.353	0.456

[a]*Experimental conditions: initial colistin concentration = 30 μmol L^{-1}; the molar ratio [Fe(VI)]/(colistin) = 30; initial pH = 7.0; temperature = 25°C, reaction duration = 0.5–60 min.*
[b]*Group mean values in a subset of the same type are displayed.*

TABLE 5 | Analysis of variance on degradation of colistin by ferrate (VI) in the presence of humic acid[a].

Concentration of humic acid (mg L^{-1})	*N*	Subset of alpha = 0.05 (Degradation rate,%)[b] 1
5.00	3	99.6582
0.00	3	99.6647
1.00	3	99.6786
15.0	3	99.6920
30.0	3	99.6925
60.0	3	99.7088
Significance		0.124

[a]*Experimental conditions: initial colistin concentration = 30 μmol L^{-1}; the molar ratio [Fe(VI)]/(colistin) = 30; initial pH = 7.0; temperature = 25°C, reaction duration = 60 min.*
[b]*Group mean values in a subset of the same type are displayed.*

were determined with *E. coli, S. aureus,* and *B. subtilis* and we used PEQ analysis as a quantitative test of the dose-response curves. A PEQ value of 1 indicated that the EC_{50} of colistin before and after treatment were not different. A reduction in the antibacterial activity of colistin results in an increase in the EC_{50} of ($colistin_t$) and a PEQ < 1. Similarly, PEQ $>$ 1 means that treatment enhances the antibacterial activity of colistin (53). All our experiments resulted in PEQ < 1 (**Figure 4**), indicating decreases in antibacterial activity. The *E. coli* strains ATCC 25922 and K88 generated PEQ < 0.02 and *S. aureus* gave PEQ $= 0.15$, implying that the antibacterial activity of colistin against them was nearly eliminated by Fe (VI) oxidation. The *B. subtilis* PEQ was 0.88 and close to 1 and was the result of high EC_{50} ($colistin_0$) and EC_{50} ($colistin_t$) values. *B. subtilis* is a Gram-positive probiotic bacterium and is widely distributed in the environment. Since colistin is a cationic polypeptide that targets lipid A that is absent in Gram-positive bacteria (14, 54), the high value of EC_{50} ($colistin_0$) against *B. subtilis* was expected. The value of PEQ close to 1 indicated that similar to colistin, the degradation products showed no obvious antibacterial activity to *B. subtilis* as well. However, it has been previously reported that colistin can destabilize the biofilm matrix structure even in species with intrinsic colistin resistance such as *S. aureus*, resulting in the release of planktonic cells that are more susceptible to antibiotics (55). In order to examine the change of antibacterial activity of colistin against both Gram-negative and Gram-positive bacteria, we included *E. coli,* the most common Gram-negative bacterium carrying plasmid-mediated resistance *mcr* genes as well as *S. aureus*, the representative Gram-positive bacteria in this study. The PEQ for *S. aureus* was higher than that for *E. coli* because the EC_{50} ($colistin_0$) against *S. aureus* was ∼10-fold greater than that against *E. coli.* Nonetheless, the EC_{50} ($colistin_t$) against *E. coli* and *S. aureus* were similar. Fe (VI) has been proven effective in eliminating the activity of other antibiotics including trimethoprim (41), β-lactams (44) and fluoroquinolone (53). In conclusion, the colistin degradation products showed almost no antibacterial activity against representative Gram-negative and Gram-negative bacteria demonstrating that the method for colistin degradation was environmentally friendly. They would not affect the microecological balance in water and posed no selective pressure on generation and spread of *mcr-1* in the environment.

Ecotoxicity Assessment

Colistin has significant side effects when used in humans and animals including nephrotoxicity that results in hematuria, proteinuria, urinary casts and increased blood urea and creatinine levels (54). In addition, this antibiotic is also neurotoxic with symptoms including paresthesia, peripheral neuropathy, muscle weakness and neuromuscular blockade resulting in respiratory paralysis (56). Colistin is also an exotoxin and is toxic to the earthworm *E. fetida* (17). However, there are no studies that assess the biological toxicity of colistin and its degradation products in water. We therefore wanted to determine whether colistin and its degradation products from Fe (VI) oxidation are non-toxic. For these experiments we measured alterations in the luminescence of *V. fischeri* with

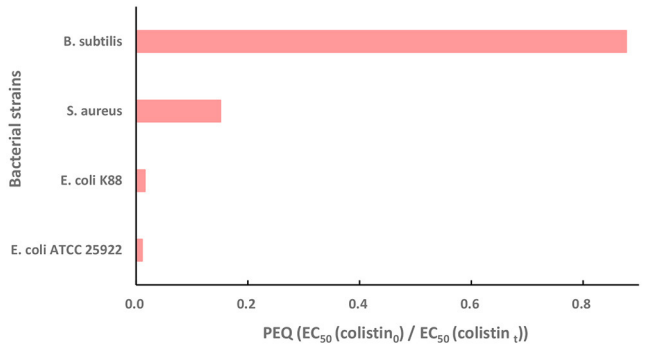

FIGURE 4 | The potency equivalent quotient (PEQ) of colistin against *E. coli* ATCC 25922, wild type strain *E. coli* K88, *S. aureus* and *B. subtilis*. Initial colistin concentration, 30 μM; [Fe(VI)]/(colistin) molar ratio, 30; initial pH, 7.0; temperature, 25°C, reaction duration, 60 min. PEQ = EC_{50} ($colistin_0$)/EC_{50} ($colistin_t$) where $colistin_0$ and $colistin_t$ represent colistin before and after Fe (VI) treatment.

exposure to colistin and the degradation products. In order to remove the interference of Fe (VI) on the growth of *V. fischeri,* Fe (VI) activity at the end of the test periods were quenched using $Na_2S_2O_3$ prior to bacterial exposure in the Microtox test.

Our results indicated that the IR% of colistin degradation products (2.99%) to *V. fischeri* was lower than that of colistin (7.38%), although these differences were not significant ($P > 0.05$). This was consistent with previous studies for the Fe (VI) oxidation of indomethacin (57), bisphenol A (48) and tetrabromobisphenol A (46). In the latter studies, the degradation products exhibited less toxicity to the *V. fischeri* bacteria compared to the parent drug. Our results also indicated that colistin and its degradation products were of low toxicity to the water environment because their IR% value were <30 (58).

One limitation of the current study is that we did not describe the degradation kinetics and identify the degradation products. Due to the large excess of Fe (VI) involved in the reactions, we found that >90% of colistin was degraded after reaction for 5 s (data not shown), indicating that most of degradation was completed nearly in an instant. The purpose of this study was to find an effective way to eliminate colistin in a short time and in turn, to reduce its risk of promoting the generation and spread of plasmid-mediated resistance *mcr* genes in the environment. The latter could cause failure as rescue treatments for human infections with multidrug-resistant bacilli rather than to compare subtle differences under different conditions. Therefore, we did not further study the degradation kinetics of colistin by Fe (VI) oxidation.

Since colistin sulfate is actually a mixture of colistin A and B as well as minor components, we were unable to identify all the components in the reaction mixtures using LC-MS/MS (data not shown). However, the final degradation products possessed minimal antibacterial activity against sensitive and non-sensitive bacteria and most had no toxicity to the luminescent bacteria. Hence, a failure to identify the products would not affect the application of Fe (VI) to remove colistin in practice under the optimal conditions proposed in this study.

CONCLUSIONS

The degradation of colistin by Fe (VI) was investigated and we found that (1) colistin can be effectively degraded by Fe (VI) oxidation (99.9%) using an Fe (VI): colistin molar ratio of 30 with an initial colistin concentration of 30 μM at pH 7.0, 25°C for 60 min. (2) The presence of humic acid had no obvious impact on the removal of colistin by Fe (VI). (3) The products of colistin produced by Fe (VI) degradation possessed no antibacterial activity against *E. coli, S. aureus* and *B. subtilis,* indicating that Fe (VI) would be effective at reducing selective pressure of colistin on environmental bacteria for generation and spread of plasmid-mediated colistin resistance genes. (4) Both colistin and its degradation products in water were of low toxicity to *Vibrio fischeri*.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LW contributed to research, wrote the first draft of the manuscript, revision, read, and approved the submitted version. SL, XW, and BL contributed to data analysis, revision, read, and approved the submitted version. ZW contributed conception, revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Bioassay Guided Fractionation of *Senna singueana* and Its Potential for Development of Poultry Phytogenic Feed Additives

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There has been burgeoning interest in plant-based feed additives following restrictions placed on the use of antibiotic feed additives in many countries. Phytogenic feed additives are recommended to have a range of useful properties to support the growth and development of poultry to a similar level as that obtained by supplementing feed with antibiotics. The aim of this study was to evaluate the antibacterial, anti-lipoxygenase and antioxidant activity, and *in vitro* safety of fractions and isolated compounds from leaves of *Senna singueana*. Antibacterial activities of the fractions and isolated compounds were determined against a panel of bacteria using a two-fold serial microdilution assay and qualitative bioautography assays. Anti-lipoxygenase activity was evaluated using the ferrous oxidation-xylenol orange (FOX) method. Antioxidant activity was assessed qualitatively and quantitatively using radical scavenging assays. Dichloromethane and ethyl acetate fractions from solvent-solvent partitioning had the best antibacterial activity with MIC values ranging from 156 to 313 μg/ml. Fractions obtained from column chromatography had significant to weak antibacterial activity with MIC values ranging from 50 to 1,250 μg/ml. Bioautography showed clear bands of bacterial inhibition, indicating the presence of a number of active compounds in several fractions. The ethyl acetate fraction and all the tested column fractions had potent anti-lipoxygenase activity with IC_{50} values of ≤2.5 μg/ml which were lower than that of quercetin (positive control), indicating anti-inflammatory potential. The ethyl acetate fraction and several column fractions had powerful antioxidant activity with IC_{50} values of ≤5 μg/ml in the ABTS assay. Cytotoxicity values against Vero kidney cells ranged from LC_{50} = 40.0–989.3 μg/ml. Bioassay-guided fractionation led to the isolation and identification of a known bioactive compound, luteolin. *S. singueana* is a promising candidate for the development of poultry phytogenic feed additives.

Keywords: antibacterial, anti-lipoxygenase, antioxidant, cytotoxicity, phytogenic, poultry feed additives, *Senna singueana*

INTRODUCTION

Phytogenic feed additives (PFAs) should have biological activity if they are to be used as alternatives to antibiotic growth promoters (AGPs). Reviews published to date have highlighted that phytogenic feed additives should have therapeutic value, such as antimicrobial, antioxidant, anti-inflammatory, immunostimulatory, anticoccidial, antiviral, and anti-ulcer (1–3). It therefore follows that compounds used in developing phytonutrient formulations for use as poultry growth promoters should preferably have multiple biological activity. They should therefore be isolated from plant parts rich in therapeutic phytochemicals.

Senna singueana (Delile) Lock belongs to the Caesalpiniaceae family and is native to tropical Africa, occurring throughout mainland tropical regions of Africa (4). Different parts of this plant species have numerous medicinal uses all over Africa. The plant is used to treat fever, malaria, pulmonary troubles, eye problems (conjunctivitis), skin disorders, venereal diseases, abdominal problems, bilharzia, impotence due to diabetes and wounds caused by leprosy, and syphilis (4, 5). It is also used as a purgative and as a lactation stimulant in both humans and animals (4, 5). In Zimbabwe, the leaves of *S. singueana* are used to treat a broad spectrum of poultry conditions such as coccidiosis, Newcastle disease, coughing, and flu-like symptoms (6).

Previous studies have shown that extracts of *S. singueana* leaves have moderate antibacterial activity against poultry pathogens, potent anti-lipoxygenase activity and powerful radical scavenging antioxidant activity (7). The bark methanol extract of *S. singueana* has also been reported to have remarkable hepatoprotective and anti-apoptotic properties (8), promoting further exploration of the plant for beneficial properties and potential uses. In view of its promising multiple biological activities, this study was designed to evaluate the antibacterial, anti-lipoxygenase, antioxidant and safety of *S. singueana* fractions and isolated compounds in order to assess the prospects of developing poultry PFAs from this plant species.

MATERIALS AND METHODS

Plant Collection and Extraction

Senna singueana (Delile) Lock leaves were collected from Chipinge district (20° 23. 300′ S, 032 29. 691′), Manicaland Province in Zimbabwe. The plant was identified by Mr. Chapano, from the National Herbarium in Harare and authenticated by Ms. Magda Nel from the Department of Plant and Soil Science, University of Pretoria, South Africa. A voucher specimen was prepared and deposited in the H.G.W.J. Schweickerdt Herbarium (PRU 0125450) at the University of Pretoria, Pretoria, South Africa.

Extraction and Solvent/Solvent Fractionation

The leaves were dried in a well-ventilated room at 25°C. Dried plant material was ground into a powder using a mill. Exhaustive extraction was carried out on powdered plant material (1,070.86 g) with 80% methanol to afford a crude extract (445.33 g). The crude extract (350.20 g) was subjected to solvent-solvent partitioning by dissolving in water (1,000 ml), and sequential partitioning with 1,000 ml each of *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. Each fraction was evaporated to dryness using a rotary evaporator (Büchi, Germany) under reduced pressure at 40°C.

Column Chromatography

Part of the ethyl acetate fraction (100.66 g) was subjected to column chromatography. Silica gel (230–400 mesh, Merck) (1,063.18 g) was placed in a column with a diameter of 10 mm and an approximate height of 70 mm. The ethyl acetate fraction was loaded on the column and eluted with a combination of chloroform:ethyl acetate:formic acid (6:4:1) in increasing polarity as shown in **Figure 1**. A total of 194 fractions of ∼50 ml each were obtained. The fractions were combined into eleven main fractions based on similarity of TLC phytochemical profiles. Fraction A4 was subjected to further column chromatography and was eluted with chloroform:ethyl acetate:formic acid (7:3:0.5) in increasing polarity (**Figure 1**) to afford eleven fractions based on TLC profiling. Sub-fraction DD2 (0.29 g) was subjected to preparative TLC using chloroform:ethyl acetate:formic acid (7:3:0.5) to yield compound 1 (yellow powder, 41.4 mg), compound 2 (116.7 mg) and compound 3 (82.7 mg).

Thin Layer Chromatography Phytochemical Profiling

TLC phytochemical profiling was done by loading 10 μl of the crude extract, fraction or compound redissolved in their respective solvents or acetone to a concentration of 10 mg/ml on aluminium-backed silica gel plates (10 × 20 cm, 60 F254, Merck, United States). They were developed in three solvent systems of different polarities, namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1)-non-polar solvent system, CEF (chloroform/ethyl acetate/formic acid (5:4:1)-intermediate polar solvent system, EMW (ethyl acetate/methanol/water (40:5.4:4)-polar solvent system (9). The separated phytochemicals were visualised under UV light at wavelengths of 254 nm and 365 nm and visible bands were marked. The TLC plates were then sprayed with freshly prepared vanillin-sulphuric acid reagent (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) and heated at 110°C until optimal colour development (10). Phytochemicals in fractions obtained from the column were analysed using the same procedure.

Antibacterial Assay by TLC Bioautography

The compounds in the crude extract and fractions, as well as the purified compounds were developed as described above but the bioautograms were sprayed with respective cultures. After development, the TLC plates were dried overnight in a stream of cold air and sprayed with an actively growing concentrated suspension of strains of either *Staphylococcus aureus* (ATCC 29213), *Salmonella enterica* subsp. *enterica* var. Enteritidis (*S. Enteritidis* ATCC 13076) or *Escherichia coli* (ATCC 25922). The plates were dried and incubated overnight at 37°C in closed, sterile, humidified plastic containers to allow growth of the bacteria on the plates. After incubation, the plates were sprayed

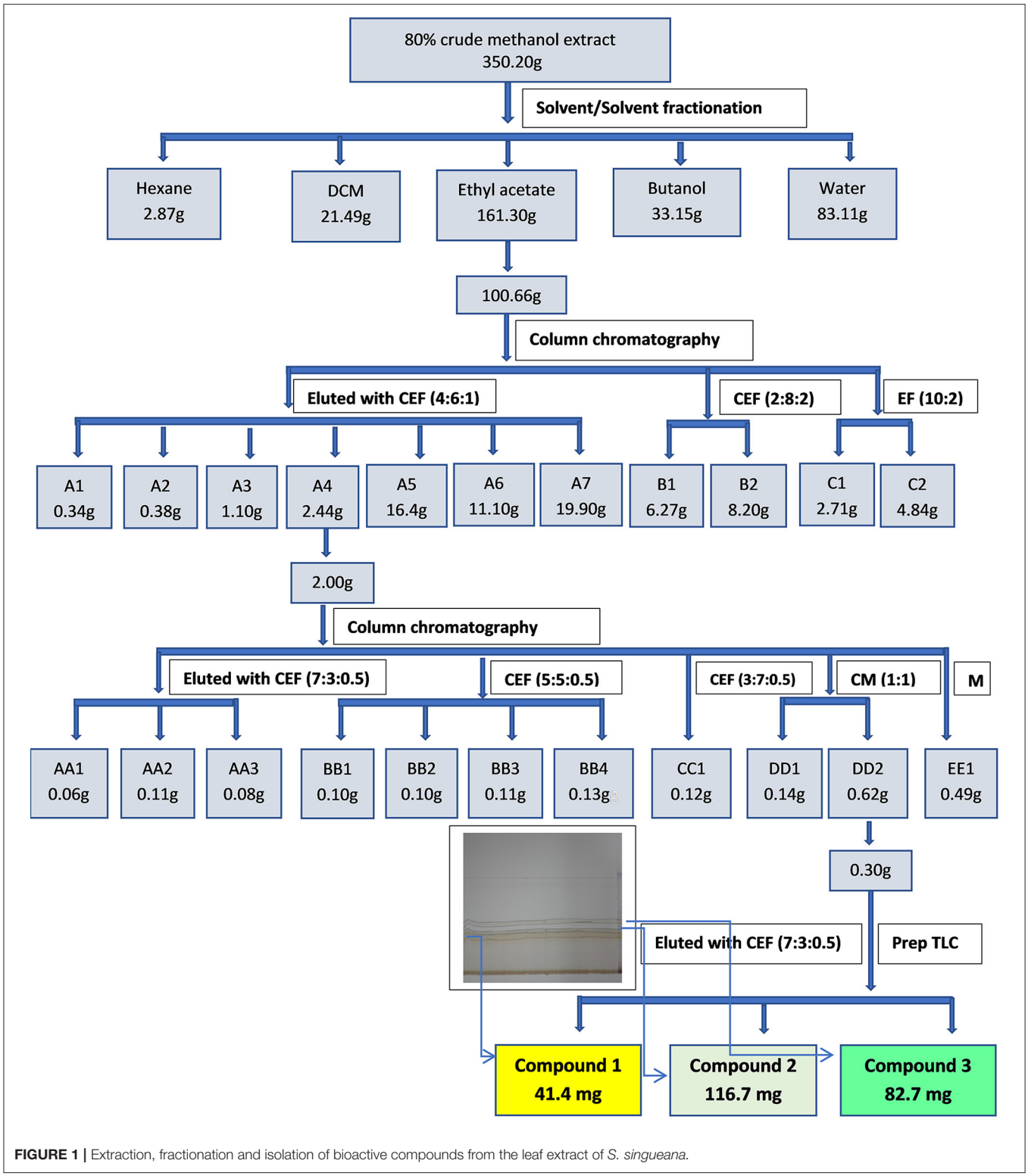

FIGURE 1 | Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *S. singueana.*

with a sterile 2 mg/ml solution of *p*-iodonitrotetrazolium (INT, Sigma-Aldrich) salt and incubated for a further 1 h. The presence of clear zones on the chromatogram after the incubation period indicated inhibition of growth as the INT is metabolised to a coloured formazan product by the actively growing cells (11). The retention factors of the bands of inhibition were calculated.

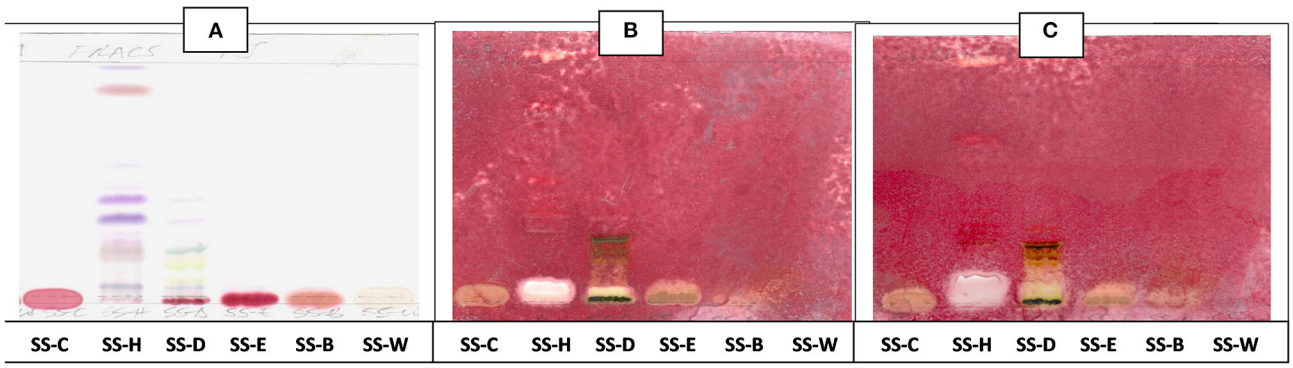

FIGURE 2 | (A) Chromatogram developed in BEA [benzene: ethanol: ammonium hydroxide (90:10:1)] of *S. singueana* fractions sprayed with vanillin. **(B)** Bioautography of *S. aureus*–TLC plate developed with BEA (90:10:1). **(C)** Bioautography of *S. Enteritidis*–TLC plate developed with BEA (90:10:1). SS-C, *S. singueana* crude extract; SS-H, hexane fraction; SS-D, dichloromethane fraction; SS-E, ethyl acetate fraction; SS-B, butanol fraction; SS-W, Water fraction. White bands indicate compounds that inhibit bacteria.

Qualitative Antioxidant Activity

TLC plates (10 × 20 cm, aluminium-backed, Merck, silica gel 60 F254) were loaded with 10 µl of the crude extract, fraction or compound (re-dissolved to 10 mg/ml) and dried before being developed in two mobile phase systems (CEF and EMW). To determine the antioxidant activity, the 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical test was performed directly by spraying the TLC plates with DPPH (0.2% w/v) in methanol to reveal the antioxidant activity of the fractions (12). A change of colour from the DPPH purple background to yellow indicated the presence of antioxidant compounds (13).

Quantitative Antibacterial Assay by Minimum Inhibitory Concentration Assay

The antibacterial activity of the samples was determined by measuring the minimum inhibitory concentration (MIC) using a serial two-fold dilution method (14). The following test organisms were used: *Staphylococcus aureus* (ATCC 29213), *E. coli* (ATCC 25922), *S.* Enteritidis (ATCC 13076), and a clinical strain of *E. coli* obtained from the Department of Veterinary Tropical Diseases, University of Pretoria.

The four bacterial cultures were prepared by inoculating a single colony from an agar plate into 10 ml of sterilised Mueller-Hinton (MH) broth (Merck, South Africa) and incubating at 37°C in an MRC orbital shaker (150 rpm) incubator (United Scientific, South Africa) for 18 to 20 h prior to the experiment. Following incubation, each bacterial strain was diluted in MH broth (Merck, South Africa) and the absorbance was measured at a wavelength of 560 nm using a spectrophotometer (Epoch microplate reader: BioTek, United States). Absorbance was adjusted to match that of a McFarland standard No 1 (corresponding to $\sim 3 \times 10^8$ colony forming units per ml, cfu/ml).

The assay was performed in microtitre plates (Lasec, South Africa) by adding 100 µl of sterile water to all wells. In the first row, 100 µl of extract, fraction or compound were added in triplicate and serially diluted two-fold to the last well, from which 100 µl were then discarded. Gentamicin (Virbac, South Africa) was used as a positive control and a sterility control containing only water was included. This was followed by addition of 100 µl of the bacterial suspension to each well (except for the sterility control). The plates were sealed with parafilm and incubated at 37°C (IncoTherm, Labotec). After 24 h, 40 µl of a 0.2 mg/ml solution of INT was added to each well and the plate further incubated for at least half an hour to ensure adequate colour development. INT is a dehydrogenase activity detecting reagent, which is converted into an intensely coloured red-purple formazan by metabolically active micro-organisms. Inhibition of growth was indicated by a clear solution or a noticeable decrease in colour reaction. This value was taken as the MIC of the sample. The experiments were conducted twice.

Cytotoxicity Evaluation

Cytotoxicity evaluation was done on fractions which showed good activity and the isolated compounds. The cytotoxic effect of the fractions and the isolated compounds was determined using an *in vitro* assay with Vero monkey kidney cells (15). The growth medium used was Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). The cells were seeded at a density of 10 000 cells/per well in 96-well-microtitre plates. The plates were incubated at 37°C in a 5% CO_2 incubator in a humidified environment for 24 h to allow cell attachment.

After incubation, the medium was aspirated and replaced with fresh MEM. The fractions/compounds (100 µl) of varying concentrations were added to the wells containing cells. The anticancer compound doxorubicin (Pfizer Laboratories) was used as a positive control. A suitable blank control with equivalent concentrations of fresh medium was also included and the plates were further incubated for 48 h in a CO_2 incubator. Subsequently, the medium in each well was aspirated from the cells, which were washed with phosphate-buffered saline (PBS) and fresh medium was then added to each well. A 30 µl aliquot of MTT (5 mg/ml in PBS) was added to each well and the plates were incubated at 37°C for 4 h. The medium was then aspirated

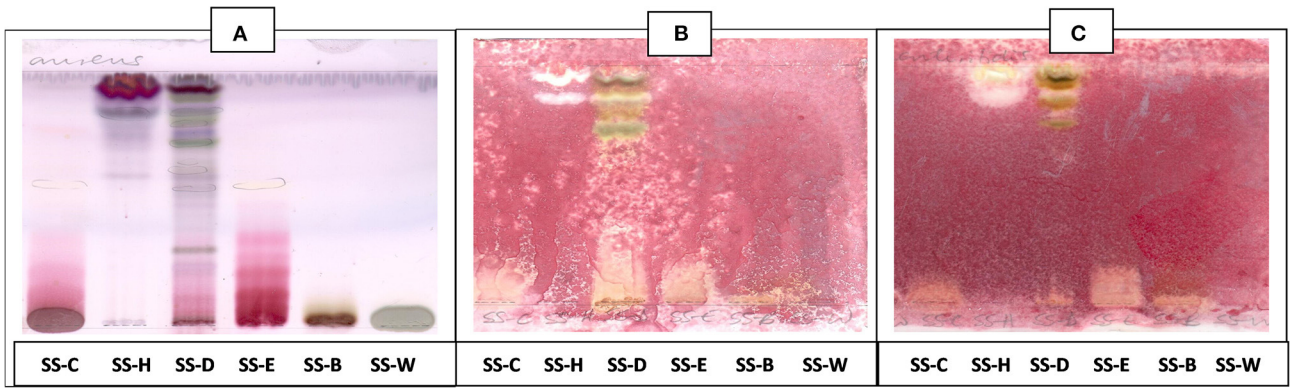

FIGURE 3 | (A) Chromatogram developed in CEF [chloroform: ethyl acetate: formic acid (5:4:1)] of *S. singueana* fractions sprayed with vanillin. **(B)** Bioautography of *S. aureus*–TLC plate developed with CEF (5:4:1). **(C)** Bioautography of *S. Enteritidis*–TLC plate developed with CEF (5:4:1). SS-C, *S. singueana* crude extract; SS-H, hexane fraction; SS-D, dichloromethane fraction; SS-E, ethyl acetate fraction; SS-B, butanol fraction; SS-W, Water fraction. White bands indicate compounds that inhibit bacteria.

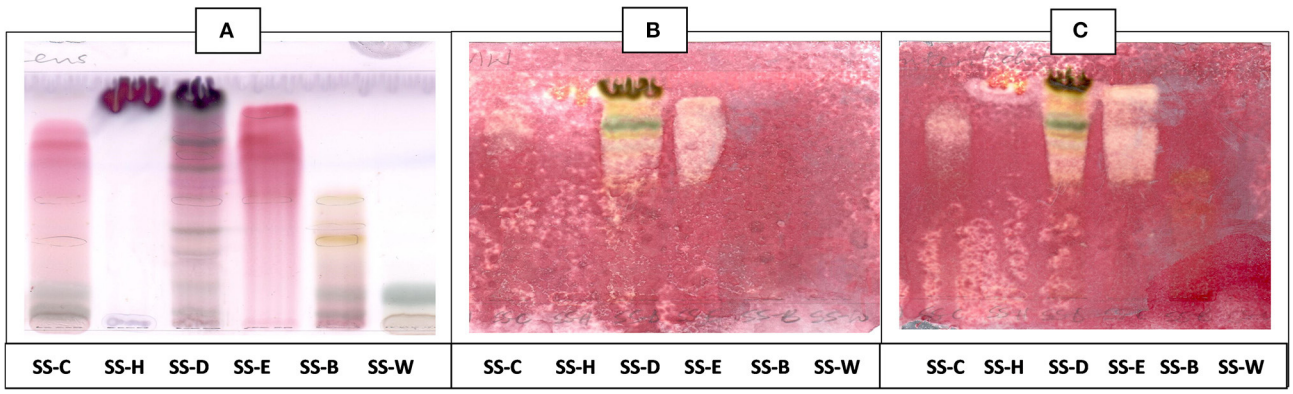

FIGURE 4 | (A) Chromatogram developed in EMW [ethyl acetate: methanol: water (40:5.4:4)] of *S. singueana* fractions sprayed with vanillin. **(B)** Bioautography of *S. aureus*–TLC plate developed with EMW (40:5.4:4). **(C)** Bioautography of *S. Enteritidis*–TLC plate developed with EMW (40:5.4:4). SS-C, *S. singueana* crude extract; SS-H, hexane fraction; SS-D, dichloromethane fraction; SS-E, ethyl acetate fraction; SS-B, butanol fraction; SS-W, Water fraction. White bands indicate compounds that inhibit bacteria.

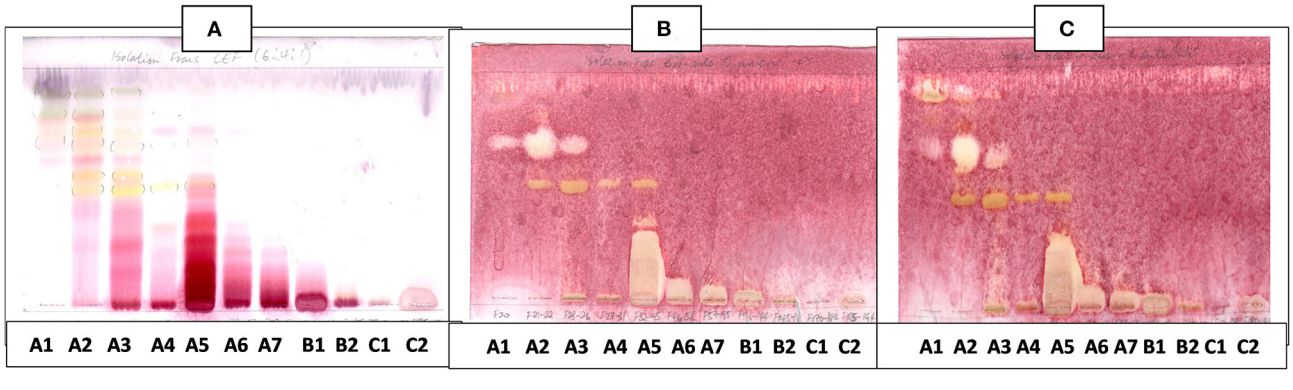

FIGURE 5 | (A) Chromatogram developed in CEF [chloroform: ethyl acetate: formic acid (6:4:1)] of 11 combined fractions obtained from the first column sprayed with vanillin. **(B)** Bioautography of *S. aureus* of the 11 combined column fractions–TLC plate developed with CEF (6:4:1). **(C)** Bioautography of *S. Enteritidis* of the 11 combined column fractions–TLC plate developed with CEF (6:4:1). Yellow and white bands indicate compounds that inhibit bacteria.

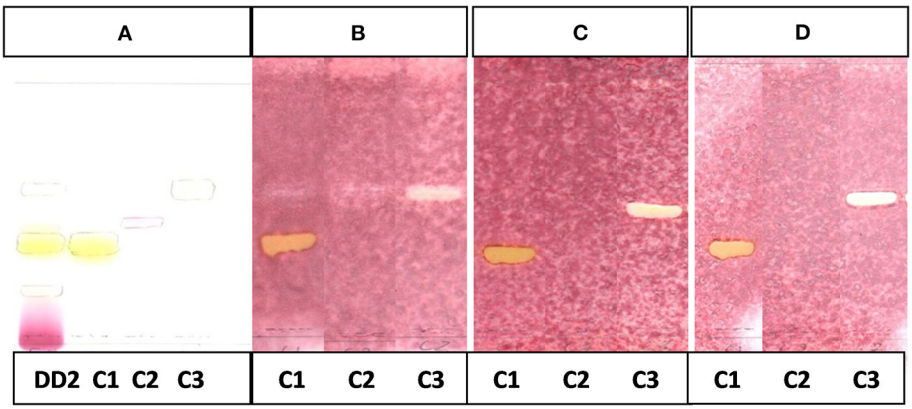

FIGURE 6 | (A) Chromatogram developed in CEF (7:3:0.5) of sub fraction DD2 and the 3 compounds sprayed with vanillin.**(B)** Bioautography of *S. aureus* of the 3 compounds. TLC developed in CEF (7:3:0.5). **(C)** Bioautography of *E. coli* of the 3 compounds. TLC developed in CEF (7:3:0.5). **(D)** Bioautography of *S. Enteritidis* of the 3 compounds. TLC developed in CEF (7:3:0.5). **(B–D)** Composite images of TLC plates run separately for each compound. C1, compound 1; C2, compound 2; C3, compound 3.

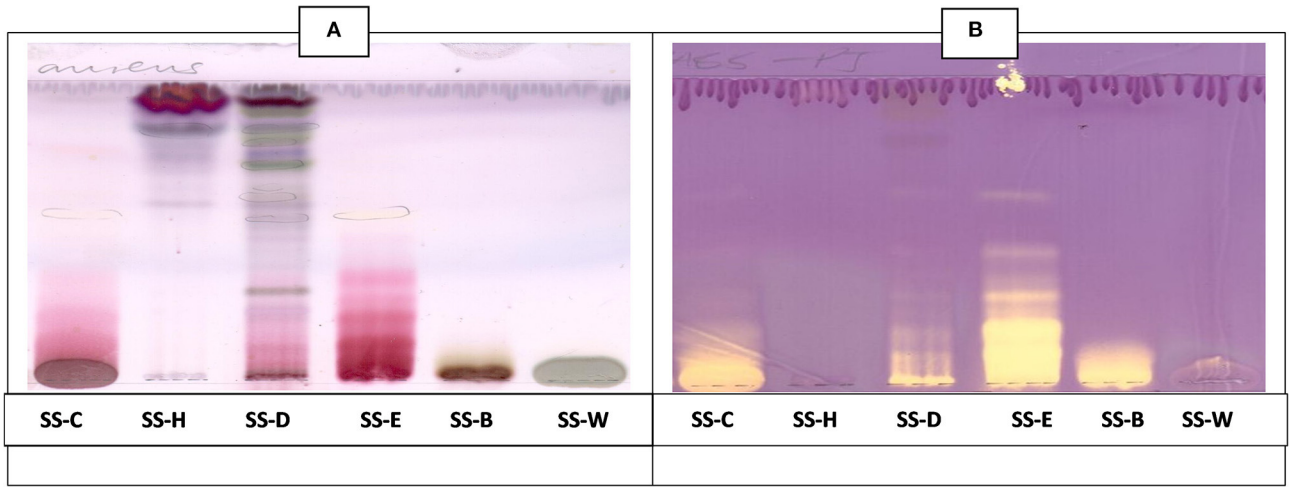

FIGURE 7 | (A) Chromatogram developed in CEF [chloroform: ethyl acetate: formic acid (5:4:1)] of *S. singueana* fractions sprayed with vanillin. **(B)** Antioxidant bioautography–TLC plate developed with CEF (5:4:1) and sprayed with DPPH. Yellowish bands indicate compounds antioxidant activity. SS-C, *S. singueana* crude extract; SS-H, hexane fraction; SS-D, dichloromethane fraction; SS-E, ethyl acetate fraction; SS-B, butanol fraction; SS-W, Water fraction.

from wells and 50 µl DMSO was added to each well to solubilise the formed formazan crystals. The absorbance was measured on a BioTek Synergy microtitre plate reader at 570 nm. Cell growth inhibition for each extract was expressed in terms of LC_{50} values. The selectivity index (SI) was also calculated. The cytotoxicity assay was repeated thrice.

Quantitative Determination of Antioxidant Activity

DPPH (1,1-Diphenyl-2-Picryl Hydrazyl) Free Radical Assay

The antioxidant activities of the samples were measured in terms of radical scavenging ability using the stable radical (DPPH) method of Brand-Williams et al. (12) with some modifications. Methanol solutions (40 µl) of the samples and positive controls (Trolox and ascorbic acid) at various concentrations (0.1–100 µg/ml) were prepared by serial dilution in a 96 well-microtitre plate. One hundred and sixty (160) µl of DPPH in methanol adjusted to an absorbance between 0.9 and 1.0 was added and the plates were incubated in the dark at room temperature (25°C) for 30 min. Absorbance was measured against a blank with a microtitre plate reader (Epoch, BioTek, United States) at 516 nm. The DPPH scavenging effect was determined using the following formula:

$$\text{DPPH Scavenging Effect}(\%) = [(A1 - A2/A1)] \times 100 \quad (1)$$

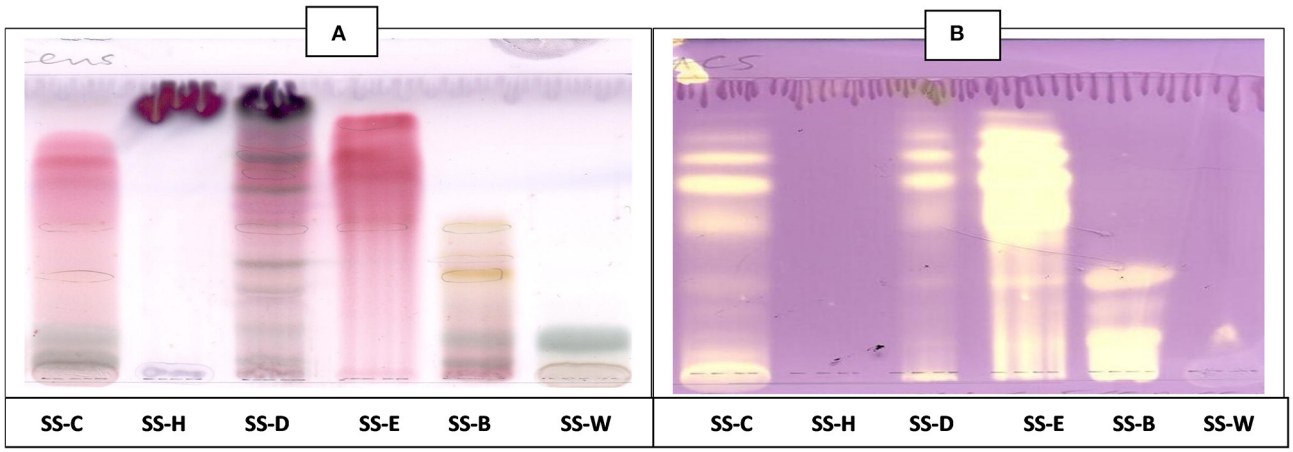

FIGURE 8 | (A) Chromatogram developed in EMW [ethyl acetate: methanol: water (40:5.4:4)] of *S. singueana* fractions sprayed with vanillin. **(B)** Antioxidant bioautography–TLC plate developed with EMW (40:5.4:4) and sprayed with DPPH. SS-C, *S. singueana* crude extract; SS-H, hexane fraction; SS-D, dichloromethane fraction; SS-E, ethyl acetate fraction; SS-B, butanol fraction; SS-W, Water.

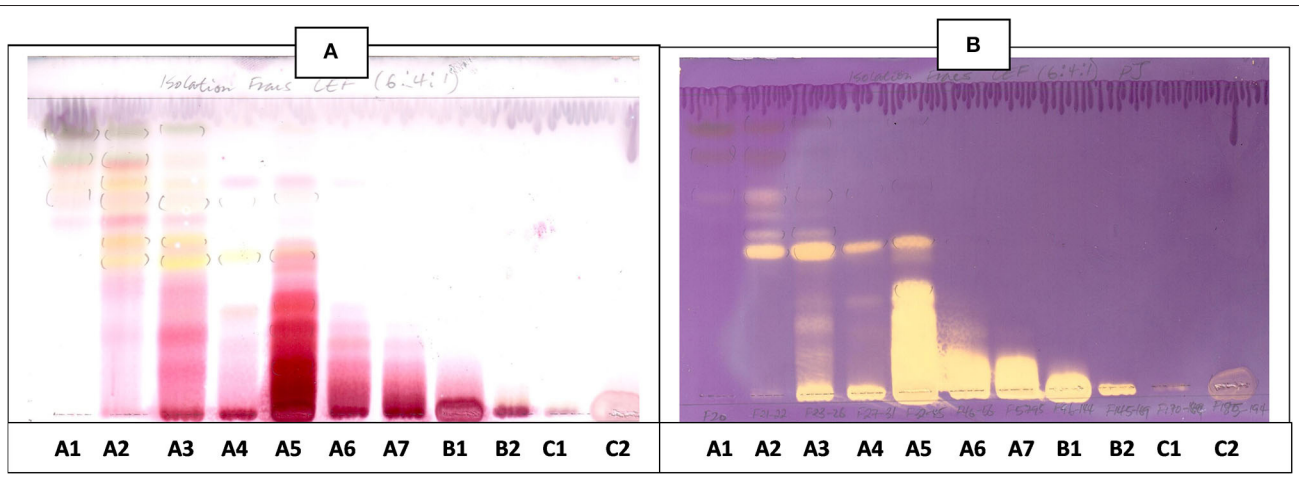

FIGURE 9 | (A) Chromatogram developed in CEF [chloroform: ethyl acetate: formic acid (6:4:1)] of 11 combined fractions obtained from the first column sprayed with vanillin. **(B)** Antioxidant bioautography–TLC plate developed with CEF (6:4:1) and sprayed with DPPH. Yellowish bands indicate compounds antioxidant activity.

Where A1 is the absorbance of the control reaction and A2 is the absorbance in the presence of the sample. Trolox and ascorbic acid were used as controls. The experiments were conducted twice.

ABTS [2,2-Azino-Bis (3-Ethylbenzothiazoline-6 Sulfonic Acid)] Free-Radical-Scavenging Assay

The free radical-scavenging activity as a measure of hydrogen donating capacity was determined by using the ABTS cation decolourization method of Re et al. (16) with some modifications. ABTS radical solution (7 μM) was prepared by dissolving 1.32 × 10^4 μg of ABTS in 10 ml of 50% methanolic solution and 7.68 × 10^4 μg of potassium persulphate ($K_2S_2O_4$) in 10 ml of distilled water. The two solutions were mixed together and made up to 200 ml with 50% methanolic solution, and kept in the dark at room temperature, 25°C for 12 h. Prior to running the assay, the ABTS radical solution was diluted with 50% methanolic solution to an absorbance between 0.7 and 0.8 at 734 nm. The samples were serially diluted (40 μl) (0.1–100 μg/ml) in 96 well-microtitre plates and 160 μl of ABTS radical solution was added to each well. The absorbance readings were taken after exactly 6 min of reaction and blanks were prepared using the respective samples without ABTS radical. The scavenging effect was calculated using the following formula:

$$\text{ABTS Scavenging Effect}(\%) = [(A1 - A2/A1)] \times 100 \quad (2)$$

The IC_{50} values were calculated from a graph plotted as inhibition percentage against the concentration. A Trolox standard curve was drawn by plotting percentage inhibition of the ABTS+ radical against the concentration of Trolox. Data from the test samples were analysed in a similar manner.

Anti-Lipoxygenase (15-LOX) Assay

Lipoxygenase (LOX) activity of the samples was determined spectrophotometrically according to published methods (17, 18). LOX inhibition was determined spectrophotometrically based on the formation of the complex Fe3+/xylenol orange as described by Pinto et al. (19). Briefly, 20 µl of Tris-HCl buffer (pH 7.4) was added to all wells of the 96-well-microplates. This was followed by the addition of 20 µl of the fractions (1 or 0.5 mg/ml) in the first row of the plate which was serially diluted. Quercetin served as the positive control, and the buffer was used as a negative control. After the serial dilution, 40 µl of the lipoxygenase enzyme (Sigma Aldrich, Germany) was added to each well and the plates were incubated at room temperature 25°C for 5 min. After incubation, 40 µl of linoleic acid (final concentration, 140 µM) prepared in Tris-HCl buffer (50 mM, pH 7.4) was added to the well (except for the blanks). The plates were incubated at 25°C for 20 min in the dark. After incubation, 100 µl of freshly prepared ferrous oxidation–xylenol orange (FOX) reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulphate (100 µM) in methanol/water (9:1)] was added to all wells. The plates were further incubated at 25°C for 30 min in the dark, 40 µl of linoleic acid was then added to the blanks. The absorbance was measured at 560 nm. The selectivity index (SI) values regarding anti-LOX activity were calculated by dividing cytotoxicity LC_{50} values by the IC_{50} values of relevant bioactivity ($SI = LC_{50}/IC_{50}$) (20). The experiments were conducted twice.

Structure Elucidation of Compounds

Structures of the isolated compounds were identified using nuclear magnetic resonance (NMR) (1D) spectroscopy. ^{1}HNMR data was acquired on a 400 MHz NMR spectrometer (Bruker Avance III 400 MHz) while ^{13}CNMR data was acquired on a 125 MHz NMR spectrometer. The structures of the isolated compound that was able to be identified was confirmed by comparison of the NMR data with those published previously. The molecular weight of the compound was confirmed using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS).

Data Analysis

Data were presented as mean ± standard deviation (SD) of the determinations. The Shapiro-Wilk Normality Test was used to check for normality of antioxidant and anti-LOX data. The hypothesis of normality was rejected when the $p \leq 0.05$. Log transformation of data was carried out on data which was not normally distributed. Statistical analyses of the antioxidant and anti-LOX data was then performed using the Tukey– Kramer multiple comparison *post-hoc* test following one way ANOVA. A $P < 0.05$ was considered statistically significant. The data were computed using IBM SPSS Statistics.

RESULTS

Qualitative Antibacterial Activity

The antibacterial activity with bioautography method (**Figures 2–4**) indicated that the *n*-hexane, dichloromethane and ethyl acetate fractions had antibacterial activity against *S. aureus* and *S.* Enteritidis (**Figures 2B,C**) with the *n*-hexane fraction having prominent bands when using BEA as mobile phase. The antibacterial compounds did not move from the point of origin.

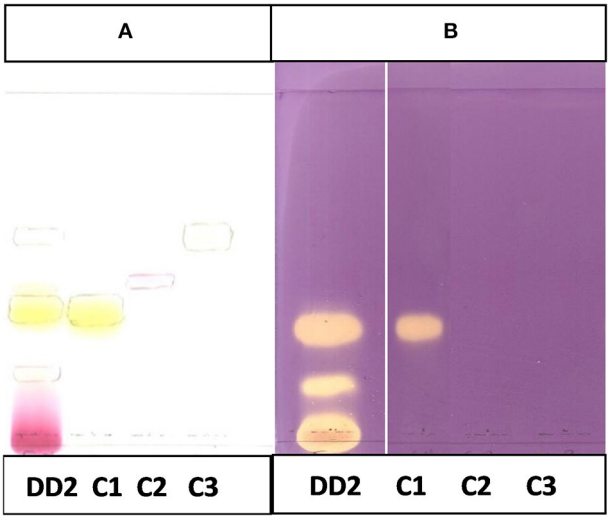

FIGURE 10 | (A) Chromatogram developed in CEF (7:3:0.5) of sub fraction DD2 and the 3 compounds sprayed with vanillin. **(B)** Chromatogram developed in CEF (7:3:0.5) of sub fraction DD2 and the 3 compounds sprayed with DPPH.

The CEF and EMW mobile phase separated antibacterial compounds in the n-hexane, dichloromethane and ethyl acetate fractions, with the dichloromethane and ethyl acetate fractions having significant bands of inhibition (**Figures 3B,C**, **4B,C**). However, the active compounds of the ethyl acetate fraction did not separate into clear bands. Fractions obtained from the column, namely A1, A2, A3, A4, A5, A6, A7, and B1 had active compounds, with the A1, A2, A3, A4 fractions showing better separation of active bands (**Figures 5B,C**). Bioautography of the isolated compounds showed that compound 1 (R_f value = 0.32) and compound 3 (R_f value = 0.51) were active against *S. aureus*, *E. coli* and *S. Enteritidis* (**Figures 6B–D**).

Qualitative Antioxidant Activity

The CEF and EMW antioxidant bioautography (**Figures 7**, **8**) showed that the dichloromethane, ethyl acetate and the butanol fraction had antioxidant activity with the ethyl acetate profile showing prominent bands of DPPH bleaching (**Figures 7B**, **8B**). The antioxidant compounds of the ethyl acetate fractions did not separate into distinct bands. Antioxidant bioautography also showed that column fractions A2, A3, A4, A5, A6, A7, B1, and B2 had bands of antioxidant activity (**Figure 9B**). Compound 1 also had antioxidant activity in the bioautography assay (**Figure 10B**).

Quantitative Antibacterial Activity

MIC values ≤ 100 µg/ml indicate significant activity, 100 < MIC ≤ 625 µg/ml moderate activity and values >625 µg/ml indicate weak activity (21). Of the fractions obtained from solvent/solvent-solvent partitioning solvent fractionation, the dichloromethane and ethyl acetate fractions had the best

TABLE 1 | Yield and minimum inhibitory concentrations (MICs), and of *S. singeuana* fractions against ATCC strains.

Sample	%yield	*Staphylococcus aureus* (ATCC 29213)	*Escherichia coli* (ATCC 25922)	*Salmonella Enteritidis* (ATCC 13076)	*Escherichia coli* (clinical strain)
		MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)
Crude extract	41.59	625 ± 0.00	235 ± 86	625 ± 0.00	469 ± 170
Hexane frac	0.82	2,500 ± 0.00	2,500 ± 0.00	1.25 ± 0.00	469 ± 170
Dichloromethane	6.14	313 ± 0.00	156 ± 0.00	313 ± 0.00	104 ± 40
Ethyl acetate frac	46.05	235 ± 86	156 ± 0.00	313 ± 0.00	117 ± 43
Butanol frac	9.47	938 ± 342	156 ± 0.00	625 ± 0.00	469 ± 170
Water frac	23.73	>2,500	>2,500	>2,500	>2,500
A1	0.34	938 ± 313	313 ± 0.00	729 ± 56	938 ± 342
A2	0.38	156 ± 0.00	**78 ± 0.00**	117 ± 42	156 ± 0.00
A3	1.09	156 ± 0.00	**52 ± 20**	**78 ± 0.00**	**78 ± 0.00**
A4	2.42	117 ± 39	**78 ± 0.00**	156 ± 0.00	**78 ± 0.00**
A5	16.29	235 ± 78	156 ± 0.00	313 ± 0.00	313 ± 0.00
A6	11.03	156 ± 0.00	**65 ± 20**	**78 ± 0.00**	**78 ± 0.00**
A7	19.77	156 ± 0.00	**78 ± 0.00**	156 ± 0.00	156 ± 0.00
B1	6.23	156 ± 0.00	**78 ± 0.00**	156 ± 0.00	156 ± 0.00
B2	8.15	313 ± 0.00	156 ± 0.00	313 ± 0.00	235 ± 86.00
C1	2.69	1,042 ± 295	938 ± 342	938 ± 0.342	625 ± 0.00
C2	4.81	1,250 ± 0.00	938 ± 342	1,250 ± 0.00	1,250 ± 0.00
Compound 1	13.80	625 ± 0.00	156 ± 0.00	1 250 ± 0.00	156 ± 0.00
Compound 2	38.90	313 ± 0.00	>2,500	>2,500	>2,500
Compound 3	27.57	313 ± 0.00	625 ± 0.00	>2,500	625 ± 0.00
Gentamicin	N/A	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00

Frac, fraction, A1–C2 indicates column fractions. MIC values ≤ 100 μg/ml indicate significant activity, 100 < MIC ≤ 625 μg/ml moderate activity and values >625 μg/ml indicate weak activity. Values in bold indicate MICs lower than 100 μg/mL.

TABLE 2 | Cytotoxicity (LC_{50} values) and selective index of the *S. singueana* fractions with respect to antibacterial activity.

Fraction	LC_{50}	Test organisms and Selectivity index (SI) = LC_{50}/MIC			
	(μg/ml)	*S. aureus* (ATCC 29213)	*E. coli* (ATCC 25922)	*S. Enteritidis* (ATCC 13076)	*E. coli* (Clinical strain)
Dichloromethane	40.0 ± 2.8	0.1	0.3	0.1	0.4
Ethyl acetate	139.3 ± 19.5	0.6	0.9	0.4	1.2
A2	63.6 ± 16.3	0.4	0.8	0.5	0.4
A3	85.2 ± 6.7	0.5	**1.6**	**1.1**	**1.1**
A4	151.5 ± 16.1	**1.3**	**1.9**	**1.0**	**1.9**
A5	989.3 ± 61.3	**4.2**	**6.3**	**3.2**	**3.2**
A6	109.6 ± 20.0	0.7	**1.7**	**1.4**	**1.4**
B1	142.2 ± 7.3	0.4	**1.8**	0.9	0.9
Compound 1	92.9 ± 1.7	0.1	0.6	0.6	0.6
Compound 2	309.5 ± 72.8	1.0	ND	ND	ND
Compound 3	79.4 ± 4.3	0.3	0.1	ND	0.1
Doxorubicin	9.00 ± 1.28	N/A	N/A	N/A	N/A

Values in bold indicate SI > 1, ND, Not determined; N/A, Not applicable.

antibacterial activity with MIC values ranging from 156 to 313 μg/ml against the three ATCC strains and *E. coli* clinical strain (**Table 1**). These two fractions had moderate antibacterial activity against the tested strains. The fractions obtained from the first column had significant to weak antibacterial activity against the bacterial strains with MIC values ranging from 50 to 1,250 μg/ml. Fraction A2, A3, A4, A6, A7, and B1 had significant antibacterial activity against the *E. coli*

TABLE 3 | Antioxidant activity of *S. singueana* fractions.

Fraction	DPPH IC_{50} (μg/ml)	ABTS IC_{50} (μg/ml)
Crude extract	6.08 ± 0.33^{a}	1.82 ± 0.77 (1.80)^{a}
Hexane	158.79 ± 31.20^{d}	126.30 ± 9.76^{c}
Dichloromethane	9.33 ± 1.17^{a}	4.06 ± 1.17^{a}
Ethyl acetate	2.69 ± 0.22^{a}	2.46 ± 0.17^{a}
Butanol	9.85 ± 0.94^{a}	3.98 ± 0.62^{a}
Water	274.41 ± 6.26^{e}	53.84 ± 22.64^{b}
A1	108.42 ± 12.52^{c}	60.09 ± 9.18^{b}
A2	8.86 ± 1.10^{a}	7.07 ± 0.87^{a}
A3	3.36 ± 0.29 (4.44)^{a}	1.88 ± 0.26 (2.37)^{a}
A4	3.05 ± 0.03 (3.38)^{a}	1.30 ± 0.12 (1.78)^{a}
A5	6.29 ± 0.67^{a}	2.16 ± 0.48 (2.60)^{a}
A6	7.50 ± 0.40 (6.77)^{a}	1.75 ± 0.24 (2.22)^{a}
A7	6.64 ± 0.12 (6.14)^{a}	1.64 ± 0.23 (2.11)^{a}
B1	6.25 ± 0.51 (5.92)^{a}	1.96 ± 0.23 (2.43)^{a}
B2	7.63 ± 1.51 (7.09)^{a}	2.55 ± 0.32 (3.02)^{a}
C1	12.55 ± 1.05^{a}	10.18 ± 3.51^{a}
C2	60.65 ± 7.23^{b}	199.79 ± 50.00^{d}
Luteolin (Compound 1)	5.92 ± 0.64^{a}	8.17 ± 0.80^{a}
Compound 2	>100	57.40 ± 1.52^{b}
Compound 3	>100	>100
Ascorbic acid	1.97 ± 0.21 (2.45)^{a}	1.90 ± 0.07 (2.45)^{a}
Trolox	3.19 ± 0.32 (4.02)^{a}	2.21 ± 0.30 (2.76)^{a}

N = 3, mean values within a column with different superscript letters are significantly different at $p < 0.05$. The values in brackets indicate IC_{50} values obtained after log transformation of data which was not normally distributed.

ATCC strain while only fractions A3 and A6 had noteworthy activity against the *S. Enteritidis* ATCC strain. Fraction A3, A4, and A6 also had significant activity against the *E. coli* clinical strain. None of the fractions had significant activity against the *S. aureus* ATCC strain, with most having moderate activity against this strain. The isolated compound, luteolin had relatively weak antibacterial activity against all the tested bacterial strains.

Cytotoxicity Results

According to the National Cancer Institute, there are four group classifications for cytotoxicity evaluation: Very active ($LC_{50} \leq 20$ μg/ml), moderately active ($LC_{50} > 20$–100 μg/ml), weakly active ($LC_{50} > 100$–1,000 μg/ml), and inactive ($LC_{50} > 1{,}000$ μg/ml) (22, 23). The ethyl acetate fraction, fractions 3 and A6, compounds 1 and 3 had moderate cytotoxicity against the Vero monkey cells whilst the dichloromethane fraction, Fraction A4, A5, A6, B1, and compound 2 exhibited weak toxicity with LC_{50} values of >100 (**Table 2**).

Quantitative Antioxidant Activity

The ethyl acetate fraction had powerful antioxidant activity with IC_{50} values of 2.69 and 2.46 μg/ml in the DPPH and ABTS assays, respectively (**Table 3**). Fractions A3, A4, A5, A6, A7, and B1 also showed remarkable antioxidant activity with IC_{50} values of <2.5 μg/ml in the ABTS assay. Fraction A4 had the best antioxidant activity in the DPPH assay with an IC_{50} value of 3.05 μg/ml.

Anti-Lipoxygenase Activity

The ethyl acetate fraction and all five fractions from the first column which were tested had potent anti-lipoxygenase activity with each having IC_{50} values of <2.5 μg/ml (**Table 4**). Similar to the antioxidant results, fraction A4 had the most potent anti-lipoxygenase activity with an IC_{50} value of 0.32 μg/ml. The selective index (SI) values regarding anti-lipoxygenase activity (20) of the dichloromethane, ethyl acetate and the five column fractions (A3, A4, A5, A6, B1) were >10.

Structure Elucidation of Isolated Compounds

NMR Results

Analyses of ^{1}H and ^{13}C NMR revealed that compound 1 was luteolin (**Tables 5**, **6**, **Figure 11**). The NMR data for compound 1 were similar to that reported for luteolin by da Silva et al. (24). Luteolin appeared as a single yellow band ($R_f = 0.32$) on spraying with vanillin. Compound 2 appeared as a light pinkish single band (R_f value = 0.42) whilst compound 3 light greenish single band (R_f value = 0.51) after spraying the TLC plate with vanillin (**Figure 6A**). The structures of compounds 2 and 3 could

TABLE 4 | Anti-lipoxygenase activity of *S. singueana* fractions.

Fraction	15-Lox IC_{50} (μg/ml)	LC_{50} (μg/ml)	Selective index
Dichloromethane	5.15 ± 0.07^{c}	40.0 ± 2.8	7.8
Ethyl acetate	2.05 ± 0.37 (1.83)^{b}	139.3 ± 19.5	**68.0**
A3	$1.14 \pm 0.48^{a,b}$	85.2 ± 6.7	**74.7**
A4	0.32 ± 0.12^{a}	151.5 ± 16.2	**473.4**
A5	$0.53 \pm 0.10^{a,b}$	989.3 ± 61.3	**1,866.0**
A6	$0.51 \pm 0.16^{a,b}$	109.6 ± 20.0	**214.0**
B1	1.79 ± 0.08^{b}	142.2 ± 7.3	**79.4**
Luteolin (compound 1)	7.39 ± 0.45^{d}	92.9 ± 1.7	**12.6**
Quercetin (positive control)	12.33 ± 0.71^{e}	N/A	ND
doxorubicin (positive control)	N/A	9.00 ± 1.28	ND

N = 2, mean values with a different superscript letters are significantly different at $p < 0.05$. Values in bold indicate SI >10. The values in brackets indicate IC_{50} values obtained after log transformation of data which was not normally distributed.

TABLE 5 | The ^{13}C NMR spectral data of luteolin isolated from *S. singueana*.

Compound ^{13}CNMR acetone-d_6, 125 MHz	Luteolin ^{13}CNMR (acetone-d_6, 150 MHz, TMS) (24)
94.70	94.7 (C-8)
99.70	99.6 (C-6)
103.85	104.2 (C-3)
105.13	105.3 (C-10)
113.88	114.1 (C-2')
116.66	116.6 (C-5')
119.97	120.1 (C-6')
123.09	123.7 (C-1')
146.87	146.6 (C-3')
150.93	150.2 (C-4')
158.75	158.9 (C-9)
163.29	163.3 (C-5)
165.18	164.9 (C-7)
165.24	165.3 (C-2)
182.98	182.9 (C-4)

not be elucidated because they decomposed before analysis. NMR results suggested that they were triterpenoids.

LC/MS Results

The LC/MS results confirmed that compound 1 was luteolin MS (*m/z*) 285.059 (M-H) (**Figure 11**) with molecular formula ($C_{15}H_{10}$ O_6) and calculated molecular weight of 286.060.

DISCUSSION

Interest in plant-based feed additives has accelerated following restrictions on the use of antibiotic feed additives in many countries. Plant-derived, or phytogenic, feed additives are being investigated as potential alternatives and to promote their commercial use, they are recommended to have various useful properties to support animal or poultry growth and development. One plant with potential for development into a PFA is *Senna singueana* and this study aimed to evaluate antibacterial, anti-lipoxygenase and antioxidant activity as well as *in vitro* safety of fractions and isolated compounds from the leaf material.

Chromatographic analysis (TLC) using three mobile phase systems did not separate the active compounds of the ethyl acetate fraction into distinct bands, suggesting that the constituent compounds may be closely related. The fact that the active compounds of the dichloromethane and ethyl acetate fractions in the BEA antibacterial bioautography profile did not move from the point of origin shows that the compounds are relatively polar. The BEA solvent system used in the study is a non-polar solvent system.

Column fractionation improved the bioactivity and safety profiles of the *S. singueana* methanol leaf extract, as the fractions were more active than the crude extract. Plant extracts or fractions with MICs of $\leq$100 μg/ml are considered to have significant activity (25). Fractions A3, A4, and A6 had significant activity against the Gramme-negative bacteria, *E. coli* and *S. Enteritidis* with MICs of <100 μg/ml. Antibacterial activity is a salient feature of PFAs as it is has been postulated that antibiotic feed additives work by modulating gut microflora of animals, thereby preventing sub-clinical infections and also through allowing efficient absorption of nutrients via the thinner intestinal wall associated with antibiotic-fed animals (26, 27). Although fractions A3, A4, and A6 had noteworthy antibacterial activity and were more active than toxic (SI > 1), their safety margins regarding antibacterial activity were low. It is generally considered that biological efficacy is not due to *in vitro* cytotoxicity when SI $\geq$ 10 (28). Fraction A5 exhibited better safety margins with SI values >3 for all the tested bacterial strains. The SI for fraction A5 regarding *E. coli* was 6.3 which is relatively good. Concerning antibacterial activity, none of the fractions had a therapeutic index of >10, with the most active fractions having selective indexes between 1 and 2.

Antioxidant bioautography revealed that column fractions A2, A3, A4, A5, A6, A7, B1, and B2 had bands of antioxidant activity. Interestingly comparison of the antibacterial and

TABLE 6 | The ^{1}H NMR spectral data of luteolin isolated from *S. singueana*.

Compound ^{1}H NMR (acetone-d_6, 400 MHz)	Luteolin ^{1}H NMR (acetone-d_6, 600 MHz, TMS) (24)	
6.23d	$J = 2.0$	6.25 (1H, d, $J = 2.1$, H-6)
6.52d	$J = 2.0$	6.53 (1H, d, $J = 2.1$,H-8)
6.56s		6.57 (1H, s, H-3)
6.96d	$J = 8.4$	7.00 (1H, d, $J = 8.4$, H-5')
7.44dd	$J = 8.4$; 2.4	7.46 (1H, dd, $J = 8.4$; 2.3, H-6')
7.48d	$J = 2.4$	7.50 (1H, d, $J = 2.3$, H-2')
13.02s		13.00 (1H, s, OH-5)

antioxidant bioautography profiles of the main fractions from the first column revealed that most of the compounds which were responsible for antioxidant and antibacterial activity were likely to be the same as they eluted in similar positions. The crude extract of *S. singueana* exhibited powerful antioxidant activity in both the DPPH and ABTS assays. An IC_{50} value of 6.08 μg/ml was recorded with the crude methanol leaf extract in the DPPH assay which was lower than a previously reported IC_{50} value of 20.8 μg/ml obtained from the bark methanol extract of *S. singueana* (8). Quantitative antioxidant analysis also demonstrated that dichloromethane, ethyl acetate and butanol fractions obtained from solvent-solvent partitioning and column fractions A2, A3, A4, A5, A6, A7, B1, B2, and C1 had strong antioxidant activity as there was no significant difference between the IC_{50} values of these fractions and those of the positive controls in both the DPPH and ABTS assays. Antioxidant activity is an important attribute of PFAs. In addition to preventing the oxidative deterioration of feed it also improves the health of the animals. One of the most prevalent poultry diseases, coccidiosis, is associated with oxidative stress caused by the production of free radical oxidative species during the host cellular response to invasion by *Eimeria* species (29). *Eimeria acervulina* oocysts have also been implicated in lipid peroxidation, increased oxidative damage and imbalances in antioxidant status of infected birds caused by disturbing the oxidative balance (30). This implies that PFAs with powerful antioxidant activities can help in the management of this condition. *In vivo* studies have also shown that plant extracts or herbal formulas rich in antioxidants can be effective anticoccidials (31, 32). It is therefore plausible that *S. singueana* fractions can be useful anticoccidials if added to chicken feed although this needs to be verified by carrying out *in vivo* studies.

The SI of the fractions concerning anti-lipoxygenase activity were very good with some having selectivity indexes of >100 with fractions A4 and A5 having very high selective indexes of 473.4 and 1,866.0, respectively. A similar study on the anti-inflammatory activity of crude acetone extract and fractions of *Grewia mollis* reported selective indexes ranging from 1.04 to 54.45 regarding anti-LOX activity (20). Therefore, fractions A3, A4, A5, A6, B1 have the potential to be developed into potent anti-inflammatory agents as they were more anti-inflammatory than toxic. The key enzyme 15-LOX is responsible for the synthesis and release of leukotrienes from poly-unsaturated fatty acids (PUFAs) (20). The *S. singueana* fractions were able to inhibit this enzyme which is involved in the synthesis of pro-inflammatory mediators. The dichloromethane, ethyl acetate and all the evaluated fractions from the first column had better anti-LOX activity than the positive control (quercetin). The IC_{50} values of these fractions were significantly lower than that of quercetin ($p < 0.5$). It has been postulated that antibiotic growth promoters work by permitting growth through an anti-inflammatory role (33). Therefore, anti-inflammatory activity is an important attribute of plant derived products with potential to replace antibiotic growth promoters. Based on their potent antioxidant and anti-lipoxygenase activity, *S. singueana* fractions have potential to be used for the development of poultry phytogenic additives. However, they might need to be blended with other phytochemicals with good antibacterial activity at non-toxic concentrations to produce phytonutrient formulations which can be used effectively as poultry PFAs.

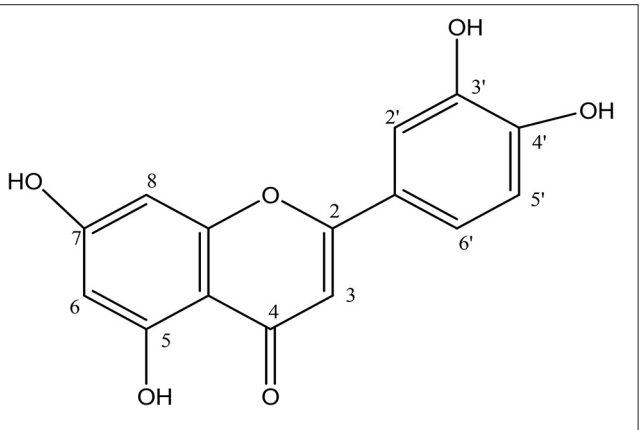

FIGURE 11 | Structure of luteolin.

Four tetrahydroanthracene derivatives which showed significant antibacterial and antiplasmodic activity namely singueanol-I and -II, torosachrysone and germichrysone have been previoulsy isolated from the roots of *S. singueana* (34). Other compounds which have been isolated from *S. singueana* include stigmasterol, stigmast-4-en-3- one, stigmast-4,22-dien-3-one, 1-heneicosanol, and hexyl heneicosanoate from the *n*-hexane leaf extract (35). The flavanoid, luteolin has also been isolated from this plant species previously (36). It was found to be responsible for broad anti-ulcer activities of *S. singueana* leaves

(36). In the current study, luteolin was also isolated. Luteolin exhibited weak antibacterial activity with MICs of >100 μg/ml being recorded. MICs of >100 μg/ml are not good enough for isolated compounds (21). The results on the antibacterial activity of luteolin were consistent with findings from previous studies which also reported MICs of >100 μg/ml against *E. coli*, *Staphylococcus* spp., and *Salmonella* spp. (37, 38). However, other researchers have reported significant *in vitro* antibacterial activity of luteolin against *S. aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *E. coli*, *Pseudomonas fluorescens*, and *Trueperella pyogenes* (39–41).

Luteolin exhibited strong antioxidant and anti-LOX activity. Previous studies confirmed the strong antioxidant and anti-lipoxygenase activities of luteolin and its mode of action (42–44). The SI of the compound regarding anti-LOX activity was >10. Previous work has also shown that luteolin inhibits cyclooxygenase-II expression (45). The compound can therefore be further investigated as an anti-inflammatory agent. Cycloogenase catalyses the committed step in the synthesis of proinflammatory mediators from arachidonic acid. In addition, luteolin suppressed synthesis of prostaglandin E_2, a proinflammatory mediator (45). Luteolin also inhibited proinflammatory gene expression in a murine intestinal cell line through the specific modulation of the NF-kB, IRF and Akt signalling pathways (46). Analogous with the *S. singueana* fractions, luteolin has the potential to be included in developing PFA preparations based on its anti-inflammatory activity via different mechanisms. However, it would need to be combined with other compounds with potent antibacterial activity against harmful pathogens, with capacity to act synergistically with it in promoting growth in poultry.

CONCLUSION

Column fractions of the ethyl acetate fraction obtained from the crude extract of *Senna singueana* leaves exhibited significant antibacterial, strong antioxidant activity and potent anti-LOX activity and were relatively safe to Vero cells. An active compound, luteolin, which has known biological activities, was isolated together with other compounds. The results of the current study support further investigation of *S. singueana* fractions and luteolin (or its derivatives) for the development of phytonutrient formulations which can be used as alternatives to poultry in-feed antibiotics. *In vivo* work on the formulations should be carried out using broiler chicken models to investigate efficacy as well as safety.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PJ conducted the experimental work, analysed the results and wrote the manuscript. FM and GF assisted with bioassays and isolation and structural elucidation of compounds. LM and GM supervised the research and edited the final version. LM provided funding and facilities and submitted the manuscript. All authors revised and edited the manuscript.

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Antibacterial and Antibiofilm Activity of Selected Medicinal Plant Leaf Extracts Against Pathogens Implicated in Poultry Diseases

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Antimicrobial resistant poultry pathogens are responsible for treatment failure and economic losses, and can also be a source of resistant zoonotic infections representing a risk to human health. In 2006 the European Union banned the use of antibiotics as growth promoters in farm animals and other regions are likely to follow suit. Alternative products and strategies are sought to help maintain animal gut health to reduce the prevalence of pathogens in the food chain. The minimum inhibitory concentration (MIC) of organic and aqueous leaf extracts of *Alchornea laxiflora*, *Ficus exasperata*, *Morinda lucida*, *Jatropha gossypiifolia*, *Ocimum gratissimum*, and *Acalypha wilkesiana* were tested against bacterial poultry pathogens including *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., and fungal species (*Aspergillus fumigatus*, *Aspergillus flavus*, and *Candida albicans)* using a 2-fold serial microdilution method. Activity of extracts against biofilms of the pathogens was done using a modified crystal violet staining *in vitro* assay. The safety of extracts was determined against Vero and Caco-2 cells using a tetrazolium-based *in vitro* assay. Acetone and cold water extracts of *M. lucida* had the best activity against three bacteria (MIC = 0.05–0.07 mg/ml) and two fungal (MIC = 0.03–0.15 mg/ml) organisms, respectively. The *E. coli* isolate and *A. flavus* were the most susceptible bacteria and fungi, respectively. Caco-2 cells generally displayed higher selectivity index (SI) values compared to Vero cells and average SI values against Vero and Caco-2 cells for both bacteria and fungi ranged from 0.01 to 4.48 and 0.005 to 16.41, respectively. All plant extracts had good anti-biofilm activity (>50%) against at least one organism. The disruption of established biofilm growth by the plant samples proved to be more difficult to achieve than efficacy against planktonic forms of bacteria. This study shows that some of the plant species are potential candidates as alternative feed additives in poultry production. In the future, a poultry feed trial to evaluate their *in vivo* efficacy as herbal feed additives will be conducted.

Keywords: antibacterial, antifungal, biofilm, cytotoxicity, poultry pathogens

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INTRODUCTION

Poultry diseases caused by a number of pathogens compromise animal health and welfare and decrease production efficiencies, causing reduced profitability, and increased levels of antimicrobial use. The contamination of poultry food products with various zoonotic pathogens is also a concern to food safety and public health, while there is increased consumer awareness and demand for organic poultry products (1). Many pathogens such as *Salmonella* spp. and *Campylobacter* spp. form biofilms, which further exacerbate diseases in poultry and resistance to antimicrobials. Biofilms are complex biological structures consisting of many bacterial cells surrounded by layers of substances produced by them, forming a barrier hindering eradication of the organisms (2).

Antimicrobial agents are used extensively in poultry production and are usually administered in the feed or drinking water. The use of antimicrobials has undoubtedly contributed to the success of the poultry industry from large numbers of small-scale farmers to a smaller number of large-scale producers who operate at high efficiency (3, 4). However, the prolonged use of antibiotics at sub-therapeutic levels as feed additives in animal and poultry feeds is a major risk factor to the emergence of drug-resistant pathogens, with major negative impacts on human, animal and environmental health (5). Also, the reliance of the poultry industry on the use of antimicrobials to prevent as well as control infectious diseases highlights the risks to the financial sustainability of the sector from the continuing growth in farm bacterial reservoirs with resistance to antimicrobial treatments (6–8). Therefore, there is a need to uphold proper antimicrobial stewardship by limiting antimicrobial use in food animals especially as prophylaxis should be decreased or stopped to limit the impact of AMR (antimicrobial resistance) on human health (9). For example, in 2006, the European Union banned the use of antimicrobials for prophylaxis in livestock while the use of colistin for animal use was recently banned in China (10).

Generally, contamination of poultry-based foods by *Salmonella* organisms has placed poultry products at a higher risk compared to other foods. In 2012, several outbreaks of *Salmonella* were associated with poultry meat and products (www.cdc.gov/salmonella/outbreaks.html). In the course of slaughtering, *Salmonella* from the gastrointestinal tract of infected chickens can contaminate the carcasses and the processing line (11). *Salmonella* species have been reported to be globally widespread food-borne pathogens, of which outbreaks are commonly associated with the consumption of contaminated food such as eggs, poultry meat and pork. In the European Union, *Salmonella* is a major cause of food poisoning (12) and *Salmonella* was recognized as one of the major food-borne pathogens in the United States, causing an estimated 1.4 million cases of illness, with ∼20,000 hospitalizations and more than 500 deaths annually (13).

In view of these challenges *vis a vis* the need to sustain profitability in livestock production without compromising public health safety, there is an urgent need for alternatives to prophylactic antimicrobial use. Phytogenic, or plant-based, additives are considered to be a promising alternative as non-antibiotic antimicrobials and potential feed additives to promote growth and increase production (14).

The use of medicinal plants as traditional medicines is well-known in rural areas of many developing countries (15, 16). Many plants have been used because of their antimicrobial traits derived from compounds which are chiefly synthesized during secondary metabolism of the plant (17). Several plant species were chosen for this study as a result of their reported antibacterial activity or traditional use to treat bacterial-related infections. *Alchornea laxiflora* (Benth) Pax and Hoffman (Euphorbiaceae) is commonly called Lowveld bead-string and is widely distributed from Nigeria to Ethiopia and down to Mpumalanga, South Africa. Akinpelu et al. (18) reported that the methanolic extract of this plant is a potent source of antibacterial and antifungal compounds. Reversal of sodium arsenate-induced liver toxicity by the hexane leaf extract in animal models was reported by Esosa et al. (19).

Ficus exasperata Vahl (Moraceae) is otherwise known as Sandpaper leaf (English), "Ewe Ipin or Eepin" (Yoruba-Western Nigeria), "Baure" (Hausa-Northern Nigeria, and "Asesa" (Igbo-Eastern Nigeria) (20). Fresh leaves are used in the local management of hypertension, rheumatism, arthritis, diarrhea, dysentery, intestinal pains and colic, epilepsy, oedema, gout, leprosy, bleeding, and wounds (21). The aqueous leaf extract of the plant had MIC values of 10, 20, and 10 mg/ml against *E. coli*, *S. aureus* and *E. faecalis,* respectively using macro broth dilution techniques (22). *Morinda lucida* L. (Rubiaceae) is a tropical West African rainforest species commonly known as Brimstone tree (23), and has been used in the traditional treatment of wound infections, diarrhea, malaria, diabetes, typhoid, abscesses, and chancre (24, 25).

Jatropha gossypiifolia L. (Euphorbiaceae) is widely distributed in countries of tropical, subtropical, and dry tropical weather as well as tropical semi-arid regions of Africa and the Americas. The leaves and bark were reported to have antimicrobial, anti-hypertensive, anti-inflammatory, analgesic, haemostatic and anti-diabetic properties (26, 27). *Ocimum gratissimum* L. (Labiateae) is found throughout the tropics and subtropics and its greatest variability occurs in tropical Africa and India (28). The extracts of leaves or whole plants of *O. gratissimum* are popular for the treatment of diarrhea and cold infusions of the leaves are used for the relief of stomach upset and hemorrhoids (29). The leaves have been reported to be rich in thymol which has antimicrobial properties (30). *Acalypha wilkesiana* (Euphorbiacae) is common in many countries, especially in the tropics of Africa, America and Asia. *A. wilkesiana* has antibacterial and antifungal properties (31, 32). The leaves of *A. wilkesiana* have various ethnomedicinal uses which include the treatment of malaria, and dermatological and gastrointestinal disorders (33).

Our previous research on some of the above-mentioned plant species revealed that *M. lucida*, *A. wilkesiana*, and *F. exasperata* leaves have appreciable amounts of macro- and microminerals, anions, sugars and organic acids, all of which are nutritional requirements of poultry. The plant species displayed appreciable levels of total phenolics and flavonoids which are most likely

related to their antimicrobial potential (34). The antimicrobial and antioxidant properties of secondary metabolites, mostly phenolics and flavonoids, will enhance the potential of these plants as phytogenic feed additives (PFAs). This study will add knowledge to the application, safety and mode of action of phytogenics which comprise a relatively new class of feed additives in animal nutrition.

The objectives of this study were to determine the antimicrobial potential of extracts of the selected plant species against the planktonic forms and biofilms of some economically important infectious poultry disease agents. Furthermore, the *in vitro* cytotoxicity of the plant extracts was determined against two mammalian cell lines.

MATERIALS AND METHODS

Plant Collection

Fresh leaves of *Alchornea laxiflora* (A.L), *Ficus exasperata* (F.E), *Morinda lucida* (M.L), *Jatropha gossypiifolia* (J.G), *Ocimum gratissimum* (O.G), and *Acalypha wilkesiana* (A.W) were collected from Ibadan Metropolis at Lagelu local Government Area of Oyo State, Nigeria in June 2017. The plants were identified by Mr Donatus Ozimede Esimekhinai. Voucher specimens were deposited after identification in the herbarium of the Department of Botany, University of Ibadan. Nigeria with numbers as follows: *A. laxiflora* (UIH-22625) *F. exasperata* (UIH-22626), *M. lucida* (UIH-2629), *J. gossypiifolia* (UIH-22627), *O. gratissimum* (UIH-22628), and *A. wilkesiana* (UIH-22793). The thoroughly cleaned and dried plants were ground into powder and kept in sealed containers in the dark until subsequent use.

Plant Extraction and Preparation

Three grams of the powdered material of each plant were weighed into 50 ml centrifuge tubes and 30 ml of acetone, methanol, ethanol, cold distilled water, and hot distilled water were added to separate aliquots and macerated for 24 h. The mixtures were centrifuged at 300 × g for 10 min and then filtered through Whatman No. 1 filter paper. The resultant extracts were transferred into pre-weighed labeled glass vials and the procedure was repeated thrice to exhaustively extract plant material. Resultant extracts were placed under a stream of air to dry completely and stored in the dark at 4°C. The resultant extracts were reconstituted in their respective solvents to the desired concentrations for the study.

Microbial Strains

Of the 18 microbial strains used in this study, eight were obtained from the American Type Culture Collection (ATCC), and 10 were clinical isolates. The ATCC strains used were *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis, ATCC 13076), *S.* Dublin (ATCC 15480), *S.* Typhimurium (ATCC 700720), *Escherichia coli* (ATCC 25922), *Campylobacter coli* (ATCC 43478), and *Campylobacter jejuni* (ATCC 33560). Clinical isolates obtained from the Department of Veterinary Tropical Diseases, University of Pretoria (UP) and included *Escherichia coli* (culture number B 3427/16), *S.* Gallinarum (B51/07), *S.* Idikan (B 1975/16), *S.* Bsilla (B 862/15), *S.* Choleraesuis (B 2209/17), *S.* Kottbus (B 297/16), *S.* Braenderup (AJ 42), *Aspergillus fumigatus* (isolated from a chicken with systemic mycosis,), and *Candida albicans* (isolated from a Gouldian finch). Avian crop candidiasis generally manifests itself as a localized infection of the mucous membranes, particularly crops (35). *Aspergillus flavus* was obtained from the Agricultural Research Council, South Africa, culture number PPRI 3954. Chickens have been reported to be exposed to feed contaminated with cyclopiazonic acid, a toxin produced by *A. flavus* which is a natural contaminant of corn (36) and peanuts (37).

In vitro Antimicrobial Serial Microdilution Assay

The antibacterial and antifungal assays were carried out using microdilution methods as described by Eloff (38) and Masoko et al. (39). All the bacteria in this study, except the *Campylobacter* strains, were prepared by inoculating a single colony of each bacterial strain from an agar plate into sterilized MH (Mueller Hinton) broth and grown overnight in a shaking incubator. *Campylobacter* strains were inoculated into BHI (Brain Heart Infusion) broth and grown overnight under anaerobic conditions at 37°C. All fungal strains were inoculated into Sabouraud Dextrose (SD) broth and placed in a shaking incubator for 24 h for *C. albicans* and 72 h for *Aspergillus* species at 30°C. Each culture was adjusted to a McFarland standard No 1 (equivalent to 3×10^8 cfu/ml). One hundred microliters of sterile water were added to each well of sterile 96-well microplates. Plant samples (100 µl) re-suspended to 10 mg/ml in sterile water for the water extracts, and acetone for the organic solvent extracts, were added to the first well of the microplates and then serially diluted along the ordinate. Gentamicin (Virbac) and amphotericin B (Sigma) were used as positive controls for the bacteria and fungi respectively while acetone and water served as negative controls. Subsequently, 100 µl of each of appropriately adjusted bacterial or fungal cultures were added to the wells of the microplates. The microplates were incubated at 37°C for bacteria and 30°C for fungi for 24 h. To each well of the incubated microplates, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium (INT, Sigma) were added to bacteria and 50 µl to the fungal plates. The plates were further incubated at 37°C for 30 min before reading the MIC for bacteria while readings were taken after 24 and 48 h for fungi. The last well with clear inhibition of bacterial and fungal growth was recorded as the minimum inhibitory concentration (MIC).

Chromatographic Analysis

Each plant extract (10 µL of a 10 mg/ml concentration) was loaded in a band of 1 cm on thin layer chromatography (TLC) Merck aluminum-backed plates (silica gel 60 F254) for chromatographic analysis. The TLC plates were later developed in three solvent systems of varying polarities (40), namely benzene: ethanol: ammonium hydroxide (90:10:1, BEA, non-polar basic), chloroform: ethyl acetate: formic acid (5:4:1, CEF, intermediate polarity, acidic), and ethyl acetate: methanol: water (40:5.4:5, EMW, polar, neutral). Separated chemical compounds were detected using acidified vanillin (0.1 g vanillin: 28 ml

methanol: 1 ml sulphuric acid) as a spray. After spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal color development.

Bioautographic Analysis

Thin layer chromatography (TLC) plates were loaded with 10 µl of each plant extract at 10 mg/ml concentration. TLC plates were prepared and developed in the three different solvent systems described above, and dried overnight under a stream of air to remove residual solvent which might inhibit organism growth. The plates were sprayed with cultures of bacteria (*E. coli*, *S.* Enteritidis, *S. aureus*, and *C. jejuni*) and fungi (*A. fumigatus* and *C. albicans*) in fresh growth medium. The moist plates were incubated at 37°C at 100% relative humidity for 24 h. The plates were then sprayed with 2 mg/ml of INT (41) and further incubated for 1–2 h. The purple-red color was an indication of cell viability while clear zones against the purple background were indicative of antibacterial and antifungal activity of separated compounds.

Anti-biofilm Assay

Inhibition of Bacterial Biofilm Formation

The inhibition of biofilm formation by acetone and aqueous (cold) extracts of the plants were assessed *via* the modified protocol by Sandasi et al. (42) and Mohsenipour and Hassanshahian (43). Two biofilm development stages were investigated, which were prevention of biofilm attachment (T0) and destruction of 24 h pre-formed biofilm (T24). The biofilm was allowed to preform for either 0 h (T0) or 24 h (T24) before the addition of samples (plant extracts) at a final concentration of 1 mg/ml. For the T0 study, 100 µl of the respective standardized bacterial culture ($OD_{590} = 0.02$ equivalent to 1.0×10^6 CFU/ml) prepared in Tryptone Soy Broth (TSB) was inoculated into sterile flat bottomed 96-well microtitre plates followed by adding 100 µl of the plant samples and incubated for 24 h at 37°C without shaking. For T24, 100 of standardized cultures were pre-incubated for 24 h for biofilm growth, before addition of plant extracts. For both T0 and T24, appropriate control included: negative control (culture + TSB), positive control [culture + TSB + antibiotics (ciprofloxacin, gentamicin and tetracycline)], sample control (sample + TSB), antibiotic control (antibiotic + TSB), and media control (TSB only). After 24 h incubation, the modified crystal violet staining (CVS) assay (42) was performed to quantify the biofilm biomass.

Crystal Violet Staining (CVS) Assay

Following incubation as described above, the wells were carefully emptied and plates were washed at least three times with sterile distilled water to remove unattached or loosely attached cells. The plates were air-dried and then oven-dried at 60°C for 45 min. Then 150 µl of 96% methanol was added to the wells for 15–20 min to fix the adherent cells. The plates were emptied, and the adhered cells stained with 100 µl of 0.1% crystal violet solution for 20 min at room temperature. Excess stain was rinsed off by washing the plates at least five times with water. Thereafter, the biofilm biomass was evaluated semi-quantitatively by re-solubilizing the crystal violet stain bound to the adherent cells with 150 µl of 100% ethanol to destain the wells. The absorbance of the plates was read at 590 nm using a microplate reader (Epoch™ Microplate Spectrophotometer) after careful and gentle shaking. The mean absorbance (OD_{590nm}) of the sample was determined and results expressed as percentage inhibition using the equation below (42).

$$Percentage\ (\%)\ inhibition = \frac{OD_{Negative\ control} - OD_{Sample} \times 100}{OD_{Negative\ control}}$$

In vitro Cytotoxicity Assay

The cytotoxicity test was carried out by screening the acetone and aqueous (cold water) extracts of the six plant species against monkey kidney cells (VERO) and human intestinal (Caco-2) cell lines using the tetrazolium-based colorimetric (MTT) assay described by Mosmann (44) and modified by McGaw et al. (45). These two extracts were chosen for their good and overall antimicrobial potentials alongside with the replication of the possible safety of mixtures of the plant powder with biological fluids (chicken gut fluids). Both cell lines were maintained in Minimal Essential Medium (MEM) supplemented with 0.1% gentamicin (Virbac) and 5% (for VERO) or 10 % (for Caco-2) fetal calf serum (Highveld Biological) at 37°C in a 5% CO_2 incubator till confluency. Cells of a sub-confluent culture were harvested and centrifuged at 700 × g for 7 min and resuspended in MEM to 5×10^4 cells/ml. Cell suspension (100 µl) was pipetted into each well of columns 2 to 11 of a tissue culture grade sterile 96 well microtitre plate and only MEM (200 µl) was pipetted in columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator to allow for cell attachment e. Different concentrations of the extracts prepared in the complete media were added to the plates in quadruplicate with 2 repeats ($n = 8$). The microtitre plates were then incubated at 37°C in a 5% CO_2 incubator for 48 h. Doxorubicin (Pfizer Laboratories) and acetone served as the positive control and negative controls, respectively. The contents of the cells were discarded and washed with phosphate buffered saline and replaced with 200 µl of fresh MEM. Then, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37°C. The medium was aspirated and MTT formazan crystals were dissolved with 50 µl dimethyl sulphoxide (DMSO). The plates were shaken gently on an orbital shaker to allow the formazan to dissolve. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader at a wavelength of 570 nm. The half maximal lethal concentration (LC_{50}) value was calculated. Selectivity index (SI) values for antimicrobial activity were calculated using the formula $SI = LC_{50}/MIC$.

Total Activity (TA)

The total antibacterial activity of extracts was calculated by dividing the quantity extracted from 1 g of plant material with the MIC values obtained against bacteria or fungi in mg/ml (46). It indicates the amount in ml to which an extract from 1 g of plant material can be diluted and still inhibit the growth of the test organism.

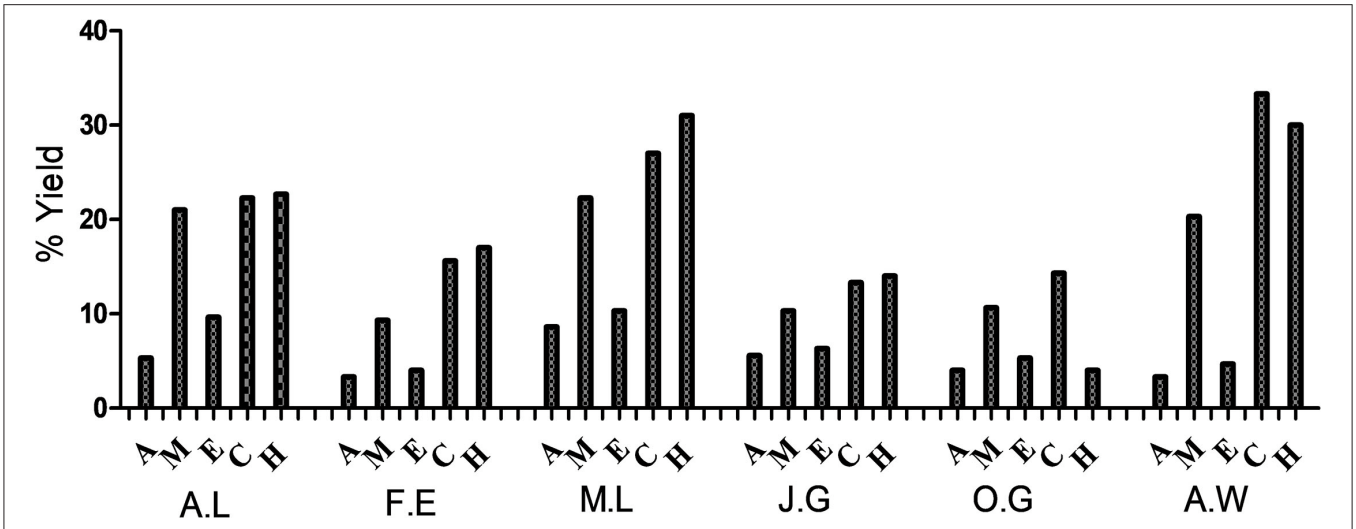

FIGURE 1 | Percentage yields of the dried plant material. A.L, *A. laxiflora*; F.E, *F. exasperata;* M.L, *M. lucida*; J.G, *J. gossypiifolia*; O.G, *O. gratissimum*; A.W, *A. wilkesiana*; A, Acetone; M, Methanol; E, Ethanol; C, Cold water; H, Hot water.

RESULTS

Plant Extract Yields

The percentage yields of the dried plant material extracted by each of the solvents (acetone, methanol, ethanol, cold water, and hot water) used in this study are represented in **Figure 1**. Cold water extracted the highest quantity of plant material. The highest quantity was extracted from *A. wilkesiana* (33%) followed by the hot water extracts of *M. lucida* (31%) and *A. wilkesiana* (30%), respectively. The yield of the acetone extracts of *F. exasperata* and *A. wilkesiana* were lowest with 3.33%, respectively.

Antibacterial Activity

Generally, among the extractants used for this study, the extracts of the tested plants prepared using acetone had the best average antimicrobial activity against the tested pathogens (**Tables 1**, **2**).

The acetone and ethanol extracts of *Alchornea laxiflora* (A.L) had the lowest average MIC values for all tested organisms compared to other extracts of this plant with MIC values of 0.26 and 0.33 mg/ml, respectively (**Table 1**). The hot and cold water extracts of A.L had poor inhibitory effect on the growth of the bacterial strains tested as their MIC values were all >0.625 mg/ml. However, the cold water extracts of this plant had the best activity against *Escherichia coli* (isolate) with MIC of 0.03 mg/ml.

The acetone and ethanol extracts of *Ficus exasperata* had the lowest average MIC value against all tested organisms compared to other extracts of this plant with MIC values of 0.27 and 1.09 mg/ml, respectively (**Table 1**). The most sensitive organisms to the plant extracts were *C. coli* and *E. coli* (isolate) while *E. faecalis*, *E. coli* (ATCC), and *C. jejuni* were relatively resistant.

The acetone and ethanol extracts of *Morinda lucida* had the lowest average MIC value for all tested organisms compared to other extracts of this plant with average MIC values of 0.18 and 1.10 mg/ml, respectively (**Table 1**). The acetone extract displayed high activity against *Salmonella* Gallinarum, *E. coli* (isolate) and *C. coli* with MIC values of 0.07, 0.07, and 0.05 mg/ml, respectively. Similarly, the cold water extract had the best activity against *E. coli* (isolate) with MIC value of 0.03 mg/ml. The acetone extract had significant activity against *C. jejuni* with MIC values of 0.04 mg /ml. Both ethanol and methanol extracts had significant activity against *S.* Gallinarum with MIC value of 0.09 and 0.09 mg/ml, respectively. In addition, the methanol extract had high activity against *C. coli* with MIC value of 0.09 mg/ml (**Table 1**).

The acetone extract of *Ocimum gratissimum* had the best average MIC values for all tested organisms with MIC values of 0.60 mg/ml. The cold water extract had the best activity against *E. coli* (isolate) while the extracts had poor activity against *S. aureus*, *E. faecalis*, *S. Enteritiditis*, and *E. coli* (ATCC).

The acetone, methanol and ethanol extracts of *Acalypha wilkesiana* had the lowest average MIC values against all tested organisms compared to other extracts of this plant with MIC values of 0.34, 0.37, and 0.41 mg/ml, respectively (**Table 1**). The acetone, methanol and ethanol extracts displayed very good activities against *E. faecalis* with MIC values of 0.03, 0.05, and 0.03 mg/ml, respectively.

The average total antibacterial activity (TAA) values of the selected plants ranged from 61 to 5 989 ml/g against all tested bacteria (**Table 3**). The highest TAA of 40 666 ml/g (**Table 3**) was produced by the methanol extract of *A. wilkesiana* against *E. faecalis* which indicates that 1 g of *A. wilkesiana* can be diluted in 40 666 ml of the solvent used and still inhibit the growth of the organism.

Antifungal Activity

The acetone and cold water extracts of *A. laxiflora*, *F. exasperata*, *M. lucida*, *J. gossypiifolia*, *O. gratissimum*, and *A. wilkesiana* had high antifungal activity with MIC values ranging from 0.03 to 0.48 mg/ml against one or more of the tested microorganisms (**Table 2**). The acetone and cold water extracts of *A. laxiflora*

TABLE 1 | Antibacterial activity of the extracts of six selected plants against both Gram-positive and Gram-negative bacteria affecting chickens.

Organisms	MIC (mg/ml)																
	Alchornea laxiflora					***Ficus exasperate***					***Morinda lucida***					**Controls**	
	A	**M**	**E**	**C.W**	**HW**	**A**	**M**	**E**	**C.W**	**HW**	**A**	**M**	**E**	**C.W**	**HW**	**Ac**	**Gent**
Staphylococcus aureus	0.46*	0.62	0.38*	2.50	2.50	0.31*	1.87	2.50	0.31*	0.70	0.15*	2.50	2.50	2.50	2.50	>2.50	<0.01
Enterococcus faecalis	0.38*	0.31*	0.38*	1.40	1.25	0.19*	1.87	2.50	>2.50	1.87	0.23*	2.50	1.25	1.32	1.25	>2.50	<0.01
Salmonella Enteritidis	0.38*	0.62	0.31*	1.87	2.50	0.23*	1.25	0.31*	0.72	1.56	0.23*	1.25	1.25	2.50	0.62	>2.50	<0.01
Salmonella Gallinarum	0.15*	0.51*	0.31*	1.25	2.08	0.22*	0.62	0.51*	1.25	2.50	**0.07**	0.51*	0.83	2.50	2.50	>2.50	0.0003
Escherichia coli (ATCC)	0.31*	0.62	0.38*	2.50	1.25	0.78	0.78	0.62	1.25	1.87	0.46*	0.62	1.25	2.50	1.25	>2.50	<0.01
Escherichia coli (isolate)	**0.05**	0.51*	0.34*	**0.03**	>2.50	**0.07**	0.15*	1.04	1.25	0.15*	**0.07**	1.66	0.51*	**0.03**	0.15*	>2.50	0.001
Campylobacter coli	0.11*	0.62	0.34*	2.50	2.50	**0.05**	2.50	0.41*	2.50	2.50	**0.05**	0.83	0.41*	0.46*	2.50	>2.50	0.62
Campylobacter jejuni	0.22*	1.66	0.23*	2.50	>2.50	0.30*	1.25	0.83	2.50	1.87	0.18*	1.04	0.83	2.50	>2.50	>2.50	0.001
Average	0.26*	0.68	0.33*	1.82	2.13	0.27*	1.29	1.09	2.50	1.62	0.18*	1.36	1.10	1.79	1.65		
	Jatropha gossypiifolia					***Ocimum gratissimum***					***Acalypha wilkesiana***					**Controls**	
Staphylococcus aureus	0.31*	0.62	0.62	0.62	0.31*	1.25	2.50	2.50	2.50	2.50	0.62	0.62	0.15*	**0.07**	2.50	>2.50	<0.01
Enterococcus faecalis	0.23*	0.62	0.93	1.25	1.25	1.25	>2.50	2.50	1.32	1.25	**0.03**	**0.05**	**0.03**	1.25	1.25	>2.50	<0.01
Salmonella Enteritidis	0.15*	0.62	0.62	0.62	0.62	0.62	1.25	>2.50	1.87	1.25	0.31*	**0.07**	**0.07**	0.66	0.66	>2.50	<0.01
Salmonella Gallinarum	0.15*	**0.09**	**0.09**	1.25	2.08	0.31*	0.83	0.31*	1.25	2.08	0.15*	**0.09**	0.31*	1.25	**0.09**	>2.50	0.003
*Escherichia coli (*ATCC*)*	0.46*	1.25	0.93	1.25	1.25	0.62	2.50	2.50	1.56	0.62	0.62	0.93	0.62	2.50	**0.07**	>2.50	<0.01
Escherichia coli (isolate*)*	0.11*	0.83	0.41*	0.62	1.25	0.15*	1.66	1.04	**0.07**	1.25	0.46*	0.46*	0.62	1.25	**0.03**	>2.50	0.001
Campylobacter coli	**0.05**	**0.09**	0.20	0.62	1.66	0.31*	0.83	0.41*	1.25	0.83	0.46*	0.46*	0.83	2.50	0.62	>2.50	0.62
Campylobacter jejuni	**0.04**	0.20*	0.20	1.25	>2.50	0.31*	0.41*	0.31*	2.50	>2.50	**0.03**	0.31*	0.62	2.50	1.25	>2.50	0.001
Average	0.19*	0.54*	0.50*	0.94	1.36	0.60*	1.56	1.51	1.54	1.53	0.34*	0.37*	0.41*	1.50	0.80		

A, Acetone; M, Methanol; E, Ethanol; CW, Cold water; HW, Hot water; Ac, Acetone; Gent, Gentamicin.

Bold values: MIC < 0.1 mg/ml (significantly active).

**Moderate activity.*

TABLE 2 | Antifungal activity of the extracts of six selected plants against chicken fungi.

Organisms	Time (hr)	MIC (mg/ml)																
		Alchornea laxiflora					*Ficus exasperata*					*Morinda lucida*					Controls	
		A	M	E	C.W	HW	A	M	E	C.W	HW	A	M	E	C.W	HW	Ac	Amp
Aspergillus fumigatus (isolate)	48	1.25*	1.25*	1.56	**0.31**	>2.50	**0.46**	0.93*	1.25*	0.62*	0.62*	1.56	1.25*	1.25*	**0.15**	2.50	>2.5	0.15
	72	1.25*	1.25*	1.56	**0.31**	1.25*	>2.50	1.56	1.25*	0.62*	0.62*	1.56	1.25*	1.25*	**0.15**	2.50	>2.5	0.62
Aspergillus flavus	48	0.62*	>2.50	2.50	>2.50	>2.50	**0.31**	>2.50	2.50	**0.15**	2.50	**0.15**	2.50	2.50	**0.31**	2.50	>2.5	0.62
	72	**0.46**	>2.50	2.50	>2.50	>2.50	**0.23**	2.50	0.51*	**0.15**	**0.38**	**0.19**	>2.50	>2.50	**0.03**	0.83*	>2.5	0.62
Candida albicans (isolate)	48	1.25*	2.50	1.87	>2.50	>2.50	>2.50	0.62*	0.62*	0.62*	0.93*	**0.46**	1.25*	2.50	>2.50	>2.50	>2.5	0.15
	72	0.93*	2.50	1.87	>2.50	>2.50	>2.50	0.93*	0.62*	0.62*	0.78*	**0.46**	2.50	2.50	>2.50	1.25*	>2.5	0.62
Average		1.00*	2.08	1.98	1.77	2.29	1.42*	1.51*	1.13*	**0.46**	0.97*	0.73*	1.88	2.08	0.94*	2.01		
		Jatropha gossypiifolia					*Ocimum gratissimum*					*Acalypha wilkesiana*					Controls	
Aspergillus fumigatus (isolate)	48	**0.31**	0.62*	0.62*	**0.31**	>2.50	2.50	1.25*	2.50	1.40*	>2.50	**0.07**	2.50	**0.15**	2.50	>2.50	>2.5	0.15
	72	**0.31**	2.50	1.25*	>2.50	>2.50	2.50	1.25*	2.50	0.62*	>2.50	0.62*	2.50	0.62*	2.50	1.25*	>2.5	0.62
Aspergillus flavus	48	0.62*	0.83*	0.62*	**0.15**	2.50	**0.03**	1.66	2.50	**0.31**	>2.50	1.25*	>2.50	**0.15**	>2.50	0.62*	>2.5	0.62
	72	0.93*	0.83*	1.25*	**0.15**	0.51	**0.15**	1.66	1.66	**0.31**	>2.50	0.62*	>2.50	0.62*	>2.50	**0.46**	>2.5	0.62
Candida albicans (isolate)	48	0.93*	1.25*	1.25*	2.50	2.50	1.25*	2.50	>2.50	1.56	0.62*	**0.15**	2.50	**0.15**	2.50	>2.50	>2.5	0.15
	72	1.25*	1.25*	1.25*	1.25*	2.50	1.25*	2.50	>2.50	1.87	0.62*	**0.15**	2.50	1.87	2.50	1.25*	>2.5	0.62
Average		0.73*	1.21*	1.04*	1.14*	2.16	1.28*	1.80	2.36	1.01*	1.87	**0.48**	2.50	0.60*	2.50	1.43*		

A, Acetone; M, Methanol; E, Ethanol; CW, Cold water; HW, Hot water; Ac, Acetone; Amp, Amphotericin B.

MIC ≤ 0.5 mg/ml—Significantly active (Bold).

**Moderate activity.*

TABLE 3 | Percentage yields and total antibacterial activity (TAA) of the extracts of six selected plants against bacteria.

Organisms	Total activity (ml/g)														
	Alchornea laxiflora					***Ficus exasperata***					***Morinda lucida***				
	A	**M**	**E**	**C.W**	**HW**	**A**	**M**	**E**	**C.W**	**HW**	**A**	**M**	**E**	**C.W**	**HW**
Staphylococcus aureus	115.93	338.70	254.38	89.33	90.67	107.50	49.91	16.00	505.38	242.86	577.78	89.33	41.33	108.00	124
Enterococcus faecalis	140.35	677.41	254.39	159.52	181.33	175.44	49.91	16.00	62.67	90.91	376.81	89.33	82.67	204.54	248
Salmonella Enteritidis	140.34	338.71	311.84	119.42	90.66	144.91	74.66	129.03	217.60	109	376.78	178.66	82.66	108.00	500
Salmonella Gallinarum	355.53	411.76	311.84	178.66	108.97	151.5	150.53	78.43	125.34	68	1,238	437.90	124.49	108	124
Escherichia coli (ATCC)	172.03	338.71	254.39	89.33	181.33	42.73	119.65	64.52	125.34	90.91	188.39	360.20	82.66	108	248
Escherichia coli (Isolate)	1,066.6	411.76	284.32	7,444.3	90.66	476.14	622.2	38.46	125.34	1,133.33	1,238	134.54	202.60	9,000	2,066.67
Campylobacter coli	484.81	338.71	284.32	89.33	90.66	666.6	37.33	97.56	62.67	68	1,733.2	269.07	252.02	586.96	124
Campylobacter jejuni	242.41	126.51	420.30	89.33	90.66	111.1	74.66	48.19	62.67	90.91	481.44	214.74	124.49	108	124
% Yield	5.33	21.00	9.66	22.30	22.67	3.33	9.33	4.00	15.60	17.00	8.60	22.30	10.30	27.00	31.00
Average	339.75	372.78	296.97	1,032.40	115.62	234.49	147.36	61.02	160.88	236.74	776.3	221.72	124.12	1,291.44	444.83
	Jatropha gossypiifolia					***Ocimum gratissimum***					***Acalypha wilkesiana***				
Staphylococcus aureus	182.79	268.82	102.15	215.05	451.61	32	42.67	21.33	57.33	66.66	53.76	327.96	311.11	4,761.90	120
Enterococcus faecalis	246.38	166.67	68.10	106.67	112	32.00	42.67	21.33	108.59	133.33	1,111.11	40,666.67	1,555.56	266.67	240
Salmonella Enteritidis	377.80	166.66	102.15	215.05	225.81	32	85.34	21.33	76.65	133.33	107.52	2,904.71	666.71	505.05	454.54
Salmonella Gallinarum	377.8	1,148.11	703.67	106.67	67.30	129.03	128.51	172.03	114.66	80.13	222.2	2,259.22	150.55	266.66	3,333.33
Escherichia coli (ATCC)	123.20	82.66	68.10	106.67	112	64.52	42.67	21.33	91.88	268.81	53.75	218.63	75.27	133.33	4285.71
Escherichia coli (Isolate)	515.18	124.49	154.46	215.05	112	266.67	64.25	51.28	2,047.57	133.33	72.46	442.02	75.27	266.66	10,000
Campylobacter coli	1,133.4	1,148.11	316.65	215.05	84.34	129.03	128.51	130.07	114.66	200.80	72.46	442.02	56.23	133.33	483.87
Campylobacter jejuni	1,416.75	516.65	316.65	106.66	56	129.03	260.15	172.03	57.33	66.66	1,111	655.90	75.27	133.33	240
% Yield	5.60	10.33	6.33	13.33	14.00	4.00	10.66	5.33	14.33	4.00	3.33	20.33	4.67	33.33	30.00
Average	546.66	452.77	228.99	160.86	152.63	101.79	99.35	76.35	333.58	135.38	350.53	5,989.64	37,075	808.37	2,394.68

A, Acetone; M, Methanol; E, Ethanol; CW, Cold water; HW, Hot water.

(A.L) had good overall antifungal activity for all tested organisms with MIC of 1.00 and 1.77 mg/ml, respectively. The cold water extract of A.L had the highest antifungal activity against *Aspergillus fumigatus* (isolate) with MIC value of 0.31 mg/ml while the acetone extract of the same plant had MIC value of 0.46 mg/ml against *Aspergillus flavus*. *Candida albicans* (isolate) was resistant to the plant extracts of A.L.

The cold water extract of *F. exasperata* had the lowest average MIC value for all the tested organisms with MIC value of 0.46 mg/ml. The acetone extract had moderate antifungal activity against *A. fumigatus* and *A. flavus* with MIC values of 0.46 and 0.31 mg/ml, respectively while the cold water extract had good antifungal activity against *A. flavus*.

The best overall average antifungal activity against all tested organisms was displayed by acetone and cold water extracts of *M. lucida* with MIC values of 0.73 and 0.94 mg/ml, respectively. Acetone and cold water extracts of *O. gratissimum* were the least active against all tested organisms with MIC values of 1.28 and 1.01 mg/ml, respectively (**Table 2**).

The acetone extract of *A. wilkesiana* had the lowest average MIC against all tested organisms with MIC of 0.48 mg/ml. This extract was active against both *A. fumigatus* and *C. albicans* with MIC values of 0.07 and 0.15 mg/ml, respectively. The acetone and cold water extracts of all the plants had antifungal activity against at least one or more of the tested fungi (**Table 2**).

Generally, the average total antifungal activity (TAA) values against tested fungi ranged from 18 to 2 281 ml/g (**Table 4**). The cold water extract of *M. lucida* had highest values of average total TAA against *A. flavus* over an incubation period of 72 h. The higher TAA indicates the levels of usefulness and economic values of the selected plant species.

Chromatographic Analysis

The **Supplementary Figure 1** revealed the chemical fingerprint of the extracts. The plates showed different compounds separated with the different solvent systems from non-polar (BEA), to intermediately polar (CEF), and polar (EMW) solvent systems. This plate gives a qualitative overview of the compounds present in the extracts using vanillin as a spray reagent.

Bioautographic Analysis

All three solvent systems separated the active bands against the tested microorganisms except against the fungi where CEF and EMW separated better than BEA. Using three solvent systems, 77 active bands were seen for the test organisms in the chromatographs of the different plant extracts. The CEF system separated 33 (42.86%) of the active bands followed by EMW with 29 (37.66%) and BEA with 15 (19.48%) which implies that most of the compounds were more polar in nature. *J. gossypiifolia* had the highest number of clear zones of inhibition representing active compounds against *E. coli* (**Supplementary Figure 2**, *S.* Enteritidis (**Supplementary Figure 3**), and *S. aureus* (**Supplementary Figure 4**). *M. lucida* had the highest number of active bands against *C. jejuni* (**Supplementary Figure 5**). However, no clear zone of inhibition was observed against *A. fumigatus* and *C. albicans* (**Supplementary Figures 6**, **7**).

Anti-biofilm Activity

The results of anti-biofilm (ABF) potential of the plant extracts against selected chicken pathogens are presented in **Table 5**. Extracts or fractions resulting in inhibition above 50% were considered to have good ABF activity (++) while those with inhibition between 0 and 50% indicated poor ABF activity (+), and the values < 0 (-) were regarded as no inhibition, or enhancement of biofilm development and growth (47). All the tested extracts, except for the aqueous extract of *M. lucida* had good inhibitory activity against the planktonic cells of *E. coli* (**Table 5**). Acetone extracts of *F. exasperata* and *O. gratissimum* had good ABF potential (>50% inhibition) against *E. coli*. Acetone extracts of *M. lucida*, *A. laxiflora*, *F. exasperata*, *O. gratissimum*, and *A. wilkesiana*, as well as the aqueous extract of *O. gratissimum*, had good inhibitory activity (>50% inhibition) against the planktonic cells of *C. coli* (**Table 5**). All the extracts enhanced the formation of biofilm by *C. coli* and *Salmonella* Gallinarum (**Table 5**). Aqueous extracts of *M. lucida*, *A. laxiflora*, *F. exasperata*, *O. gratissimum*, *A. wilkesiana*, *J. gossypiifolia*, and the acetone extract of *J. gossypiifolia* had good ABF activity (>50% inhibition).

The results of the percentage inhibition of acetone and aqueous extracts against biofilm formation of eight *Salmonella* serovars is presented in **Table 6**. Acetone extracts of *M. lucida* had good inhibitory activity (>50% inhibition) against planktonic cells of all the organisms except *S.* Typhimurium. Acetone extracts of *M. lucida* showed good ABF activity (>50% inhibition) against *Salmonella* Cholerasuis, *S.* Idikan, *S.* Kottbus, and *S.* Enteritidis. Similarly, aqueous extracts of *M. lucida* also exhibited good ABF activity (>50% inhibition) against *S.* Dublin, *S.* Idikan, *S.* Kottbus, and *S.* Typhimurium. Also, the acetone extract of *M. lucida* had good ABF activity (> 50 % inhibition) (**Table 6**). The above results showed that the inhibition of biofilm formation by the extracts at T_0 was higher compared to inhibition at T_{24}, since the cells are still floating at T_0 and not properly attached compared to those at T_{24}, which reflects a more established biofilm.

Cytotoxicity and Selectivity Index (SI)

The cold water extract of *A. laxiflora* had the highest LC_{50} value (lowest toxicity) of 0.709 mg/ml followed by the cold water extract of *Morinda lucida* with LC_{50} of 0.333 mg/ml (**Table 7**). The acetone extract of *J. gossypiifolia* was the most toxic (LC_{50} = 0.023 mg/ml) against Vero cells. The cold water extract of *Morinda lucida* had the highest LC_{50} value (lowest toxicity) of 0.580 mg/ml followed by the cold water extract of *F. exasperata* with LC_{50} value of 0.575 mg/ml while the acetone extract of *J. gossypiifolia* was the most toxic with LC_{50} value of 0.003 mg/ml against Caco-2 cells (**Table 7**). Both acetone and cold water extracts of *A. laxiflora*, *F. exasperata*, *Morinda lucida*, *J. gossypiifolia*, *O. grattisium*, and *A. wilkesiana* were not toxic to Caco-2 cells except for the acetone extract of *J. gossypiifolia*.

The average SI values against Vero and Caco-2 cells for both bacterial and fungal organisms ranged from 0.01 to 4.48 and 0.005 to 16.41, respectively (**Tables 7**, **8**). The cold water extract of *A. laxiflora* had the highest SI of 26.33 against *E. coli* isolates for Vero cells. The acetone extract of *A. wilkesiana* had the highest SI

TABLE 4 | Percentage yields and total antifungal activity (TAA) of the extracts of six selected plants against chicken fungi.

Organisms	Time (h)	Total activity (ml/g)														
		Alchornea laxiflora					*Ficus exasperata*					*Morinda lucida*				
		A	M	E	C.W	HW	A	M	E	C.W	HW	A	M	E	C.W	HW
Aspergillus fumigatus (isolate)	48	42.66	168	61.97	720.42	90.66	72.46	100.35	32	252.69	274.19	55.55	178.66	82.66	1,800	124
	72	42.66	168	61.97	720.42	181.33	13.33	59.83	32	252.69	274.19	55.55	178.66	82.66	1,800	124
Aspergillus flavus	48	86.02	84	38.67	89.33	90.66	107.52	37.33	16	1,044.47	68	577.73	89.33	41.33	870.97	124
	72	115.93	84	38.67	89.33	90.66	144.91	37.33	78.43	1,044.47	447.37	456.11	89.3	41.33	9,000	373.50
Candida albicans (Isolate)	48	42.66	84	51.70	89.33	90.66	13.33	150.53	64.52	252.69	182.80	188.39	178.67	41.33	108	124
	72	57.34	84	51.70	89.33	90.66	13.33	100.35	64.52	252.69	217.94	188.39	89.33	41.33	108	248
% Yield		5.33	21.00	9.66	22.30	22.67	3.33	9.33	4.00	15.60	17.00	8.60	22.30	10.30	27.00	31.00
Average		64.54	112	50.78	299.69	105.77	60.81	80.95	47.91	516.62	244.08	253.62	133.99	55.11	2,281.16	186.25
		Jatropha gossypiifolia					*Ocimum gratissimum*					*Acalypha wilkesiana*				
Aspergillus fumigatus (isolate)	48	182.81	166.66	102.15	430.10	56	16	85.33	21.33	102.38	66.66	476.14	81.33	311.13	18.67	120
	72	182.81	41.33	50.66	53.33	56	16	85.33	21.33	231.18	66.66	53.76	81.33	75.27	18.67	240
Aspergillus flavus	48	91.40	124.49	102.15	888.87	56	1,333.33	64.25	21.33	462.35	66.66	26.66	81.33	311.13	18.67	483.87
	72	60.94	124.49	50.66	888.87	274.51	266.67	64.25	32.13	462.35	66.66	53.76	81.33	75.27	18.67	652.17
Candida albicans (isolate)	48	60.94	82.66	50.66	53.33	56	32	42.66	21.33	91.88	268.80	222.2	81.33	311.13	18.67	120
	72	45.34	82.66	50.66	106.66	56	32	42.66	21.33	76.65	268.80	222.2	81.33	24.96	18.67	240
% Yield		5.60	10.33	6.33	13.33	14.00	4.00	10.66	5.33	14.33	4.00	3.33	20.33	4.67	33.30	30.00
Average		104.37	103.72	67.82	403.53	92.42	282.67	64.08	23.13	237.80	134.04	175.79	81.33	184.82	18.67	309.34

A, Acetone; M, Methanol; E, Ethanol; CW, Cold water; HW, Hot water.

TABLE 5 | Anti-biofilm activity of acetone and aqueous extracts of plant extracts against selected poultry pathogens.

Plants	Solvents	% inhibition (*E. coli*)		% inhibition (*C. coli*)		% inhibition (*C. jejuni*)		% inhibition (*S.* Gallinarum)	
		T_0	T_{24}	T_0	T_{24}	T_0	T_{24}	T_0	T_{24}
M. lucida	Acetone	++	-	++	-	+	-	++	-
	Aqueous	-	-	-	-	-	++	-	-
A. laxiflora	Acetone	++	-	++	-	++	-	++	-
	Aqueous	++	-	-	-	-	++	-	-
F. exasperata	Acetone	++	++	++	-	++	-	++	-
	Aqueous	++	-	-	-	-	++	-	-
O. gratissimum	Acetone	++	++	++	-	++	-	++	-
	Aqueous	++	-	++	-	-	++	++	-
J. gossypiifolia	Acetone	++	+	-	-	++	++	-	-
	Aqueous	++	-	-	-	-	++	+	-
A. wilkesiana	Acetone	++	-	++	-	-	++	++	-
Gentamicin		-	++	++	-	-	++	++	1.04
Ciprofloxacin		++	++	-	-	-	-	++	-
Tetracycline		++	++	++	-	++	-	++	125.58

Good (++) ABF activity (> 50% inhibition); Poor (+) ABF activity (more than 0–50% inhibition); No (-) ABF activity (0 % or less); Aqueous: Cold water.

TABLE 6 | The percentage inhibition of acetone, aqueous extracts of *M. lucida* against biofilm formation of eight *Salmonella* serovars.

Organisms	% inhibition							
	T_0				T_{24}			
	Morinda lucida		Positive controls*		*Morinda lucida*		Positive controls*	
	Acetone	Aqueous	Gent	Cipro	Acetone	Aqueous	Gent	Cipro
S. Gallinarum	++	-	++	++	-	-	+	-
S. Dublin	++	-	-	++	-	++	-	-
S. Choleraesuis	++	-	-	++	++	-	-	++
S. Braenderup	++	-	+	+	-	-	++	-
S. Idikan	++	+	+	-	++	++	++	-
S. Kottbus	++	-	-	+	++	++	++	-
S. Typhimurium	-	-	-	-	-	++	-	-
S. Enteritidis	++	-	-	+	++	-	-	+

**Positive controls: Gent, gentamicin; Cipro, ciprofloxacin. Good (++) ABF activity (> 50% inhibition); Poor (+) ABF activity (more than 0–50% inhibition); No (-) ABF activity (0 % or less).*

of 52 against *C. jejuni* for Caco-2 cells. The SI of *M. lucida* (cold water) against Vero and Caco-2 cells for *E. coli* isolates were 11.10 and 19.33, respectively (**Tables 7**, **8**). The higher the SI the safer the plant extracts are potentially, but this has to be confirmed using *in vivo* tests.

DISCUSSION

Antimicrobial Activity

In general, cold water offered the best yield of plant material while acetone rendered the lowest amount of plant constituents. Generally, in this study, aqueous solvents offered the best yield in most of the tested plant species, but this does not necessarily translate to efficient extraction of antimicrobial substances. According to several authors, organic solvents like acetone remain better extractants of antimicrobial substances compared to other solvents like water (48). Acetone was noted to be the best extractant for screening and isolation of antimicrobial compounds from plants (49). This is because acetone has high capacity to extract compounds with a wide range of polarity. This does not imply that other solvents are not equally useful, as results obtained from extracts made with methanol, ethanol, and water were similarly promising.

There are no generally accepted standard MIC end-points for *in vitro* testing of plant extracts. However, Kuete (50), proposed that the antibacterial activity of a plant extract is considered significant when MIC values are below 0.1 mg/ml, moderate when $0.1 \leq \text{MIC} \leq 0.625$ mg/ml and weak when MIC > 0.625 mg/ml. In this study, Gram-negative organisms (*Salmonella*, *Escherichia* and *Campylobacter* species) were more susceptible

TABLE 7 | Cytotoxicity (LC_{50} in mg/ml) and selectivity index (SI) of plant extracts and selectivity index against Vero kidney and Caco-2 cells.

Plant species	Extracts	Selectivity index																	
		Vero cells									Caco-2 cells								
		LC_{50}	Sa	Ef	Se	Sg	Ec (T)	Ec (iso)	Cc	Cj	Average	LC_{50}	Sa	Ef	Se	Sg	Ec (T)	Ec (iso)	Cc	Cj	Average
A. laxiflora	Acetone	0.026	0.06	0.07	0.07	0.17	0.08	0.52	0.24	0.12	0.17	0.026	0.06	0.07	0.07	0.17	0.08	0.52	0.24	0.12	0.17
	Cold water	0.709	0.28	0.51	0.38	0.57	0.28	**26.33**	0.28	0.28	**3.61**	0.417	0.17	0.30	0.22	0.33	0.17	**13.90**	0.17	0.17	**1.93**
F. exasperata	Acetone	0.099	0.32	0.52	0.43	0.45	0.13	**1.41**	**1.98**	0.33	0.70	0.561	**1.81**	**2.95**	**2.44**	**2.55**	0.72	**8.01**	**11.22**	**1.87**	**3.95**
	Cold water	0.164	0.53	0.07	0.23	0.13	0.13	0.13	0.07	0.07	0.17	0.575	**1.85**	0.23	0.80	0.46	0.46	0.46	0.23	0.23	0.59
M. lucida	Acetone	0.033	0.22	0.14	0.01	0.47	0.07	0.47	0.66	0.18	0.28	0.041	0.27	0.18	0.18	0.59	0.09	0.59	0.82	0.23	0.37
	Cold water	0.333	0.13	0.25	0.13	0.13	0.13	**11.10**	0.72	0.13	**1.59**	0.580	0.23	0.44	0.23	0.23	0.23	**19.33**	**1.26**	0.23	**2.77**
J. gossypiifolia	Acetone	0.023	0.07	0.08	0.15	0.15	0.05	0.21	0.46	0.58	0.22	0.003	0.01	0.01	0.02	0.02	0.01	0.03	0.06	0.08	0.03
	Cold water	0.144	0.23	0.12	0.23	0.12	0.12	0.23	0.23	0.12	0.18	1.000	1.61	0.80	1.61	0.80	0.80	1.61	1.61	0.80	**1.21**
O. gratissimum	Acetone	0.117	0.09	0.09	0.19	0.38	0.19	0.78	0.38	0.38	0.31	0.180	0.14	0.14	0.29	0.58	0.29	1.20	0.58	0.58	0.48
	Cold water	0.134	0.05	0.10	0.07	0.11	0.09	**1.91**	0.11	0.05	0.31	0.041	0.02	0.03	0.02	0.03	0.04	0.59	0.03	0.02	0.10
A. wilkesiana	Acetone	0.132	0.21	**4.40**	0.43	0.88	0.21	0.29	0.29	**4.40**	**1.39**	1.560	**2.52**	**52.00**	**5.03**	**10.40**	**2.52**	**3.39**	**3.39**	**52.00**	**16.41**
	Cold water	0.070	**1.00**	0.06	0.11	0.06	0.03	0.06	0.03	0.03	0.17	0.470	**6.71**	0.38	0.71	0.38	0.19	0.38	0.19	0.19	**1.14**
Doxorubicin	-	0.022	-	-	-	-	-	-	-	-	-	0.0004	-	-	-	-	-	-	-	-	-

Sa, Staphylococcus aureus; Ef, Enterococcus faecalis; Se, Salmonella Enteritidis; Sg, Salmonella Gallinarum; Ec (T), Escherichia coli (ATCC); Ec (Iso), Escherichia coli (Isolate); Cc, Campylobacter coli; Cj, Campylobacter jejuni; $LC_{50} \leq 0.03$ mg/ml is toxic (American National Cancer Institute NCI). Selectivity Index values > 1 regarded to be more toxic to bacterial cells than mammalian cells (Bold).

than Gram-positive organisms (*S. aureus* and *E. faecalis*). This is contrary to the general belief that Gram-positive organisms are more susceptible than Gram-negative ones because of their weaker and less complex cell wall (51). Furthermore, apart from their cell membrane permeability, the observed resistance by the Gram-positive organisms could be ascribed to genetic factors such as dissemination of resistant genes (52). Interestingly, the ATCC strains of tested pathogens were more resistant than the chicken isolates, while the most sensitive chicken isolates were *Salmonella* Gallinarum and *Escherichia coli* (isolate). The most susceptible of the tested pathogens was the *E. coli* isolate and according to Ogundare and Onifade (53), the inhibition of establishment of *E. coli* by methanol extract of *M. lucida in vitro* and *in vivo* using agar well diffusion method and albino rats respectively showed good antibacterial activity with 25 mg/ml of the extract inhibited *E. coli* with a zone of inhibition measuring 5 mm. In a similar manner, Ndukwe et al. (54) reported appreciable activity of the aqueous root extract of *M. lucida* against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* at MIC < 2.5 mg/ml using the agar dilution method.

Generally, *A. laxiflora*, *F. exasperata* and *M. lucida* had better antibacterial activity than *J. gossypiifolia*, *O. gratissimum*, and *A. wilkesiana* against all tested pathogens. In a similar study, Akinpelu et al. (18) found that the hydromethanolic leaf extract of *A. laxiflora* had some activity against some bacteria and fungi strains. Using different antimicrobial assays, the antimicrobial activities of different parts of *F. exasperata* and other *Ficus* species has been reported. Suresh et al. (55) reported good antibacterial activities of the bark extracts of *F. racemosa* against standard strains and clinical isolates using micro broth dilution. In addition, the ethanol extract of the leaf of *F. exasperata* has been reported to have inhibitory activity (300 mg/ml) against *E. coli* using the well diffusion assay (56). Likewise, the methanol extract of the bark of *F. religiosa* was active against enterotoxigenic *E. coli* using disc diffusion (57). These reports on the antimicrobial activities of *F. exasperata* support the results from this present study.

The aqueous extracts of the leaves of *O. gratissimum* contain substances with antibacterial properties (58) this is in conformity with some our findings. The benzene extract of *J. gossypifiola* has been reported to have maximum antibacterial activity (zone of inhibition 13.05 ± 0.02 mm) against *E. coli* and *B. subtilis* while minimal efficacy (zone of inhibition 2.04 ± 0.02 mm) was observed with the aqueous extract (59). It was earlier reported that the range of MIC of methanol, ethanol and aqueous extracts of *A. wilkesiana* against *E. coli*, *S. aureus*, *K. pneumoniae*, *S. typhi*, *B. cereus*, and *S. dysenteriae* is 10–30 μg/ml using broth dilution methods (60).

Escherichia coli, *Salmonella* and *Campylobacter* organisms are best-known among human intestinal microbial flora and are versatile gastrointestinal pathogens. Several *E. coli* strains that have been incriminated in the cause of diarrhea have a distinct mode of pathogenesis (61). Campylobacteriosis is commonly associated with eating raw or undercooked poultry. *Escherichia coli* is the most common cause of bacterial diarrhea, affecting an estimated 2.4 million people each year in the United States (62). Our findings in this study showed the potential of the selected

TABLE 8 | Cytotoxicity (LC_{50} in mg/ml) and selectivity index (SI) of plant extracts and selectivity index against Vero kidney and Caco-2 cells.

Plant species	Extracts	Selectivity index								
		Vero cells								
		LC50	*A. fumigatus*		*A. flavus*		*C. albicans*		Average	
			48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
A. laxiflora	Acetone	0.026	0.02	0.02	0.04	0.06	0.02	0.03	0.03	0.04
	Cold water	0.709	**2.29**	**2.29**	0.28	0.28	0.28	0.28	0.95	0.95
F. exasperate	Acetone	0.099	0.23	0.04	0.33	0.43	0.04	0.04	0.20	0.17
	Cold water	0.164	0.26	0.26	**1.09**	**1.09**	0.26	0.26	0.54	0.54
M. lucida	Acetone	0.033	0.02	0.02	0.22	0.17	0.07	0.07	0.10	0.09
	Cold water	0.333	2.22	2.22	**1.07**	**11.10**	0.13	0.13	**1.14**	**4.48**
J. gossypiifolia	Acetone	0.023	0.01	0.01	0.04	0.02	0.02	0.02	0.02	0.01
	Cold water	0.144	0.46	0.06	0.96	0.96	0.06	0.12	0.49	0.38
O. gratissimum	Acetone	0.117	0.05	0.05	3.90	0.78	0.09	0.09	**1.35**	**0.31**
	Cold water	0.134	0.10	0.22	0.43	0.43	0.09	0.07	0.21	0.24
A. wilkesiana	Acetone	0.132	**1.89**	0.21	0.11	0.21	0.88	0.88	0.96	0.43
	Cold water	0.070	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Doxorubicin	-	0.022	-	-	-	-	-	-	-	-

Plant species	Extracts	Selectivity index								
		Caco-2 cells								
		LC50	*A. fumigatus*		*A. flavus*		*C. albicans*		Average	
			48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
A. laxiflora	Acetone	0.026	0.02	0.02	0.04	0.06	0.02	0.03	0.03	0.04
	Cold water	0.417	**1.35**	**1.35**	0.17	0.17	0.17	0.17	0.56	0.56
F. exasperate	Acetone	0.561	**1.22**	0.22	**1.87**	**2.44**	0.22	0.22	**1.10**	0.96
	Cold water	0.575	0.93	0.93	**3.83**	**3.83**	0.93	0.93	**1.90**	**1.90**
M. lucida	Acetone	0.041	0.03	0.03	0.27	0.22	0.09	0.09	0.13	0.11
	Cold water	0.580	**3.87**	**3.87**	**1.87**	**19.33**	0.23	0.23	**1.99**	**7.81**
J. gossypiifolia	Acetone	0.003	0.01	0.01	0.004	0.003	0.003	0.002	0.006	0.005
	Cold water	1.000	3.23	0.40	6.66	6.66	0.40	0.80	**3.43**	**2.62**
O. gratissimum	Acetone	0.180	0.07	0.07	**6.00**	**1.20**	0.14	0.14	**2.07**	0.07
	Cold water	0.041	0.03	0.07	0.13	0.13	0.03	0.02	0.06	0.07
A. wilkesiana	Acetone	1.560	**22.29**	**2.52**	**1.25**	**2.52**	**10.40**	**10.40**	**11.31**	**5.15**
	Cold water	0.470	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Doxorubicin	-	0.0004	-	-	-	-	-	-	-	-

A. fumigatus, Aspergillus fumigatus; A. flavus, Aspergillus flavus; C. albicans, Candida albicans. $LC_{50} \leq 0.03$ *mg/ml is regarded as toxic (American National Cancer Institute NCI). Selectivity index > shows selective toxicity to fungal cells than to mammalian cells (Bold).*

plants as a good candidate for testing against a wide range of diarrhea-causing chicken bacterial diseases.

With regard to antifungal activity, according to Aligiannis et al. (63) and Hamza et al. (64) MIC values of 0.5 mg/ml or less are considered to be significantly active, moderate with MIC between 0.6 and 1.5 mg/ml and weak with MIC above 1.6 mg/ml. In this study, *A. flavus* was the most susceptible fungus to most extracts of the six plants studied at 48 and 72 h incubation. Acetone and cold water extracts of all the plants displayed the best antifungal activity against at least one or more tested fungi, and are most likely to be generally fungistatic as growth of the pathogens appeared to resume after 72 h of incubation with INT (**Table 2**).

However, *A. flavus* and *C. albicans* were more susceptible to these extracts at the longer incubation period (72 h) so whether a longer contact period with fungal pathogens can potentiate bioactive constituents of the plants is a subject of further studies. The aqueous extract of *M. lucida* had excellent antifungal activity against all the tested chicken fungi this is similar to findings from previous studies by Banerjee et al. (65) and Jainkittivong et al. (66) who reported the morphological conversion of *C. albicans* and the germination of *Aspergillus nidulans* by the water-soluble components in *Morinda citrifolia* (Noni) which made it an option for anti-fungal therapy for candidiasis and aspergillosis in humans. Likewise, good antifungal activity of alizarin−1-methyl ether, anthraquinone isolated from dichloromethane extract of the roots of *M. lucida* against *Aspergillus fumigatus* and *Trichophyton mentagrophytes* at MIC values of 100 and 50 μg/ml, respectively has been reported (67).

The total antimicrobial activity indicates the volume to which the amount extracted from 1 g of the plant can be diluted with retention of activity. The higher the total activity of a plant extract, the more potent it is and the higher its usefulness and economic value (40). In this study, the significantly higher average total activity against bacterial pathogens than fungal pathogens indicated better potency against bacterial than against fungi.

The results from the present study showed *in vitro* antimicrobial activity of selected plant species as a possible candidate for testing against bacterial and fungal pathogens implicated in causing infectious diseases in poultry. However, *in vivo* data is necessary in future to determine the potential usefulness of these plants for management of infectious diseases as factors such as absorption, metabolism and enzymatic activation influence *in vivo* efficacy (68).

Bioautography

In the bioautography analysis, most of the antimicrobial compounds were visible in the extracts prepared using polar solvents, and *S. aureus* was the most susceptible organism. All the extracts had weak activity against the fungi. *J. gossypiifolia* had the highest number of bands indicating presence of active compounds against all the tested organisms while *A. wilkesiana* had the lowest number of bands. The observed antibacterial activity of *J. gossypiifolia* may be attributed to general toxicity to both animal and bacterial cells as indicated by its toxicity to both Vero and Caco-2 cells, therefore care should be exercised in its use as a feed additive or it can be suggested for external use, although cautiously. In this study, little to no activity observed in some crude plant extracts may be ascribed to very low concentrations of the active compounds in the crude plant extracts (69). Furthermore, all the test plant extracts had fewer zones of inhibition against *A. fumigatus* and *C. albicans*. However, absence of activity could be attributed to factors including evaporation of active compounds, photo-oxidation or a low quantity of active compound.

Generally, the acetone extracts of the six plant species had good to moderate activity against both bacterial and fungal organisms. The cold water extracts of the six plant species had weak activity against the tested bacterial organisms but better antifungal activity with very low toxicity to both mammalian cell lines compared to other extracts. The aqueous extracts (cold water) are more relevant to the clinical application of the powdered leaves as alternative feed additives. The bioactive constituents of the feed additives will be released into the chicken gut fluids before final absorption into the general circulation. The low antimicrobial activity of the aqueous extracts may be attributed to their inability to extract the bioactive compounds in the plants compared to acetone (48). It is also possible that antimicrobial effects of these aqueous extracts are not mediated through direct inhibition on microbial growth but rather through immunostimulation, or the bioactive compounds may need metabolic activation by certain enzymes *in vivo*. The choice of aqueous (cold water) extracts agreed with the traditional applications of these plant species as antimicrobials coupling with their good and overall antimicrobial potentials alongside with the replication of the possible safety of mixtures of the plant powder with biological fluids (chicken gut fluids). Also, it was observed that cold water produced the best yield from the extraction process. In order to fulfill the main objective of the overall study to which these experiments contribute, which is the production of feed additives from powdered leaves, the aqueous extracts were chosen for further studies.

Anti-biofilm Activity

Aqueous extracts of the majority of plant species tested in this study had good ABF activity (>50% inhibition) against *C. jejuni* compared to that of acetone extracts. Good inhibitory activity (>50% inhibition) against planktonic cells of *E. coli*, *C. coli*, *C. jejuni*, and *S.* Gallinarum was exhibited by most of the extracts at T_0 indicating that prevention of biofilm attachment and growth proved to be easier to achieve than inhibition of pre-formed biofilms (T_{24}) because the cells at T_0 are not fully attached compared to those of T_{24}.

Similarly, Mohsenipour and Hassanshahian (43), while evaluating ABF activity of the alcoholic extract of *A. sativum* against *E. coli* observed higher values (%) in the inhibition of biofilm formation than the values (%) for the destruction of already formed biofilm. In this study, all extracts and fractions enhanced the formation of biofilms of *C. coli* and *S.* Gallinarum and were expressed as 0% inhibition. This promotion of biofilm growth could be attributed to the presence of metabolites or production of conditioning films for microbial adhesion that may enhance the growth and development of biofilms (42). Sandasi

et al. (42) made a similar observation in their investigation of the ABF activity of selected herbs, spices and beverages against *Listeria monocytogenes*. Furthermore, the presence of an EPS (glycocalyx) and negative charge on the EPS are among factors that have been linked to the ability of pathogens to form biofilms, while the negative charge limits the infiltration of molecules by charge attraction, thus causing resistance (70). In addition, plant lectins have been reported to improve the adsorption of cells onto a surface by acting as receptors of bacterial glycan, thereby enhancing cell attachment (71). Resistance and persistence of *Salmonella* has been attributed to their ability to form biofilms in abiotic surfaces outside the host, such as in farms, the food processing industry, kitchens or toilets, on plant surfaces, or even in animal epithelial cells (72).

In view of the role of the *Salmonella* genus in antimicrobial resistance (AMR), the anti-biofilm potential of extracts of *M. lucida* were further evaluated against eight *Salmonella* serovars that are relevant in livestock infections. Out of all the *Salmonella* serovars tested, the best ABF activities (>50% inhibition) were observed against *Salmonella* Enteritidis by the acetone extract of *M. lucida*, which may be attributed to the differential solubility of the bioactive compounds with solvent polarity (73). Good inhibitory activity (>50% inhibition) was exhibited against at least four of the *Salmonella* serovars by acetone and aqueous extracts of *M. lucida*, which is similar to the findings of Vijayan et al. (74) on the ability of the aqueous extract of another plant, *T. conoides* in the prevention of biofilm formation.

Cytotoxicity and Selectivity Index

Acetone and cold water extracts of the six plant species were tested against Vero kidney and Caco-2 cell lines for cytotoxicity. According to Makhafola et al. (75), no crude plant extracts or natural products are regarded as safe for use until they are subjected to cellular toxicity tests. It is imperative to determine the cytotoxicity of a plant extract by using more than one cell line because the establishment of the safety and usefulness of a plant extract using only one cell line might be misleading. It is expected that the sensitivity of the cell lines to the extracts will be different because of different metabolic activities and uptake capabilities (76). The human intestinal cell line (Caco-2) has been reported for its known uptake capabilities (77). Therefore, the choice of Caco-2 cells in addition to Vero cells was made owing to its uptake capabilities and ability to serve as an absorptive surface for the bioactive ingredients in the feed additives. A study comparing the toxin Cylindrospermopsin (CYN) toxic effects in four different cell lines indicated that Caco-2 cells were one of the most sensitive to the toxin (78).

In another study by Pinto (79), it was demonstrated that Caco-2 cells, upon differentiation, expressed several morphological and biochemical characteristics of small intestinal enterocytes. In order to reduce the use of experimental animals for toxicity testing, the Caco-2 cell model has been considered for the development of alternative *in vitro* toxicity tests. In addition, the gastrointestinal tract is relevant for the absorption and biotransformation of xenobiotics due to the extensive area of exposure to orally ingested drugs, feed additives and contaminants. Moreover, the gastrointestinal tract can be a direct target for several toxicants (80). The extracts with $LC_{50} > 0.1$ mg/ml are considered to have negligible cytotoxicity (50). Also, the American National Cancer Institute (NCI) proposed that crude extracts are highly cytotoxic at $LC_{50} \leq 0.03$ mg/ml following incubation with cells between 48 and 72 h (81).

The aqueous extract of *A. laxiflora* and *M. lucida* had the highest LC_{50} values (lowest toxicity) against Vero and Caco-2 cell lines respectively while the acetone extract of *J. gossypiifolia* had the lowest LC_{50} values (most toxic) against Vero and Caco-2 cell lines. Therefore, the antimicrobial activity of the *J. gossypiifolia* might be attributed to general toxicity. The antiproliferative activity of the aqueous extract of *M. lucida* against human promyelocytic leukemia (HL-60) cell lines has been reported (82). Our findings in this study showed that all the tested extracts were relatively safe against both Vero and Caco-2 cell lines except for the acetone extract of *J. gossypiifolia*. This plant has been previously reported to be toxic although its toxic nature has been mostly associated with the latex and seeds (83).

The selectivity index (SI) expresses the correlation between the antimicrobial and cytotoxic activities of the plant extracts on bacterial and normal cells so that the biological activity of the plant extract is not attributed to constituent toxic principles. Kudumela et al. (84) and McGaw et al. (85) reported that SI values greater or equal to 10 indicate a promising hit for product development, necessitating *in vivo* studies. Generally, SI above 1 is an indication that the biological activity of the plant extracts or natural products is higher than their cellular toxicity. The aqueous extract of *M. lucida* had the highest SI values against both Vero and Caco-2 cell lines therefore the aqueous extract of *M. lucida* was the safest of all the tested extracts in this *in vitro* study. Caco-2 cells were generally less susceptible than Vero cells to the tested extracts.

CONCLUSION

The selected plant extracts had varying antimicrobial activity against relevant poultry bacteria and fungi which indicate the potentials of the plant species as a candidate for future testing *in vivo* in form of natural feed additives against relevant poultry pathogens. The cold water extract of *M. lucida* had the lowest MIC against *E. coli* (isolate) and *A. flavus*, respectively. Generally, the acetone extract of *M. lucida* exhibited the best ABF activities against *S.* Enteritidis while the aqueous extracts of same plant displayed good inhibitory activity (>50% inhibition) against at least four of the *Salmonella* serovars. Due to the promising activity of *Morinda lucida*, further study in an *in vivo* chicken feed trial as a potential candidate for development as a feed additive is recommended in future. The findings from this study will provide researchers and chicken farmers with useful information on the use of additives which are not only cost effective but also of herbal origin.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

OO conducted the experimental work, analyzed the results, and wrote the manuscript. All authors revised and edited the manuscript. LM and IF supervised the research and edited the final version. LM provided funding and facilities and submitted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Ex Vivo Pharmacokinetics and Pharmacodynamics Modeling and Optimal Regimens Evaluation of Cefquinome Against Bovine Mastitis Caused by *Staphylococcus aureus*

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Cefquinome, the fourth-generation cephalosporin applied solely for veterinary medicine, is commonly used for bovine mastitis caused by *Staphylococcus aureus*. The present study aims to establish an optimal dose and provide a PK/PD Cutoff value (CO_{PD}) for cefquinome against *S. aureus* based on *ex vivo* pharmacokinetics and pharmacodynamics (PK/PD) integration. This study investigated the pharmacokinetics (PK) of cefquinome when administered as three consecutive intramammary (IMM) doses of cefquinome in three healthy dairy cows at 75 mg/gland. Drug concentration was determined by HPLC-MS/MS assay. The *ex vivo* pharmacodynamics (PD) of cefquinome were evaluated by using a milk sample from a PK experiment. The relationship between the AUC/ MIC of cefquinome and bacterial loading reduction was simulated using a Sigmoid Emax model. The cefquinome concentration in milk attained a maximum level of 1.55 ± 0.21 mg/mL at 1.8 h after the third administration. The mean value of the area under the concentration-time curve (AUC_{0-24}) was 26.12 ± 2.42 mg·h/mL after the third administration. The elimination half-life was 10.6 h. For PD profile, the MICs of cefquinome in milk were 2–4 times higher than those in the broth. *In vitro* time-killing curve shows that initial bacterial concentration has a huge impact on antibacterial effect on three strains. The antibacterial effect was weakened with the initial bacterial concentration increasing from 10^6 to 10^8 CFU/mL. The AUC_{0-24h}/MIC index correlated well with *ex vivo* efficacy both for the initial inoculum of 10^6 CFU/mL and 10^8 CFU/mL ($R^2 > 0.84$). According to the inhibitory sigmoid E_{max} model analysis, the PK/PD surrogate (AUC_{0-24}/MIC) values were 8,638, 1,397, and 3,851 for bactericidal effect ($E = -3$) with an initial inoculum of 10^6 CFU/mL, while the corresponding values were 12,266, 2,295, and 5,337, respectively, with the initial inoculum of 10^8 CFU/mL. The *ex vivo* PK/PD based population dose prediction indicated a target attainment rate (TAR) of 90% of 55 mg/gland/12 h. The CO_{PD} for cefquinome against *S. aureus* was 2 μg/mL under the

recommended dose of 55 mg/gland/12 h. However, it should be validated in clinical practice in future investigations. These results contribute to the rational use of cefquinome for mastitis treatment in clinical veterinary medicine.

Keywords: cefquinome, *S. aureus*, *ex vivo*, PK/PD modeling, cutoff, bovine mastitis

INTRODUCTION

Bovine mastitis is known as a serious disease in the dairy industry due to deterioration in the quality of milk, veterinary care expenses, and prohibitive labor costs for producers. Bovine mastitis usually results from bacterial, yeast, and even fungal or algae infection that accounts for almost 90% of all diagnoses (1). *Staphylococcus aureus* is one of the most common etiologic agents, which could result in chronic, contagious, and intractable bovine mastitis (2). *S. aureus* infection is extremely difficult to control (3), as it can release exotoxin, and survive in the intracellular where the drug concentration is often low. Until now, antimicrobial approaches have been the best way to control bovine mastitis. However, the resistance rate of *S. aureus* had been raised obviously and the number of multiple drug resistance (MDR) *S. aureus* has increased sharply, meaning the treatment of mastitis will become more difficult in the future (4, 5).

Cefquinome (CEQ) is a fourth-generation cephalosporin, solely for veterinary use. It has been used in bovine mastitis treatment for many years (6). It is highly stable when exposed to β-lactamases that are produced by the most clinically important bacteria. The pharmacokinetic (PK) characteristics of cefquinome have been studied in various animals, such as sheep, goats, cattle, buffalo calves, and camels via intravenous (i.v.) or intramuscular (i.m.) administration. The PK profiles of CEQ after local intramammary administration have also been performed in lactating cows and buffalo (7, 8). Previous reports have suggested that the intramammary recipe was more successful than systemic therapy, especially for mastitis caused by *S. aureus* (2, 9).

It has been reported that a rational antibacterial dose regimen based on pharmacokinetic and pharmacodynamic (PK/PD) modeling could balance the therapeutic effect and the emergence of resistance, and could also reduce the emergence and spread of drug resistance (10). PK/PD integration is also a key method to evaluate the clinically relevant relationship between time, drug concentration, and effect. The integration of the PK/PD model has now been widely applied in the evaluation of antibacterial activity and optimization of dosing regimens. In previous reports (11, 12) mouse or rat mastitis models were used for PK/PD evaluation in a mastitis *in vivo* PK/PD study, because establishing a mouse or rat mastitis model is easier than in other animals. However, this mastitis model cannot accurately reflect the mastitis in target animals such as a lactating cow. To date, few *in vivo* PK/PD model studies in cows have been reported. Although the PK fate of cefquinome and its efficacy in clinical treatment has been widely studied. There is no complete research linking the PK parameters to the PD effectiveness on mastitis therapy of various intramammary administration dosing regimens in dairy cows. It cannot be denied that there are differences between the *ex vivo* PK/PD model and the *in vivo* target animal intramammary infection model. The *ex vivo* model could not only ensure animal welfare but also save the cost of experimental intramammary infection in cows.

CO_{PD} is a significant parameter that assists in the definition of susceptibility breakpoints from the perspective of the exposure-response relationship (13). CO_{PD} was determined by Monte Carlo simulation (MCS), considering pharmacokinetic variation in target animals and PK/PD indices. This method has also been used by regulatory agencies such as CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), in defining the susceptibility breakpoints (14).

In this study, the *ex vivo* PK/PD relationship of cefquinome against *S. aureus* in dairy cows through intramammary infusion was investigated. A 10,000-subject Monte Carlo simulation was performed to derive a PK/PD cutoff based on three aspects: MIC distributions of CEQ against *S. aureus*; pharmacokinetic/pharmacodynamics (PK/PD) indices; and pharmacokinetics of CEQ in cows obtained. Then, a rational regimen of CEQ against dairy mastitis caused by *S. aureus* was determined.

MATERIALS AND METHODS

Antimicrobial Agents

Cefquinome intramammary infusion was obtained from Merck Animal Health (Cobactan LC®; INTERVET). Cefquinome reference standard is purchased from Solarbio Life Sciences Co. Ltd. (Beijing, China). Test solutions of the antimicrobial agent were freshly prepared prior to use.

Bacterial Strains and Animals

Sixty-three *S. aureus* strains isolated from clinical bovine mastitis individuals in Jiangsu China were evaluated in this study. ATCC 29213 was stored in our laboratory. B4030RH-31.4 and 2014RQG-33.15 were screened from 63 strains above. Brain-Heart-Infusion (BHI), Mueller-Hinton (MH) broth, and MH agar were purchased from Qingdao Hope Bio-Technology CO., Ltd. (Qingdao, China).

Three healthy Holstein dairy cows with weights ranging from 700 to 800 kg, were housed individually and fed with antimicrobial-free mixed ration and water *ad libitum*. Cows were milked twice daily during lactation.

Antimicrobial Susceptibility

Susceptibility tests were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (15), using the following antibiotic: cefquinome (CEQ), streptomycin (STR), doxycycline (DOX), meropenem (MEM), ciprofloxacin (CIP), florfenicol (FFC), cefoxitin (FOX), tetracycline (TET), apramycin (APR), clindamycin (CLI), cloxacillin (CLO), and penicillin (PEN). CEQ MIC in milk was determined according to the

CLSI micro broth dilution method, using milk as a bacteria medium. The MIC in milk was measured by sampling the mixture of drugs and bacteria and then 10-fold serial dilution and plating onto MH agar for colony counts calculation. The MIC in milk was defined as the minimum concentration where the bacteria loading equal to the initial concentration or grow $<1 \times \log_{10}$ CFU/mL. The MIC_{50} and MIC_{90} values of CEQ were calculated, which represented the MIC value inhibiting the growth of at least the corresponding 50 and 90% of isolates. *S. aureus* American Type Culture Collection (ATCC) 29213 and *Escherichia coli* ATCC25922 strains were used as quality control. All determinations were performed in triplicate.

In vitro Time-Killing Curves

An overnight culture in BHI broth of three *S. aureus* isolates including ATCC 29213, B4030RH-31.4, and 2014RQG-33.15 was diluted appropriately to attain two different concentrations of 10^6 and 10^8 CFU/mL. Pathogens were exposed to CEQ with different concentrations (0×, 0.5×, 1×, 2×, 4×, and 8× MIC) in MH broth and milk incubation at 37°C. An aliquot of 100 μL mixture was sampled at 0, 2, 6, 12, and 24 h and subjected to 10-fold serial dilution and then plated onto MH agar for visible counts calculation. The detection limit was 10 CFU/mL. All the MH agar plates were cultured at 37°C for 22 to 24 h before colony counting.

Pharmacokinetics

The PK trials were performed in three healthy lactating bovines. CEQ was administered with 75 mg per udder every 12 h 3 times. A thirty second massage was applied to make the drug absorb homogeneously. After first intramammary (IMM) infusions, milk samples (about 50 mL at each time) were collected at time point: 0, 0.083, 0.167, 0.5, 1, 2, 4, 8, 12 h. After third IMM infusions, milk sample (about 50 mL at each time) were collected at the time points: 0, 0.083, 0.167, 0.5, 1, 2, 4, 8, 12, 24, 30, 36, and 48 h. The drug concentrations in milk were determined using the high-performance liquid chromatography-Mass Spectrometry (HPLC-MS/MS) method reported previously (16). Matrix matched calibration standards gave linear responses from 0.005 to 10 μg/mL ($R^2 > 0.999$), with limits of quantification (LOQ) of 0.005 μg/mL. All samples with drug levels > 10 μg/mL were diluted proportionally with the extraction of blank milk. The PK parameters were calculated by the Winnonlin software (version 6.3, Pharsight, St. Louis, MO, USA).

Ex vivo PK/PD Time-Killing Curve and PK/PD Analysis

Ex vivo time-killing curves were established using inactivated skimmed milk collected at specified time points from 0 to 84 h after IMM infusion administration. An overnight culture of *S. aureus* ATCC29213, B4030RH-31.4, and 2014RQG-33.15 were 10-fold diluted appropriately to attain two different concentrations of 10^6 and 10^8 CFU/mL. Pathogens were exposed to the milk sample of each time point after third administration and bred at 37°C. The method was the same as *in vitro* time-killing curves. The *ex vivo* antimicrobial effect (E) at a given CEQ concentration was expressed as the change in $\log_{10}$ CFU/mL after 24 h of incubation. The detection limit was 10 CFU/mL.

AUC_{0-24h}/MIC values of CEQ against *S. aureus* were calculated for each sample. The effect (E) was the bacteria reduction during *ex vivo* time–killing assays. Data were analyzed using the sigmoid E_{max} model (17, 18). WINNONLIN software as follows:

$$E = E_0 - \frac{E_{MAX} \times C_e^N}{EC_{50}^N + C_e^N}$$

where E_0 is the change in log10 CFU/mL after 24 h incubation in the control sample (absence of CEQ) compared with the initial inoculum; E_{max} is the difference in effect between the greatest amount of growth (as seen for the growth control, E_0) and the greatest amount of kill; C_e is the AUC_{0-24}/MIC in the effect compartment; EC_{50} is the AUC_{0-24}/MIC value producing a 50% reduction in bacterial counts from the initial inoculum, and N is the Hill coefficient that describes the steepness of the AUC_{0-24}/MIC-effect curve.

Monte Carlo Analysis and Determination of Pharmacodynamics Cutoff Value

A Monte Carlo simulation (MCS) with 10,000 iterations was conducted using Crystal Ball software (version 7.2.2) (Oracle, United States) based on MIC distribution, PK parameters for CEQ in milk after IMM infusion, indices of PK/PD targets obtained in the *ex vivo* PK/PD modeling (19–22). The MIC data of CEQ against *S. aureus* were collected according to the previous study reported data (23–27), and its distribution was shown in **Supplementary Figure S1**. The AUC_{0-24} was assumed to be log-normally distributed for the mean values and confidence intervals (CI). The distribution of the AUC_{0-24h}/MIC was calculated through MCS. Then a target value of Bactericidal activity AUC_{0-24}/MIC derived from *ex vivo* PK/PD modeling was set to calculate the target attainment rate (TAR) of the corresponding dosing regimen. CO_{PD} was defined as the MIC at which the PTA reached up to 90% under an existing dose or a recommended dose regimen, according to the CLSI guidelines described in previous reports by Turnidge and Paterson (28) and Zhang et al. (29).

RESULTS

MIC Results

The MIC distributions of antimicrobials against *S. aureus* (n = 63) isolated from clinical mastitis in Jiangsu China are shown in **Supplementary Table S2**. The MIC_{50} and MIC_{90} values of CEQ are 0.5 and 2 μg/mL in MH broth and 1 and 2 μg/mL in milk. MIC_{90} of CEQ in milk was one dilution higher than their MIC_{50}. The resistance rate of tetracycline was most serious, attaining 30%, and the resistance rate of FOX and CLO was as high as 17% (**Figure 1**).

The MICs of CEQ in milk were two to four times more than in MH broth. The MIC of CEQ in the broth for B4030RH-31.4, 2014RQG-33.15, and ATCC 29213 were 4, 1, and 0.5 μg/mL,

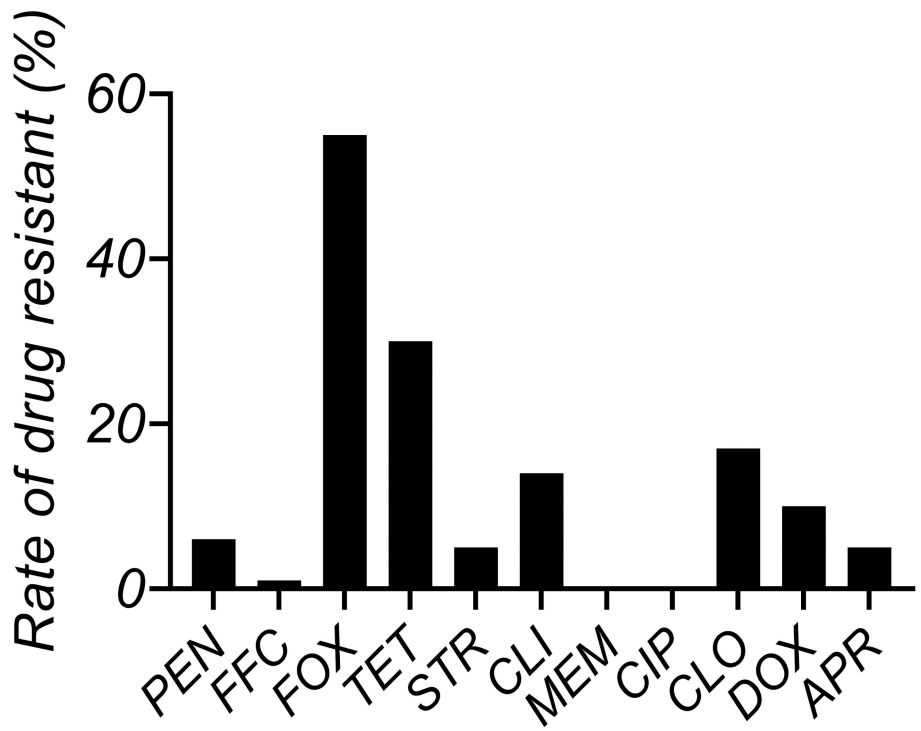

FIGURE 1 | *In vitro* susceptibility assays of 63 *Staphylococcus aureus* isolates used in this study.

respectively, and in the milk they were 16, 2, and 1 μg/mL, respectively (**Supplementary Table S3**).

In vitro Time-Killing Curves

The *in vitro* time-killing curves against *S. aureus* ATCC 29213, B4048RH-3.14, and 2014RQG-33.15 with two initial bacterial loads in artificial medium and milk are shown in **Figure 2**. Bactericidal activity was observed in the broth at 1 × MIC CEQ concentration against ATCC 29213 with bacteria titer of 10^6 and 10^8 log CFU/mL (**Figures 2A1,A2**), but the concentration of CEQ was 4 × MIC in the milk matrix, which could achieve the same effect (**Figures 2A3,A4**). The bactericidal activity was observed at 8 × MIC against B4030RH-3.14 in the broth (**Figures 2B1,B2**). However, in milk, CEQ cannot inhibit the growth of the same strain B4030RH-3.14 even at 8 × MIC (**Figures 2B3,B4**). The drug killing mode against 2014RQG-33.14 was similar to ATCC29213. The bactericidal was achieved at 1 × MIC CEQ concentration in broth (**Figures 2C1,C2**), while the corresponding concentration was 2 × MIC in milk against 2014RQG-33.14 (**Figures 2C3,C4**).

Milk Pharmacokinetics for CEQ

The PK curve of CEQ in milk is shown in **Figure 3**. After the first CEQ IMM administration, the CEQ concentration in milk attained a maximum level of 1.80 ± 0.64 mg/mL at 1.71 h. The AUC_{0-12} was 15.53 ± 5.64 mg·h/mL. Then, after the third IMM administration, the CEQ concentration in milk attained a maximum level of 1.55 ± 0.21 mg/mL at 1.8 h. The drug elimination half-life was 10.6 ± 4.01 h, the AUC_{0-24} was 26.12 ± 2.42 mg·h/mL. The mean residue time was 22.58 ± 7.83 h (**Table 1**). These results suggested that CEQ was eliminated slowly and maintained a long period of effective concentration in milk.

Ex vivo PK/PD Integration

In the *ex vivo* time-killing curve, bacteria number changes with the drug concentration of the milk sample. The higher the drug concentration, the stronger the bactericidal activity. The initial inoculum concentration will also affect the antibacterial activity of CEQ, that is, the bactericidal effect will be reduced under high initial inoculum amount (10^8 log CFU/mL). The CEQ antimicrobial activity is different when in a different medium. The relationships between PK/PD parameters and the *ex vivo* antibacterial effects in milk with different initial titers of *S. aureus* (1×10^6 CFU/mL, 1×10^8 log CFU/mL) are shown in **Figure 4**. According to **Figure 4**, the bactericidal effect of CEQ in milk is weak compared to in the broth. The AUC_{0-24h}/MIC index correlated well with *ex vivo* efficacy with the initial bacteria loading of 10^6 cfu/mL ($R^2 > 0.88$). While for the initial bacteria loading of 10^8 cfu/mL, the R^2 was about 0.84, indicating that bacterial loading, in other words, the severity of infection poses an effect on the PK/PD modeling results. The PD parameters of E_0, E_{max}, and PK/PD parameters required for various degrees of antibacterial activity and the Hill coefficient N are presented in **Table 2**. According to the inhibitory sigmoid E_{max} model analysis, the AUC_{0-24}/MIC were 8,638, 1,397, and 3,851 to attain

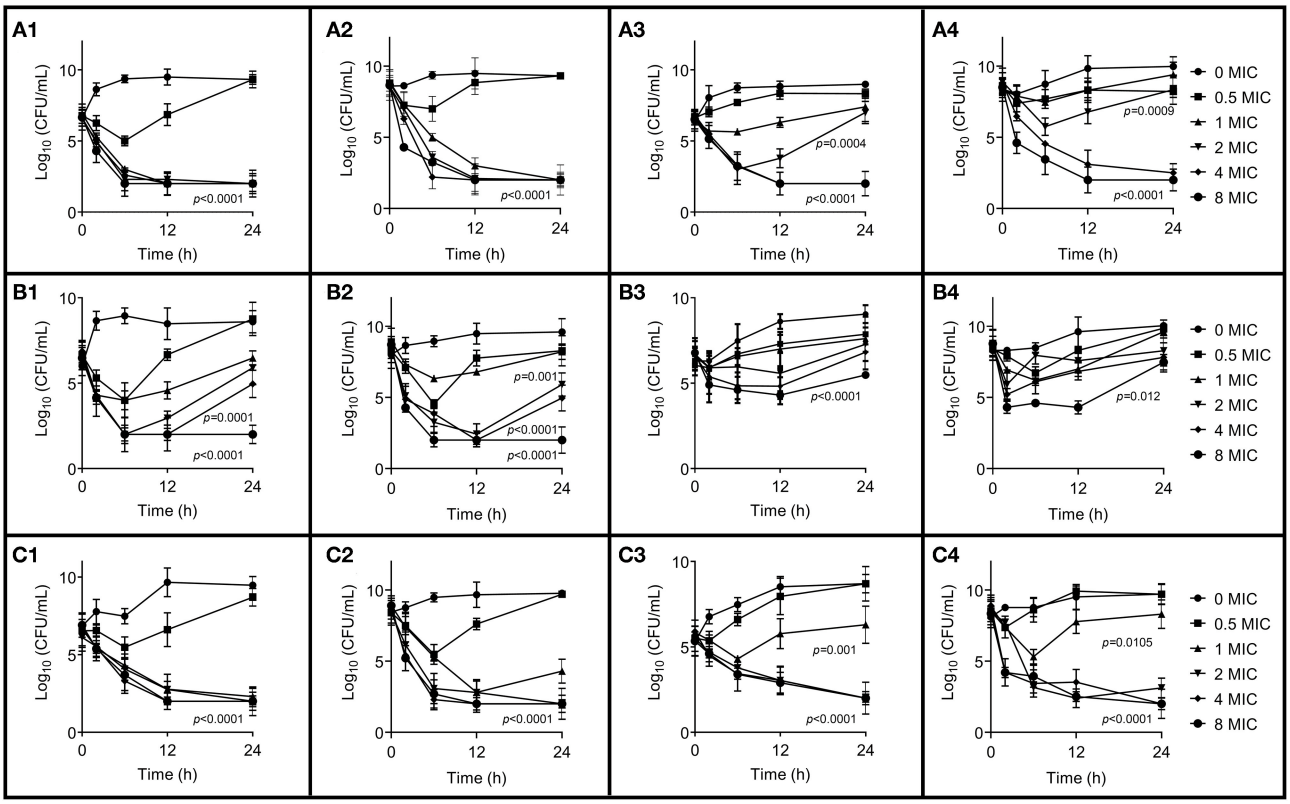

FIGURE 2 | *In vitro* time-killing curves of cefquinome in broth and milk against *S. aureus* ATCC 29213, B4048RH-31.4, and 2014RQG-33.15, respectively. **(A1–A4)** Represents the *S. aureus* ATCC 29213, **(B1–B4)** represents the *S. aureus* B4048RH-31.4, and **(C1–C4)** represents the *S. aureus* 2014RQG-33.15. **(A1,B1,C1)** Show antibacterial activity against an initial bacterial load of 10^6 $\log_{10}$CFU/mL in broth, and **(A2,B2,C2)** show antibacterial activity against t initial bacterial load 10^8 $\log_{10}$CFU/mL in broth. **(A3,B3,C3)** Show antibacterial activity against an initial bacterial load of 10^6 $\log_{10}$CFU/mL in milk, and **(A4,B4,C4)** show antibacterial activity against an initial bacterial load of 10^8 $\log_{10}$CFU/mL in milk. Error bars represent the SD ($n = 3$) and p-value were calculated by Student's t-test.

bactericidal effect against ATCC 29213, B4030RH-31.4, and 2014RQG-33.15 with the initial inoculum of 10^6 CFU/mL, while the corresponding AUC_{0-24}/MIC values were as high as 12,266, 2,295, and 5,337 with the initial inoculum of 10^8 CFU/mL.

Dose Assessment and PK/PD Cutoff Determination

The MIC values of CEQ against *S. aureus* were collected from previously reported data (23–27) and this study. The MIC distribution of CEQ against *S. aureus* is shown in **Supplementary Figure S1**, MIC_{50} and MIC_{90} were 1 and 2 μg/mL, respectively. Through Monte Carlo simulation, the optimal dosage for slight infection mastitis (10^6 CFU/mL) to bactericidal ($E = -3$) activity against *S. aureus* was 38 mg/gland/12 h, and for severe infection mastitis (10^8 CFU/mL) for bactericidal ($E = -3$) activity against *S. aureus* it was 55 mg/gland/12 h (**Table 3**).

The PTAs following CEQ administration at a dose of 38, 55, 75 mg/gland/12 h are shown in **Figure 5**. For the recommended dose and clinical recommended dose (38, 55, 75 mg/gland/12 h), the PTA > 90% could only be achieved for MIC <2 or 1 μg/Ml, which means, the PK/PD cutoff for CEQ against *S. aureus* was 1, 2, 2 μg/mL, respectively. The recommended dose of 55 mg/gland/12 h was lower than 75 mg/gland/12 h, which not only reaches bactericidal effect even in severe infection mastitis but reduces the risk of drug resistance. Therefore, the recommended dose regimen of 55 mg/gland/12 h in this study is the optimal dose regimen for clinical mastitis therapy.

DISCUSSION

According to previous research, the PK/PD modeling of CEQ against mastitis was studied in the mouse mastitis model (30) instead of bovine. To our knowledge, this study is the first to address the PK/PD modeling and CO_{PD} of CEQ against *S. aureus* in lactating cows. In this study, we investigated pharmacokinetic and *ex vivo* PK/PD modeling of CEQ after IMM administration in healthy cows, and the rational dose regimen and CO_{PD} were evaluated.

According to the MIC result, PEN, CLO, and CLI are common use drugs in the clinical treatment of most dairy farms for mastitis. This could be the reason why the resistance rate is higher than other drugs. However, TET displays the highest resistance

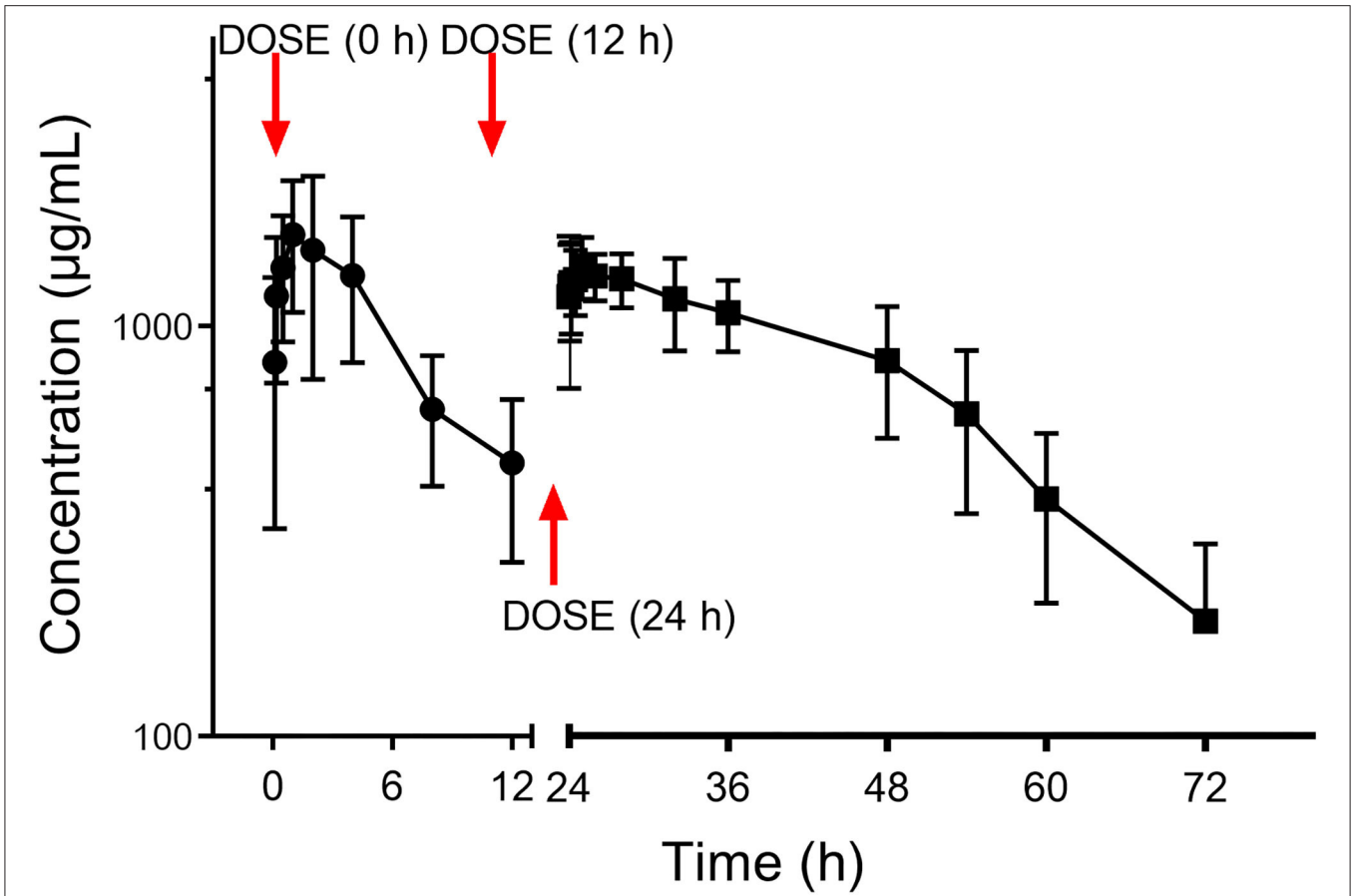

FIGURE 3 | Milk concentration vs. time curve of cefquinome following three consecutive IMM administration of 75 mg/gland.

TABLE 1 | Pharmacokinetic parameters of cefquinome in milk following the administration of three consecutive IMM of 75 mg cefquinome in each breast of 3 cows (Mean ± SD, $n = 12$).

Parameters	Unit	Dosage (75 mg/gland)	
		First	Third
C_{max}	mg/mL	1.80 ± 0.64	1.55 ± 0.21
T_{max}	h	1.71 ± 1.18	1.8 ± 2.93
AUC_{last}	mg·h/mL	15.53 ± 5.64	42.25 ± 12.3
AUC_{0-24}	mg·h/mL	–	26.12 ± 2.42
MRT	h	–	22.58 ± 7.83
CL/F	L/kg	–	2.07 ± 0.38
Vz/F	L/h/kg	–	31.09 ± 11.04
$T_{1/2\beta}$	h	–	10.6 ± 4.01

rate except for FOX. The possible reason for this is high-frequency TET-resistant gene horizon transfer and low fitness cost in *S. aureus* (31–33). The result of MIC in milk is different from in broth, and most of the MIC data in milk were two to four times higher than those in broth. Milk as the culture medium could provide a bacterial growth environment, especially for mastitis-related bacteria. Data of MIC in milk can better reflect the real situation in the bovine gland. As indicated in **Figure 2**, CEQ displayed a discrepancy in antimicrobial effect because of different mediums for the same strain. The high bacterial load group (8 $\log_{10}$CFU/mL) exhibited a weak bactericidal effect in the same medium. Although a 6 $\log_{10}$CFU/mL bacteria loading is usually used as the initial inoculum *in vitro* killing trials, in this study bacterial loads of 6 and 8 $\log_{10}$CFU/mL were employed to simulate slight and severe mastitis. In this study, significantly, CEQ bactericidal effect against *S. aureus* was influenced along with the bacterial initial load. In the high bacterial concentration (8 $\log_{10}$CFU/mL) group, CEQ bactericidal effect is weaker compared to normal bacterial concentration (6 $\log_{10}$CFU/mL) group. The amount of initial load will contribute to a determining factor. The reason is that when the initial concentration of *S. aureus* was high, the reproduction was low due to the limit of space, nutrition, and so on. The result suggested that the milk as a medium could show the real drug effect better than in broth. Compared with previous work (30), wild *S. aureus* strain including B4030RH-31.4 and 2014RQG-33.15 in the *in vitro* time-killing of this study displayed different bactericidal characteristics from standard strain ATCC2592. This is because of its complicated origin background, so treatment of wild

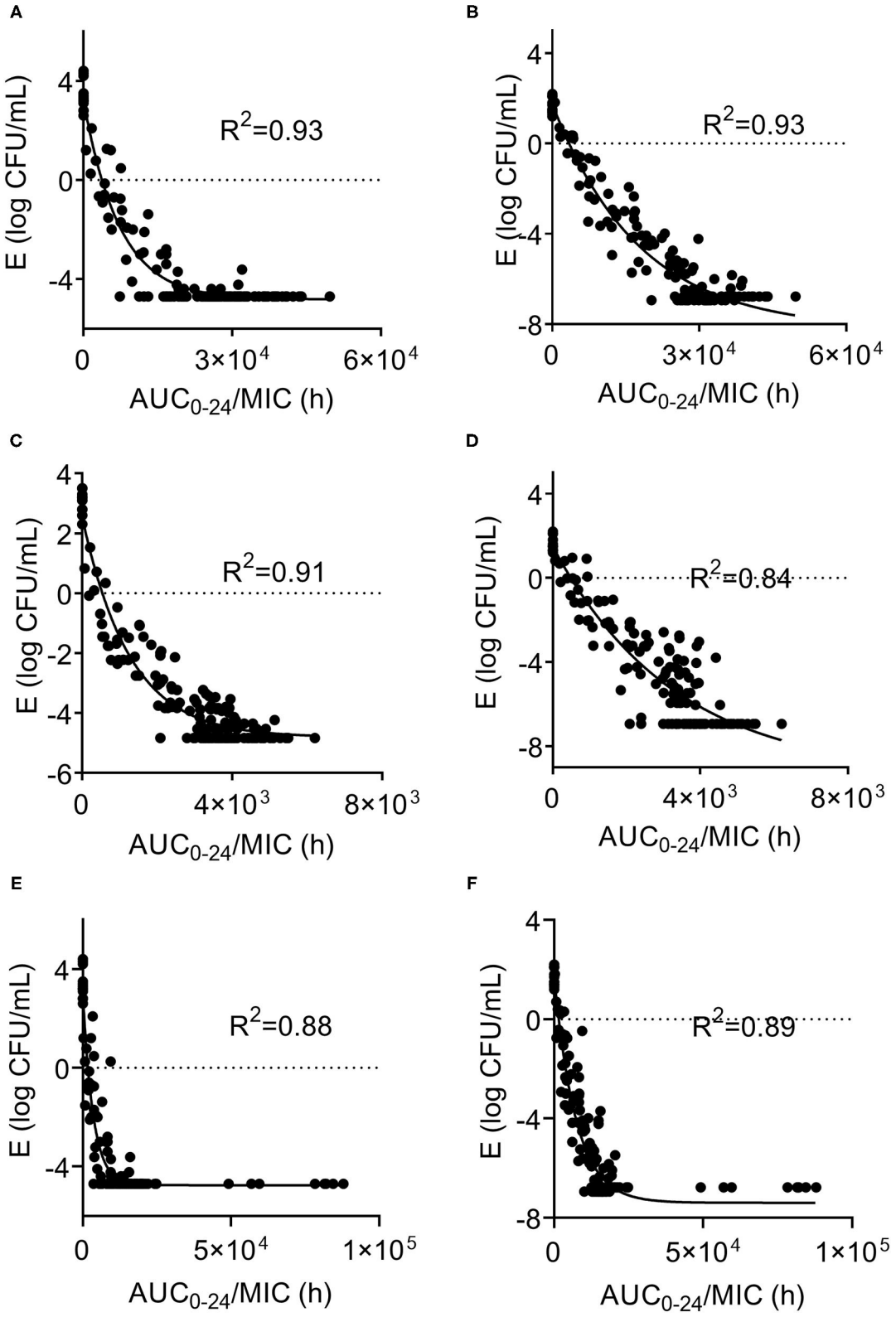

FIGURE 4 | Sigmoid E_{max} relationships between *ex vivo* antibacterial effect and PK/PD indexes of cefquinome against *S. aureus* ATCC29213, B4048RH-31.4, 2014RQG-33.15. **(A,B)** Showed Sigmoid E_{max} relationships against *S. aureus* ATCC29213 with an initial bacterial load of10^6 and 10^8 log10CFU/mL. **(C,D)** showed Sigmoid E_{max} relationships against *S. aureus* B4048RH-31.4 with an initial bacterial load of 10^6 log_{10}CFU/mL and 10^8 log_{10} CFU/mL. **(E,F)** Showed Sigmoid E_{max} relationships against *S. aureus* 2014RQG-33.15 with an initial bacterial load of 10^6 log_{10}CFU/mL and 10^8 log_{10}CFU/mL.

TABLE 2 | Integration of *ex vivo* PK/PD after administration of three consecutive IMM of cefquinome against in *S.aureus*.

Parameter	ATCC 29213		B4048RH-31.4		2014RQG-33.15ATCC29213	
	10^6CFU/mL	10^8CFU/mL	10^6CFU/mL	10^8CFU/mL	10^6CFU/mL	10^8CFU/mL
Emax	9.61	11.35	8.4	9.144	9.01	9.13
EC_{50}	5,437	15,489	1,040	1,158	2,263	5,405
E_0	3.2	1.6	3.6	2.2	3.2	1.41
Bacteriostatic activity	3,172	5,177	974	1,564	1,695	2,138
Bactericidal activity	8,638	12,266	1,397	2,295	3,851	5,337
Bacterial elimination	12,691	15,227	1,734	2,608	5,665	6,886
N	1.29	1.65	4.4	3.3	1.75	1.15

TABLE 3 | Predicted dose of cefquinome against *S.aureus* to reach 50%TAR, 90%TAR under different bacterial load (mg/gland/12 h).

Predicted dose (mg)		Target rations (10^6)		Target rations (10^8)	
		50%	90%	50%	90%
29213	Bacteriostatic ($E = 0$)	7	14	11	23
	Bactericidal ($E = -3$)	18	38	26	55
	Eradication ($E = -4$)	27	57	31	70
B4048RH-31.4	Bacteriostatic ($E = 0$)	34	36	54	57
	Bactericidal ($E = -3$)	49	51	79	87
	Eradication ($E = -4$)	60	63	91	98
2014RQG-33.15	Bacteriostatic ($E = 0$)	3	8	4	10
	Bactericidal ($E = -3$)	8	17	11	24
	Eradication ($E = -4$)	12	25	15	31

pathogens may call for a brand new dose regimen, like more frequent dosing intervals or large doses.

In the present investigation, three consecutive IMM CEQ administrations (75 mg/gland) produced an AUC of 42.25 mg·h/mL, the milk concentration vs. time curve is shown in **Figure 3**. The absorption of CEQ was rapid, drug reached its peak concentration at 1.8 h (T_{max} =1.8 h, C_{max} =1.55 mg/mL). This value was very similar to that in the cattle of Li's report (44.74 mg·h/mL, T_{max} =2 h, C_{max} =1.05 mg/mL) (34). The half-life of CEQ in this study was 10.6 h, which is similar to those described in cattle (6.3 h) following three consecutive IMM administrations (34) and in healthy lactating goats (12.9 h) (6).

According to a previous study, β-lactam antibiotics were time-dependent drugs (35) and showed bactericidal effect when the concentration in the target organ was above the MIC of the pathogen (36). According to previous research (30), %T>MIC and AUC_{0-24}/MIC were the PK/PD index for CEQ (37). In this study, %T>MIC could not be obtained accurately. The AUC_{0-24}/MIC in a previous study showed a good linear correlation with the antimicrobial effect of CEQ as the optimal PK/PD index (38). Thus, the PK/PD surrogate AUC_{0-24h}/MIC was chosen as the optimal PK/PD index. The linear correlation coefficient of AUC_{0-24h}/MIC and CEQ antimicrobial effect was higher for the initial inoculum of 10^6 than 10^8 CFU/mL (0.91 vs. 0.84). This result exhibited the impact of initial inoculum on PK/PD modeling result. The specific cause of impact needs further study. The EC_{50} of the 2014RQG-33.15 strain was 2,263 for the initial inoculum of 10^6 CFU/mL, and the value of AUC_{0-24h}/MIC for the bactericidal effect of ATCC29213 for the initial inoculum of 10^8 CFU/mL was 12,266. This result was similar to that derived in an *in vivo* PK/PD integration in a lactating mouse model where the EC_{50} and AUC_{0-24h}/MIC were 2,483 and 13,492, respectively (11), but the PK/PD parameters vary for other strains in this study. The possible reasons were as follows: (1) the PK/PD model is different. In this study, the *ex vivo* PK/PD model was used: previous work adopted the *in vivo* PK/PD model, and there was a distinction between the *ex vivo* and *in vivo* PK/PD models. (2) The dose of CEQ in mouse mammary gland tissues was lower than in the bovine gland. The concentration of CEQ mammary gland tissue was obtained that could be lower than in milk. Thus, the target animal and dose regimen was important for PK/PD modeling. (3) In this study, lactating bovine was used as a target animal rather than laboratory animals to reflect CEQ antimicrobial activity in clinical treatment.

Population dose prediction derived from MCS indicated that the existing dose of CEQ (55 mg/gland/12 h) was sufficient for cow mastitis caused by *S. aureus*. Under the recommended dose (55 mg/gland/12 h), even severely infected mastitis (10^8 CFU/mL) could be cured, much less the slight infection mastitis (10^6 CFU/mL). It was higher than that recommended in Shymaa's study (37.5 mg) (39) because in this study the severe infection (8 $\log_{10}$CFU/mL) was concerned. In addition, the recommended dose (55 mg/gland/12 h) was lower than the present using dose regimen (75 mg/gland/12 h) without reducing the antibacterial effect. Its application could reduce economic costs.

CO_{PD} is an important tool to set susceptibility breakpoint, and it was also used by regulatory agencies, such as EUCAST and VAST, to refine the susceptibility breakpoint (40–42). Monte Carlo simulation provides a great advantage using drug exposure–effect relationship (43), which considers

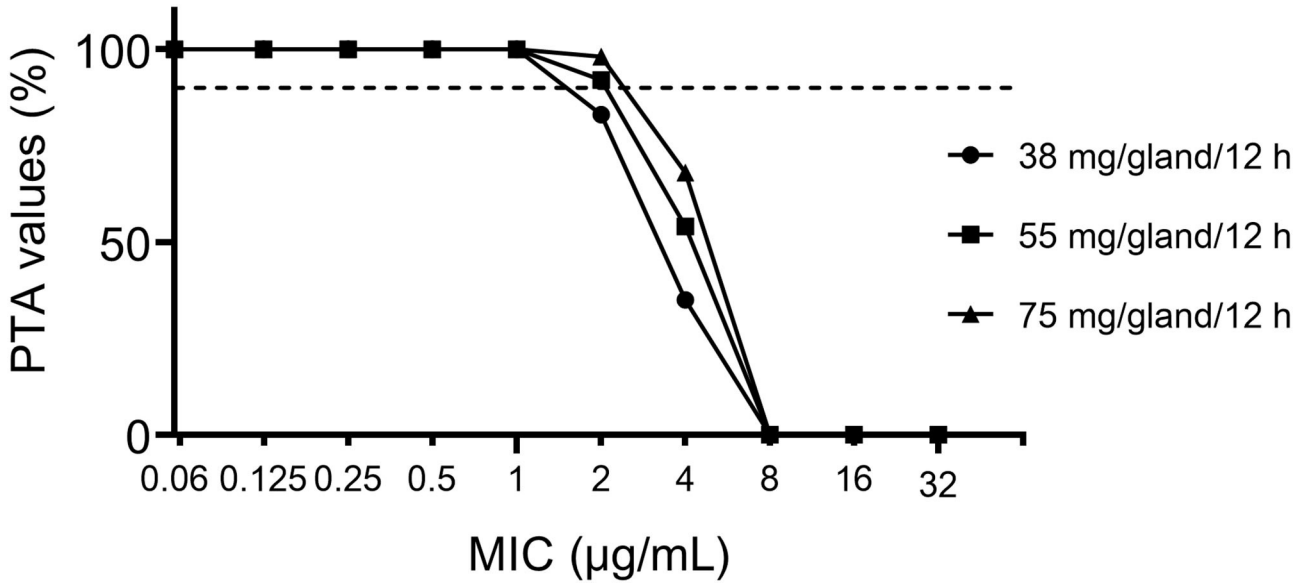

FIGURE 5 | Probability of target attainment (PTA) of cefquinome against bovine mastitis caused by *S. aureus* at the dose of 38, 55, 75 mg/gland/12 h.

pharmacokinetic variation in target animals, MIC distribution, and PK/PD indices in defining the PK/PD cutoffs. Currently, no breakpoint data of CEQ was established for animal infections caused by *S. aureus*. In the present study, the CO_{PD} of CEQ against *S. aureus* in bovine were determined to be 2 μg/mL at the recommended dose (55 mg/12 h/gland) and clinical dose regimen (75 mg/12 h/gland) based on Monte Carlo analysis, which was equal to the CLSI clinical CO_{PD} values of cefalotin (2 μg/mL), cefazolin (2 μg/mL), cefpodoxime (2 μg/mL), ceftiofur (2 μg/mL), and lower than cefoxitin (4 μg/mL) against *S. aureus* (15). However, the relatively conservative CO_{PD} should be verified in a larger number of bacteria and clinical practices.

Based on the current PK study, MIC distribution, and specific PD targets, if the dose is given at 55 mg/gland/12 h, the 10,000-subject Monte Carlo simulation showed that the bactericidal effect could be achieved under recommended dosage against *S. aureus* isolates in this study. CEQ 55 mg/gland/12 h is estimated to be effective against *S. aureus* infection in bovine. For the dose of 55 mg/gland/12 h recommended in this study, the PK/PD cutoff was 2 μg/mL. To confirm this value, CO_{PD} derived from *in vivo* PK/PD modeling should be investigated further in the future (40).

CONCLUSION

This is the first study to assess *ex* vivo PK/PD of CEQ against *S. aureus*. (1) Milk as a medium can reflex the real mastitis progress in clinical therapy. (2) Initial bacterial concentration has a huge impact on antibacterial effect on all the tested three strains. (3) The *ex vivo* PK/PD based population dose prediction indicated a target attainment rate (TAR) of 90% of 55 mg/gland/12 h. (4) The PK/PD Cutoff value (CO_{PD}) for CEQ against *S. aureus* was 2 μg/mL under the recommended dose of 55 mg/gland/12 h.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Jiangsu Administrative Committee for Laboratory Animals (SYXKSU-2007-0005).

AUTHOR CONTRIBUTIONS

XX and Z-qW conceived and designed the experiments, manuscript reviewing, and editing. L-jJ, K-xY, and TD performed the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.837882/full#supplementary-material

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REVIEW
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Combating Bovine Mastitis in the Dairy Sector in an Era of Antimicrobial Resistance: Ethno-veterinary Medicinal Option as a Viable Alternative Approach

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Bovine mastitis (BM) is the traditional infectious condition in reared cattle which may result in serious repercussions ranging from animal welfare to economic issues. Owing to the high costs associated with preventative practices and therapeutic measures, lower milk output, and early culling, bovine mastitis is accountable for most of the financial losses suffered in cattle farming. *Streptococcus agalactiae, Staphylococcus aureus, Streptococcus dysgalactiae* and coliform bacteria are the predominant pathogens for bovine mastitis. In addition, the occurrence of BM has been linked to lactation stage and poor management, in the latter case, the poor stabling conditions around udder hygiene. BM occurs throughout the world, with varying rates of *Streptococcus agalactiae* infection in different regions. Despite the modern techniques, such as the appropriate milking practices that are applied, lower levels of pathogen vulnerability may help to prevent the development of the disease, BM treatment is primarily reliant on antibiotics for both prophylactic and therapeutic purposes. Nevertheless, as a result of the proliferation of bacterial agents to withstand the antibiotic effects, these therapies have frequently proven ineffectual, resulting in persistent BM. Consequently, alternative medicines for the management of udder inflammation have been researched, notably natural compounds derived from plants. This review focuses on BM in terms of its risk factors, pathogenesis, management, the molecular identification of causative agents, as well as the application of ethno-veterinary medicine as an alternative therapy.

Keywords: mastitis, ethnobotany, antibiotics, milk output, infectious conditions

INTRODUCTION

Bovine mastitis (BM) is associated with the swelling of the udder which is caused by various factors, such as contagious pathogens, poor nutrition, and ineffectual management conditions on the farm (1, 2) (**Figure 1**). Its effects range from inferior animal welfare to mediocre industrial economic conditions, some of which encompass, without bounds, reduced milk yield, increased therapeutic costs, and premature culling (3–5). Moreover, different microorganisms, including bacteria, can cause mastitis. Pathogens that are most frequently encountered in a mastitic condition are *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Escherichia coli,* and other coliforms (6). Vanderhaeghen et al. (7) state that the clinical presentation of bovine mastitis assumes two distinct forms (clinical or subclinical) (8), which are distinguished by a rise in the pathological cell number. Clinical mastitis is marked by evident alterations in the quality and composition of the milk, including the level of coagulants, and is often followed by pervasive indicators and impairments, *inter alia* high temperatures, in the animal. On the other hand, BM caused by environmental pathogens is characterized by an increase in the somatic cell count (SCC), resulting in significant cost implications, especially on account of the increased cell count in milk samples (2, 8). According to Giesecke et al. (9), annual milk wastage owing to BM on farms in South Africa amounted to approximately ZAR 29.68 million. More recent data on the prevalence of BM in South Africa are unavailable, probably due to the fact that the condition is not commonly and regularly reported (10).

Antibiotics have long been considered the first line of defense against bacterial infections in dairy cattle, especially in the case of mastitis, where antibiotic residues occur in the milk and there is the risk that microbial resistance will spread to the environment (5, 11). Owing to the proliferation of multiple antibiotic-resistant (MAR) bacteria, which is a pressing public health concern for animal and human health, food security, and development, the use of antibiotics in animal production has been studied with great caution (12, 13). From the one-health perspective, mastitis-causing bacteria have broken through a number of hierarchical barriers, allowing for zoonotic transmission from bovines to people *via* milk and meat, thus putting public health at risk. Hence, there is a significant risk of microbial contaminants from the animal to the pastures and the equipment, to the effect that the consumption of raw milk may cause food-borne infections. Pasteurization of milk is thus essential to guarantee its quality and to extend its shelf-life (14).

In this age of the proliferation of microbial resistance to antimicrobials, it is imperative to source alternative medicines for the management and prevention of bovine mastitis. Hence, this review focuses on BM in terms of risk factors, pathogenesis, control and treatments, the molecular identification of causative agents, as well as the application of ethno-veterinary medicine (EVM) as an alternative therapy for BM.

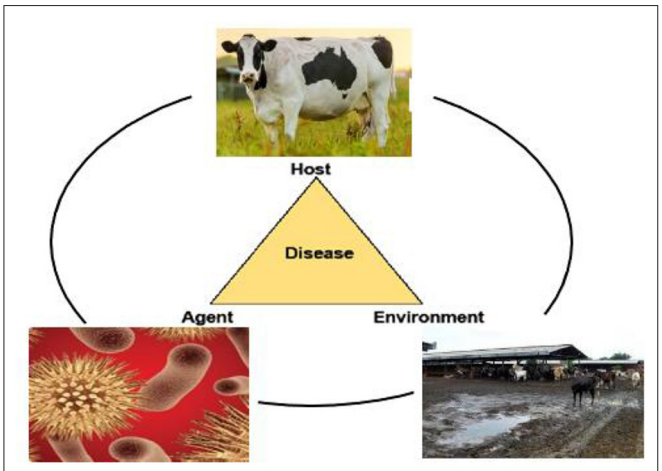

FIGURE 1 | Factors contributing to bovine mastitis.

METHOD FOR SELECTION OF LITERATURE

Keywords such as bovine mastitis, ethno-veterinary medicine, livestock and antimicrobial resistance were used to promote the literature search technique. In addition, phrases such as ethno-botany, alternative medicine, anti-inflammatory and antibacterial effect were employed to generate data for the biological activity and the phytochemical characteristics discussed in the review. These keywords were used alone and in combination to find relevant material from electronic data base such as Google Scholar, Pubmed, Science Direct, and Web of Science. To be considered for inclusion in the review, an article or study should have included and identified information about the usage of a specific medicinal plant in the ethno-medicinal context for treating BM within the research period (i.e., up until October 2021).

The following data were obtained for each study: the plant's scientific name, plant part, life form, disease treated, dose, preparation and administration. Articles reporting on the ethno-veterinary applications (**Table 2**) and the preparation and administration of medicinal plant products against antimicrobial resistant BM pathogens (**Table 3**) met the inclusion criteria. Review and research papers not involving the ethno-veterinary medicinal approach in combating antimicrobial resistance of mastitis pathogens were excluded. Finally, all papers that met the inclusion criteria were retrieved for the collection of adequate information. All scientific plant names were cross-referenced on the international databases, namely The World Flora Online (http://www.worldfloraonline.org/) and The Plant List (www.theplantlist.org).

BURDEN OF MASTITIS ON DAIRY FARMS

Udder inflammation is often regarded as the ultimate threat to the dairy sector, resulting in both financial losses and harmful impacts on public health. Mastitis is also

the foremost source of mortality in dairy cows. Mastitis-related financial losses are difficult to quantify; however, financial losses are attributed to the cost of medications, veterinary services, laboratory fees, and additional labor for the farmer. Losses also include those incurred on account of lost milk, culling/deaths and treatment costs (29). Amuta et al. (30) reported that the majority of farmers covered in their study in the North-West region of Nigeria considered clinical mastitis (CM)-related milk production losses to be significant (55%).

In Germany, milk wastage, ranging from 27 to 52%, (median rate; 37%) and rejected milk, ranging from 13 to 30%, (median value; 20%), were the most expensive charges associated with mastitis (29). Replacement expenditures, which were due to culling, and mastitis mortality, ranging from 3 to 28% with a median value of 22%, were the next biggest losses reported (median 22%). More so, Beyene and Tolosa (31) reported an average yearly drop of 59,719.08ETB, equivalent to USD 2,949.8, on account of mastitis, and a milk loss of 22.3% in crossbred cattle and of 2.24% in local zebu cattle in Ethiopia. The productive impact of subclinical mastitis in Colombian cattle ranches was estimated at about USD 800,000 per year, with milk production losses ranging from 1.3 to 13.5% per farm (32).

The losses in the value of milk and veterinary expenses in Central India on account of mastitis were reported to be about 49 and 37%, respectively (33). According to a study released by the University of Glasgow (https://www.fortunebusinessinsights.com/bovine-mastitis-market-103482), mastitis is predicted to cost the global dairy industry between USD 19.7 and 30 billion every year. Furthermore, another associated cost is a shortening of the lactation term of the animals. According to Khan and Khan (34), each infected animal's lactation period is reduced by about 57 days, while Seegers et al. (35) described reductions in mean milk output to be 375 kg for each lactation.

RISK FACTORS AND PREVALENCE OF BOVINE MASTITIS

The harmful factors associated with BM are classified as intrinsic and extrinsic and include, amongst others, species, age, parity, suckling period, antiquity of condition, state of habitat, and common handling conditions (36). These were all found to be remarkably related to BM. Conversely, milk-gland washing and drying and antiquity of condition had no remarkable outcome on the occurrence of BM (37). Nearly 30% of Saharan countries currently register instances of BM, leaving the rest of the continent in the dark (38). At the cow stage, the overall mastitis prevalence recorded on dairy farms in some parts of Africa is $< 54\%$, with CM at $< 14\%$ and subclinical mastitis at 16 to 88%. Mastitis was found to be more than 50% prevalent in previous research studies conducted in various parts of the southern region of Saharan Africa, with subclinical mastitis ranging from 16 to 80% (39). Mastitis is also common in Asian countries, with a prevalence of over 50% (40). Kvapilik et al. (41) published the global prevalence of sub-CM as ranging from 15 to 75%. Also, with the increase in the counts of cattle farms, the frequency of BM is escalating tremendously on a yearly basis.

THE PATHOGENESIS AND PREVALENCE OF BOVINE MASTITIS

Invading pathogens penetrate the irritated udder through the teat canal, which is located at the anterior margin of the cow mamilla (42, 43). In addition to physical stress and chemical irritants, bacterial pathogens grow and produce toxins that cause harm to the milk-producing tissue. The quantity of leukocytes in the milk increases as an effect of these factors. The initial line of defense is presented by the bovine mammary epithelial cells (bMECs). Thus, the bMECs play a significant role in that they provide broad and unique protection against resistant pathogenic microorganisms (42). More so, the udder may be contaminated by a number of microbial pathogens, which kill these cells and result in mastitis (44, 45).

A sphincter of smooth muscles covers the mamilla of the milk duct and works to ensure that it is closed from the outside (46). By preventing milk from escaping, it also prevents pathogens from entering the teat. Keratin generated from the stratified squamous epithelium lines the mamillary duct from the inside. The effectiveness of the keratin in the teat canal is to suppress the bacteria to a limited extent and to prevent their proliferation (47). Keratin is a waxy substance made up of phospholipids and fibrous proteins. Keratin fiber proteins are able to bind electrostatically to mastitis pathogens in the teat canal, thus altering the bacterial cell wall and making it more vulnerable to osmotic pressure. Invading pathogens are inhibited and killed when osmotic pressure is not maintained (48). During the milking process, germs near the teat's opening find a way into the teat canal, causing shock and damage to the keratin and mucous membranes surrounding the teat sinus (49). Also, after milking, the teat canal may remain partially open for 1 to 2 h, allowing infections to freely enter the teat canal (50).

The global prevalence rates of subclinical (SCM) and clinical mastitis (CM) are 42 and 15%, respectively (51). The SCM prevalence was found to be higher when compared to CM prevalence in the countries of the World. In most of the studies, CMT and its clinical manifestation were used for diagnosing SCM and CM, respectively. The pervasiveness rates for SCM and CM were found to be 45 and 18%, respectively in India. As stated by the College of Veterinary Medicine, Cornell University, clinical mastitis affects from 0 to 200 cows per 100 annually. Several studies from around the world have estimated the yearly occurrence of clinical mastitis as 25 to 30 cases per 100 cows (https://www.fortunebusinessinsights.com/bovine-mastitis-market-103482). In an Ethiopian study, the overall mastitis occurrence was reported to be 73.7% (283/384), of which 28.9% (82/283) was of clinical importance and 71.02% (201/283) was of subclinical significance (52). A Japanese research study stated the prevalent rates of CM and SCM to be 28 and 13%, respectively, while a closely-related margin of 23.6% was reported from an American study (53).

DIAGNOSIS OF BOVINE MASTITIS

When the udder of a cow is inflamed, there are several pointers or bio-signatures which are freed and/or influenced as a result of the changes in the milk. These indicators are set out as testing pointers for mastitis (54). Rossi et al. (55) have outlined several testing techniques, ranging from common examinations (somatic cell counts, the California mastitis test) (8) to molecular techniques (polymerase chain reactions) (56), and other sophisticated methods, such as loop-mediated isothermal amplification (LAMP) (57), genetic (58), proteome, as well as transcriptome (59, 60) analyses, nano-assembly and the micro-modeling of compact or mobile gadgets (61) which are used to detect these markers.

Conventional Screening Techniques for Bovine Mastitis

Regardless of its drawbacks, which include culturing time and costs (62), the microbiological investigation of milk is regarded as the gold standard for detecting bovine mastitis. However, during the inflammatory phase, leukocytes migrate in large numbers to the location site of the inflammation (63). Consequently, the cellularity of the milk can be used to assess the health of the mammary gland of the animal. Techniques including SCC, CMT and Somaticell® are the secondary ways for identifying BM (64).

Somatic Cell Count

Somatic cell count (SCC) is a technique that quantitatively detects the various categories of cells in milk (62). Using this method, figures between 100,000 and 272,000 cells mL^{-1} connote the presence of mastitis-causative agents (62). SCC can be carried out using an automated system (Somacount 300 automatic somatic cell counter) or manually by viewing stained microscope slides with appropriate reagents and then viewed using a microscope.

The California Mastitis Test

CMT is an elementary, low-cost, quick evaluation that can be conducted within the cattle ranch and is commonly used for identifying subclinical mastitis during milking ((64); 8). The California Mastitis Test is one of the most effective ways to identify mastitis (CMT). To perform the test, only a four-compartmentalized paddle and the CMT reagent are needed. Milk is appropriately collected in the CMT paddle and an equivalent amount of the indicator is added using a horizontal swirling motion for about 30 s. Results are then interpreted accordingly. A strong gel formation that tends to adhere to the paddle and with a leucocyte count of over five million per milliliter of milk sample would point to a strong positive result. A distinct gel formation with a leucocyte count of between 800,000 and 5,000,000,000 mL−1 of milk sample would also indicate a positive result. Distinct precipitate formation with a paddle movement but without gel formation and with a leucocyte count of 400,000 to 1,500,000 per milliliter of milk sample would show a weak positive result. On the other hand, a trace result, denoted by a slight precipitate, which would tend to disappear with paddle movement and which is associated with a leucocyte count of 150,000 to 500,000, would be representative of a mixture without a precipitate and would point to a negative test result (https://extension.missouri.edu/publications/g3653).

Somaticell®

This is a numerical assessment technique comparable to the Wisconsin Mastitis check that was created with the goal of producing outcomes matching those of the SCC, yet having an edge of portability (65). This technique is also advised for usage in bulk tank milk as a pilot test to determine whether it satisfies the current legal limits. A single-use graded plastic vial with a capped preset scale of SCC is used in this test (63).

Automatic Digital Diagnostic

These are newer testing techniques for the examination of mastitis in bovine. They are very easy, fast and field applicable methods (66). These techniques detect the physical, chemical, and biological changes of milk, or quantify the biomarkers in relation to mastitis. The methods include the DeLaval cell counter, the Afimilk mastitis detector, and the Draminski mastitis detector (67). The DeLaval cell counter is suitable for determining the somatic cell count.

Other newer techniques in diagnosing mastitis include the sensor-based system, infra-red thermography (IRT), and proteomic applications. Sensor-based detection systems are usually employed on large farms. A Portuguese study reported the use of magnetic nanoparticles for the actual identification of several staphylococci and specifically *Staphylococcus aureus*. The proteomic approach is helpful in diagnosing, preventing, and forecasting mastitis (68). It aids in the detection of the biomarkers of the intramammary infection (IMI). Hettinga et al. (69) reported the observation of some protein-related substances in relation to the host's reaction to intramammary infection.

Molecular Techniques for Bacterial Identification for Improved Treatment

Although culture-based approaches for diagnosing intramammary infection (IMI) remain the gold standard in many diagnostic centers, culture-independent methods for identifying bacterial infections in milk have grown more widespread in recent years (8).

When compared to culture-based approaches, molecular-based techniques have been proven to be more sensitive and more rapid since their findings may be ready in only a few hours (8). Bacterial taxonomy and phylogeny are based on conserved gene sequences, particularly the ones that denote ribosomal ribonucleic acids (rRNA). They are used as universal molecular chronometers since they contain sections of varying evolutionary degrees (some extremely conserved; others highly changeable and all-pervasive) (70).

Whole Genome Sequencing

This technique yields data on (a) pathogen naming, (b) the characterization of resistant genes (c) epidemiological typing, (d) the selection of peculiar types, regardless of primer development (to track genetic changes in organisms, their adaptations and modifications), and (e) the instant drafting of polymerase chain

reaction (PCR) probes based on WGS figures (70). WGS has provided valuable information that has aided advances in present screening techniques, including the detection of the mecC gene (a homolog of the mecA gene that is responsible for methicillin resistance in MRSA) which have elicited the reworking of PCR tests to boost reactivity and circumvent fallacies (71). When prognostics drawn from WGS figures were compared to phenotypic drug vulnerability, WGS was found to be very insightful and specific, as well as to show significant similarities with phenotypic antibacterial susceptibility/resistance approaches (72).

CONVENTIONAL TREATMENT OPTIONS FOR BOVINE MASTITIS

Prophylaxis

Bovine mastitis is characterized by the epidemiological triad; host, pathogen, and environment. The expression, "Better prevent than cure," accurately describes the disease mastitis, as there are many changes, such as harm to the teat and teat canal and udder alveoli that cannot be cured. These are the effects of howl arising from the condition. Mastitis can be circumvented following good acceptable procedures (73, 74). Reduced new infections (NI) and pathogen transmission through improved management standards, the segregation of the animal, and mitigations in the exacerbation of subclinical to clinical mastitis through a regular supply of nutrients are all possible preventative approaches (75). The maintenance of udder health is a process that is always being improved upon and which has already progressed to a high level in that it allows for additional improvements to reduce antimicrobial usage, albeit in a slow and methodical manner (75). Researchers have been working on efficient vaccinations to prevent bovine mastitis for decades, but established vaccines, such as those for *E. coli* or *S. aureus* intramammary infections (IMI) provide only limited protection (76).

Therapeutic Measures

Dry-off Treatment

The dry phase linking two milking periods is critical for teat well-being in dairy cows. It offers an encouraging way to treat current teat contamination, but also provides for conditions where the udder would be likely to incubate a new intramammary infection (IMI). Infections caused by the dry phase often cause increased somatic cell counts (SCC) in early breastfeeding. Management of udder health during the dry season is critical for a smooth transition into the lactation period (75). Epithelial cells that have been damaged or have become senescent are biologically replaced (77). The regeneration and phagocytosis of pathogens cure a substantial amount of IMI without interference, and antimicrobial dry cow therapy is highly effective because the medication is not milked out, thereby allowing for a greater and more consistent concentration of antibiotics in the udder. Thus, most IMIs heal (78).

Use of Antibiotics

The goal of managing mastitis with antibiotics is to eradicate the pathogens from the cattle, or worse still, those from the udder. Antibiotics are often used to manage mastitis in lactating cows, as well as to prevent intramammary infections in non-lactating cows (4, 5). More or less 60–70% of the antimicrobials used in cattle are for the prevention and treatment of mastitis (79). Antibiotics, such as the beta-lactams (penicillin G) and macrolides (erythromycin), are generally accepted for the management of bovine mastitis (2, 80). Nevertheless, the non-selective use of antibacterials without the *in-vitro* sensitivity testing of causative organisms is one of the major reasons for failures in the treatment of mastitis (81). Bacterial isolation and antibiotic sensitivity tests are always required for effective antibiotic therapy. According to Holko et al. (82), over 62 percent of the examined mastitis bacterial pathogens were insusceptible to one or more antibacterial drugs. In the majority of instances, the identified microbes were insusceptible to cephalexin, neomycin, penicillin, and streptomycin. Resistance was discovered in 86% of *S. uberis* isolates and 79% of *E. coli* isolates.

Mode of Action of Antibiotics

The course of efficacy of chemotherapeutics can be classified on the basis of the functions that the agents influence which commonly include the inhibition of cell wall synthesis, the suppression of nucleic acid synthesis, the repression of ribosome function, cell membrane function, and folate metabolism inhibition (83) (**Figure 2**).

Development of Antimicrobial Resistance

The overuse of antibiotics in clinical practice has led to bacterial resistance to antimicrobial medicines (84). As it accounts for several losses, MR is a significant challenge in the management of microbial infections in the food and dairy sector and also in the treatment of human diseases. Antibiotic inactivation, target alteration, altered permeability, and the "bypassing" of metabolic pathways are some of the biochemical resistance mechanisms exploited by bacteria (84). The basic anatomy of a Gram-positive bacterium has a cytoplasmic membrane which is surrounded by a thick and stiff mesh, termed the cell wall. Conversely, Gram-negative bacteria have a thin cell wall that is bordered by a second lipid membrane, termed the outer sheath. In Gram-negative bacteria, the sheath is an extra protective layer that inhibits numerous chemicals from entering the organism (84). A growing number of antibiotic-resistant bacteria are jeopardizing the effectiveness of antimicrobials (83). Resistance can be discussed in two broad terms:

- ✓ **Intrinsic resistance** occurs when microorganisms lack natural antimicrobial target sites and the antibiotic has no effect on them.
- ✓ **Acquired resistance** occurs when a naturally susceptible bacterium develops a method to resist an antibiotic. Antibiotic resistance in bacteria can be acquired through mutations in regular genetic codes or the uptake of alien insusceptible genetic codes, or both. The presence of an enzyme that

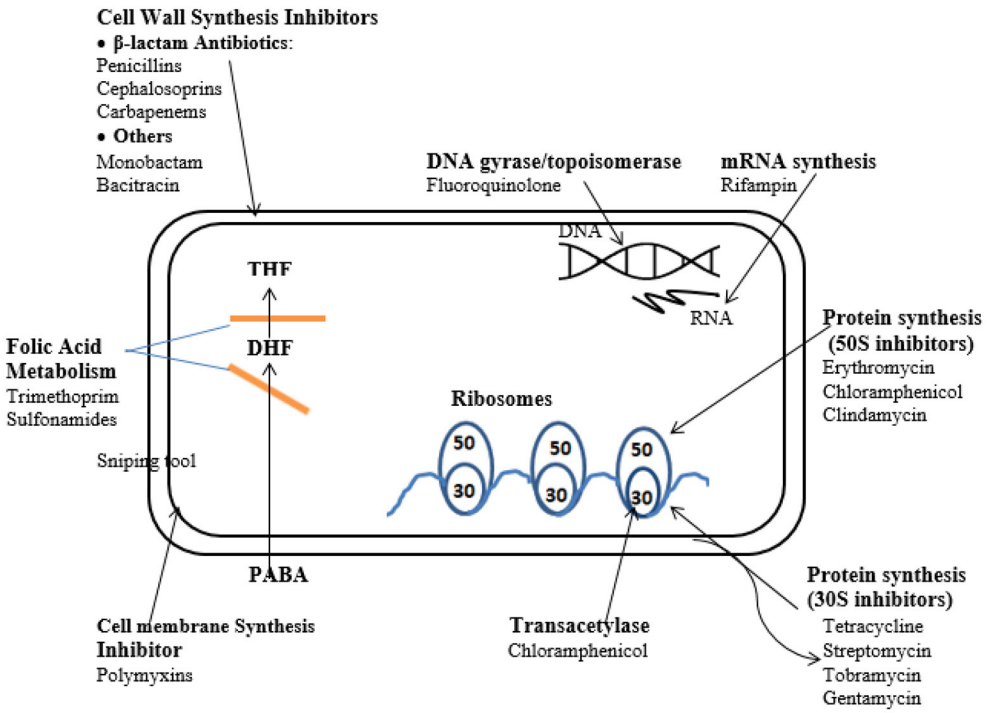

FIGURE 2 | Mode of action of antimicrobial agents.

causes inactivity in the antibacterial drug, that reduces the uptake of the antimicrobial agent, and the proteinaceous transportation of the anti-infective agent, are all examples of acquired resistance mechanisms (85).

Genetics of Antimicrobial Resistance

Bacterial non-susceptibility is a complicated condition that begins with the existence of genes encoding resistance in plasmids or chromosomal genetic material. Considerably aided by the proliferation of flexible genetic features, such as plasmids, transposons, and integrons, there has been a rapid transmission of antibiotic resistance across the various human bacterial groups (86). This is considered to be a matter for serious veterinary concern. Antimicrobial resistance genes (ARGs) have been found to abound on transposable segments (**Table 1**). Hence, an exclusive occurrence at the genetic level can transmit multiple-drug support traits to a vulnerable receiver. The amplification of integrase genes (intI 1, intI 2 and intI 3) can be used to determine the presence of integrons (98). A couple of researchers have reported some ARGs and their location sites in some isolates of animal origin (**Table 1**).

Challenges of Treatment With Antibiotics

The shortcomings of this strategy include low cure rates, the increased occurrence of antibiotic-resistance; pressing public health concerns, reduced food safety, security, and their upshots (12, 13), as well as the appearance of flakes in cow products (5, 11, 99). Milk tainted with antibiotics can cause sensitive reactions, alterations in the microbial makeup of the gut, and the growth of antibiotic-resistant strains in the general public (100). Because antibiotics aren't allowed to reach them, bacteria that can live intracellularly within the udder and that cause sores to develop are laborious to treat. This is evident with *S. aureus*, where the cure rate, based on the generally accepted antibiotics (e.g., pirlimycin), is about 10–30% (76). Consequently, these pathogens have developed resistance to antibiotic treatment. Thus, the World Health Organization (WHO) has released guidelines for limiting antibiotic use in livestock farming (101, 102).

More so, from the one-health perspective, mastitis-causing bacteria have broken through a number of hierarchical barriers, allowing for zoonotic transmission from bovines to people *via* milk and meat, either locally or remotely, and as a result, disease control has been quite challenging (103).

ALTERNATIVE TREATMENT TO CONVENTIONAL ANTIBIOTICS

Ethno-Veterinary Medicine

Ethno-veterinary medicine (EVM) is the application of humankind's expertise, abilities, practices, habits, and credence in managing and safekeeping the well-being of their animals (104). These hands-on skills are shared or transferred from age to age only through the oral medium (105). Both the scientific and consumer communities are increasingly turning to herbal plants as health promoters (106). In the interests of the foremost availability of these products and the cheap manufacturing costs

TABLE 1 | Mode of action, mechanisms of resistance of antibiotics and some antimicrobial resistance genes (ARG) of animal origin.

Family	Mode of action	Mechanism of resistance	Resistant gene	Location[a]	Reference
β-lactams	Cell wall synthesis inhibitors. Binds transpeptidase also known as penicillin binding proteins (PBPs) that help form peptidoglycan	Beta-lactamase production primarily-*bla* genes	*mec*C (mecALGA251) *mec*A *blaZ*	C C	(83, 86, 87)
	Inactivates the enzyme; beta-lactamase	Expression of alternative PBPs		Tn, P, C	
β-lactamase inhibitors	Hydrolysis of the beta-lactam ring	Production of extended spectrum beta-lactamases (ESBLs)	bla_{SHV} bla_{TEM}	P, C P, C	
Fluoroquinolones	Binds DNA-gyrase or topoisomerase II and topoisomerase IV; enzymes needed for supercoiling, replication and separation of circular bacterial DNA.	Target modification Decreased membrane permeability Efflux pumps	*qnr*A *qnr*B *qnr*S 9, 10	P P P	(85, 88)
Macrolides, Licosamides and Streptogamin (MLS)	Binds the bacterial 50 S ribosomal subunits; inhibit protein synthesis	Target site modification	*erm*A, (*erm*B), *erm*C, *erm*F, *erm*T, *erm* (73) *erm* (83)	C, Tn, P, (Tn,P) P, C P, P C	(89, 90)
		Active drug efflux	*msr* (A) except lincosamides	C, P	
Aminoglycosides	Bind to the bacterial 30 S ribosomal subunit thus inhibit bacterial protein synthesis	Target site modification [*via* the action of 16 S rRNA methyltransferases (RMTs)]			(89, 91, 92)
		Enzymatic drug modification (adenylation, acetylation and phosphorylation)	aacA-aphD *aad*D	Tn, P, C P, C	
		Efflux systems	*aad*E *str*	Tn, P P	
Tetracyclines	Bind reversibly to the 30 S ribosomal subunit as such blocks the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex	Efflux systems Target modification Inactivating enzymes Ribosomal protection	*Tet* (K), *tet* (K) *Tet* (M), *tet* (O)	P Tn/C, C	(93, 94)
Sulfonamides (Folate pathway inhibitors)	Inhibit the bacterial enzyme dihydropteroate synthetase (DPS) in the folic acid pathway, thereby blocking bacterial nucleic acid synthesis	Excessive bacterial production of dihydrofolate reductase (DHFR) Reduction in the ability of the drug to penetrate the bacterial cell wall Production of altered forms of the dihydropteroate synthetase (DPS) enzyme with a lower affinity for sulfonamides Hyperproduction of PABA, which overcomes the competitive substitution of the sulfonamides	*sul*1 *sul*2 *sul*3	P, C	(95–97)

[a] *C, chromosomal DNA; P, plasmid; Tn, transposon.*

around these items, studies using local plants must of necessity encompass multiple geographical locations. Kalayou et al. (28) investigated the antibacterial potency of several plant species against mastitis, with Croton aurea, Croton macrostachyus, Achyranthes aspera, Nicotiana tabacum and *Vernonia* species producing the most promising effects of all tested plants. Findings by Serunkuma et al. (107) established the efficacy of the acetone extract of the *Acacia nilotica* bark and the *Tetradenia riparia* flower against bacterial species cultured from mastitis samples from a farm. Across the board, the development of *S. agalactiae, S. uberis, E. coli* and *S. aureus* was suppressed by a methanol extract derived from *Spathodea campanulata* (108). Globally, the use of diverse plants from distinct geographical terrains to treat livestock infections remains common (**Table 2**). *In-vitro* assays into medicinal plants reveal their potency as antibacterial, anti-inflammatory or immune-modulatory agents (109).

Application of Ethno-Veterinary Medicine in the Treatment and Management of Mastitis

Ethno-veterinary medicine is a local animal healthcare system that incorporates traditional beliefs, knowledge, skills, methods, and practices. It includes the traditional treatment of veterinary illnesses, as well as the spiritual aspects of the treatment (24). Depending on the active substances to be extracted, the delivery route, and the medicinal purpose (prophylaxis

TABLE 2 | Ethno-veterinary medicinal plants used in the dairy and livestock farming.

Scientific name	Family	Life form	Plant part used	Animal	Use/disease	Reference
Erysimum melicentae Dunn.	Brassicaceae	Herb	Whole plant	Cattle and sheep	For general health improvement	(15)
Becium obovatum (E. Mey. Ex Benth. In E. Mey.) N.E. Br.	Lamiaceae	Herb	Root	Livestock	Mastitis, Black leg, listeriosis/encircling disease, diarrhea	(16)
Malva parviflora	Malvaceae	Herb	Whole Plant	Cattle	Mastitis	(17)
Brucea antidysenterica	Simaroubaceae	Tree	Leaf	Cattle	Mastitis	(18)
Acorus calamus L.	Acoraceae	Herb	Rhizome	Cows, Sheeps, Goats, Donkeys, Camels, Buffaloes	Mastitis, Anaplasmosis, constipation, heal wounds, dysentery, body tonic, gastric problems, bloating, indigestion, urinary disorder	(19)
Prosopis juliflora (Sw.) DC.	Mimosaceae	Shrub	Leaf	Cattle	Infections	(20)
Triticum sp.	Poaceae	Herb	Aerial parts, Bran	Livestock	Mastitis, breast lumps, difficulty of birth, retained placenta, increasing egg production	(21)
Arachis hypogea L.	Fabaceae	Shrub	Seed and seed oil	Goat and cattle	Increased milk production	(22)
Peganum harmala L.	Zygophyllaceae	Herb	Leaf, branches	Buffalo, Cattle, Dog	Mastitis	(23)
Citrus limon (L.) Osbeck	Rutaceae	Tree	Fruit	Buffalo, Cattle, Goat	Mastitis	(23)
Withania somnifera (L.) Dunal	Solanaceae	Shrub	Root	Buffalo, Cattle, Goat	Mastitis	(23)

or therapeutics), the method used to make ethno-veterinary medications differs. Infusions, decoctions, powders, drips, fumes, pastes, and ointments are made from medical plants, animal products, minerals, and other inorganic ingredients accessed by livestock owners and ranchers. These might be treated topically with drenches, or intra-nasally with smoke, vaccines, or suppositories, vapors, or massages (24).

Abbasi et al. (23) reported that paste made from the fruit juice of *Citrus limon* and sugar fed to animals and applied topically to the mammary glands for 10–15 days is used to treat mastitis in buffalo, cattle and goats. Also, the application of a paste of 200 g of fresh crushed roots of *Withania somnifera* to the udder of a cow and goat respectively for up to a week successfully treats the diseased condition. It is also noteworthy to state that the preparation and application of the smoke of the leaves and branches of *Peganum harmala* over a period of about 5 days successfully treats mastitis in cattle and horses. The anti-inflammatory effects of the topical application of the extract of fresh leaves of *Rumex nepalensis* to the affected part for about 5 days has also been reported. A poultice of young twigs of *Calotropis procera* applied to a swollen udder also relieves pain and inflammation (23). The treatment of BM using several of the EVM currently available is highlighted below (**Table 3**).

An herbal mixture of *Aloe vera* (L.) Burm. -F., *Curcuma longa* L. and calcium hydroxide, as documented by a traditional healer, was reported to be efficient against BM causing pathogens (110). Since the components of the mixture had a synergistic effect, their activity against the clinical mastitis pathogens was successful. The formulation was found to be antimicrobial and anti-inflammatory and to have wound cleansing and healing properties.

Natural Products (Plants): the Sleeping Giant of the Modern Pharmaceuticals

Globally, the plant domain renders a diversity of species utilized as treatments for various ailments (111). The World Health Organization (WHO) report states that traditional medicines, including those used for plant decoctions or functional compounds are used by a high percentage of the population of the world (112). An understanding of the plant parts used historically as treatments is the most essential requirement in the ethno-pharmacological approach (**Figure 3**). Chinese herbal medicine and Ayurveda are the prominent traditional medicines used in treating various diseases, but where no texts are available, the ethno-botanical survey is the only way in which to learn about the traditional uses of medicinal plants.

Natural products and/or organic by-product forms continue to play a pivotal role in the medication upshot process. As a result, biological diversity offers an endless supply of new chemical entities (NCEs) with their potential as therapeutic leads. These NCEs are derived productsgenerated by plants to shield them from herbivores and pathogens, or to attract pollinators (111).

The efficacy of secondary metabolites in plants is an indication of the strength of natural products as potent modern pharmaceuticals (113, 114). Researchers, including

TABLE 3 | Ethno-veterinary medicinal products, preparation and administration for the treatment and control of mastitis.

Product	Dose rate	Indication	Reference
Kali mur 6x + Calc Flur 6x	B.I.D. for 1 week to 10 days	Presence of clots in milk	(24)
Calundula Q + Belladonna 30+ Dulcamara Q + Echinaea 30 aa 1 ml Made upto 20 ml with distilled water	10 ml B.I.D. intra mammary injection for 2 to 3 days. Massage the udder to disperse the medicine uniformly	Inflammation of the udder with loss of appetite, fever congestion and injury	(24)
Silicea 1M + Calc. Sulph 200	Q.I.D. for 2–7 days	Mastitis without anorexia, udder is hard and with clots	60
Phytolocca 200 + Calc. Fluor 200 + Silicea 30 + Belladonna 30 + Arnica 30 + Conium 30 + Ipeca 30 aa 0.5 ml. Made up to 30 ml vimeral	B.I.D. for 2–4 days	In acute, subacute and chronic mastitis.	(24)
Kali Mur 30	Q.I.D. for 2–5 days	Mastitis without anorexia, hardness of udder and white or gray or cream color clots	(24)
Ferrum Phos 6x	Q.I.D. for 2–5 days	Mastitis without anorexia, blood in milk with or without bad smell	(24)
Plant	Plant part	Administration/dosage for cows	Reference
Allium sativum L.	Rhizome	250 g, grinded with butter and administered orally for 7 days	(25–27)
Allium cepa L.	Bulb	Heated in oil, given as food supplement once per day during 2 or 3 days or until the animal gets better (topical application and vaginal washes)	(27)
Asphodelus tenuifolius Cav.	Aerial part	Heated with barley peels (topical application)	(27)
Amomum subulatum Roxb.	Fruit	25 g, given orally for 3 days.	(25)
Brassica compestres + *Curcuma longa*	Seeds + root	250 g seeds are grinded with 50 g root and administered orally for 5 days	(25)
Brucea antidysenterica JF. Mill	Seed	Add 1 L of water to the ground fresh seed given orally once per day for 3 days	(26)
Peganum harmala + *Triticum sativum*	Fruit + stem crushing (hay)	50 g + 2 Kg, fumigation of harmal by putting it on fired hay under the affected udder for 4 days	(25)
Capsicum annuum	Fruit/whole plant	50 g, given orally for 8 days	(25)
Sesamum indicum	Seed oil	250 ml, mixed oil in 1.5 L of milk whey, and given orally for 7 days	(25)
Citrus limon	Extract	With raw sugar given orally for 5 days	(25)
Osyris quadripartita Decn.	Root	Pound the fresh root and mix with water, filter and administered orally for 6–7 days, daily	(26)
Gossypium hirsutum L.	Flowers	250 g, boiled in 1 L water to 250 ml, then drenched for 3 days	(25)
Galium aparine L.	Vine	500 g, given as decoction drench for 3 days	105
Chenopodium ambrosioides L.	Leaf	After grinding the fresh leaf, mix with water to prepare (liquid) 1 L then it is given orally once	(26)
Solanum spp.	Leaf	The fresh leaf and root are chewed by the local healer and spit to the mouth of the animal for 2 days	(26)
Artemisia herba-alba Asso	Aerial part	Heated with barley peels and/or aggaya (topical application)	107
Ricinus communis L.	Leaf	Pound about 50 g of fresh leaf and mix with 1 L of water then administered orally 1 L/day (every morning) for 2 days	(25, 26)
Cynomorium coccineum L.	Whole plant	Washes with decoction water	(27)
Hordeum vulgare L.	Seed	Roasted seeds mixed with water	(27)
Ziziphus spina-christi	Leaf	The leaves are ground and applied on the affected teat quarter	(28)
Achyranthes aspera + *Commicarpus podunculosus*	Root + leaf	The fresh root of an A*chyranthes* aspera is chopped and bounded together with a leaf of *Commicarpus podunculosus*. This will be mixed with water and given orally	(28)

Smyth et al. (115) and Wu et al. (116), have issued reports on sample preparation and characterization. However, these reviews primarily focus on a chemosystematics-disposal method: the plant type chosen for testing is known to contain specific derivatives (amino acids, alkaloids and steroids); hence, the best extraction/purification/separation of these compounds is achieved by using the most appropriate extraction methodology and analytical technique (111, 117).

The antimicrobial peptides (AMPs) of plant origin are a source of biocontrol agents against bacteria, fungi, nematodes, insects, and pests (118). They are classified into different groups to include the type of charge, the disulfide bonds present, the cyclic structure, and the mechanism of action. The common types

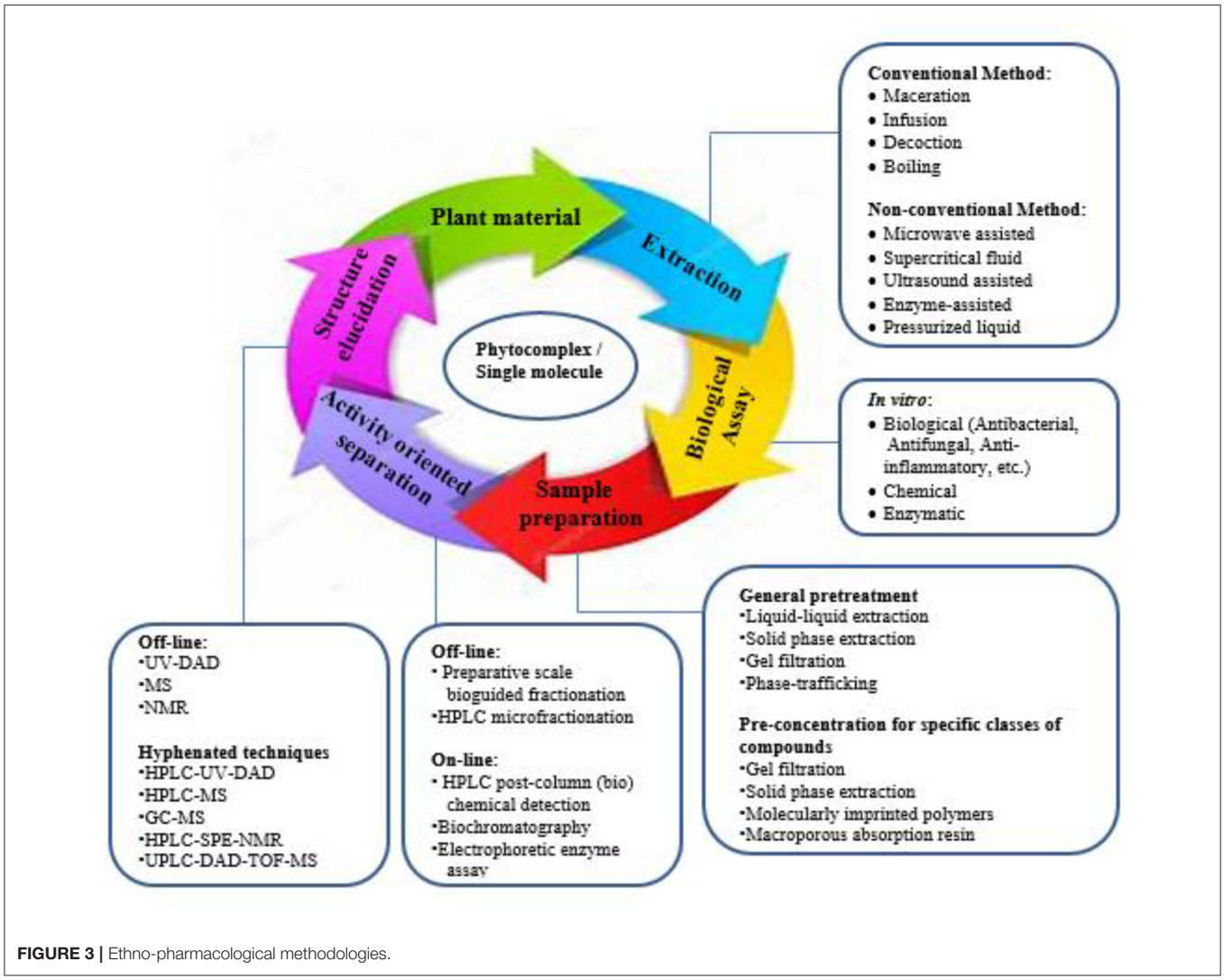

FIGURE 3 | Ethno-pharmacological methodologies.

include cyclotide, defensins, hevein-like proteins, knotin-type proteins, lipid-transfer proteins, protease inhibitors, snakins, and thionins (119). Researchers have reported the isolation and identification of peptides, defensins, protease inhibitors, lectins, thionin-like peptides, and vicilin-like peptides from solanaceae (120, 121). Particularly peptides and peptide-rich extracts of plants have been found to promote antibacterial activity against various strains of bacteria (121). Although their mechanism of action remains unclear, their antibacterial activity could be attributed to changes in membrane permeability (119).

It is clear that peptide contact with the cell membrane induces changes in the structure and aggregation state of the peptides which are adapted by membrane lipids *via* changes in their lipid conformation and packing structure (122). Given that the outer membranes of Gram-negative bacteria or the cell walls of Gram-positive bacteria have negatively-charged surfaces, no fundamental difference was found in the way in which AMPs worked on them. Moreover, the cell wall of Gram-positive bacteria have pores (40 to 80 nm) through which multiple AMPs may readily pass to interact with the target location (123).

Characterization of Secondary Metabolites in Plants

Plants produce an extensive array of chemical molecules including those that are indirectly involved with development and growth and which are termed secondary metabolites (124). The polar difference of the fragment and the solvent utilized determine the extraction procedure for bioactive substances. More so, depending on the qualities of each biomolecule of interest, plant-derived particles from unrefined extracts can be additionally segregated, isolated, and refined using a mix of separation procedures and various approaches (125). Several of such techniques are presented below:

High Performance-Thin Layer Chromatography

HPTLC is a sophisticated form of TLC which yields superior separation efficiency results. For fingerprint analysis, TLC is the most frequent method. Capillary action pulls the

energetic section into the immobilized part. The polarity of each component is used to distinguish samples. The HPTLC characterization is mostly carried out to investigate substances with small to medium polar differences. The approach is generally used in the process upshot, the observation of impurities in the herbal by-products, the identification of the pesticide, as well as its content, and in a standard check of herbal mixtures and wellness foods (126).

Unrefined extracts are used in conjunction with standard molecules, and software is available to assess the quantities of chemicals available in the test material (125).

High Performance Liquid Chromatography

Previously known as high pressure liquid chromatography, this is a more sophisticated method than HPTLC. A liquid sample is injected into a stream of solvent (mobile phase), and passes through a separation medium-filled column (stationary phase). As the sample components pass through the column, a process called differential migration separates them (127). The stationary phase usually involves a column filled with minute porous matter, with the liquid vigorous part being injected *via* the column. Currently, the most effective technology is a merger of LC/MS and HPLC (128).

Ultra-Performance Liquid Chromatography

This is a comparably recent approach in liquid chromatography that opens up new possibilities, particularly in terms of time and solvent consumption. This technique takes total advantage of chromatographic principles to stimulate separations using columns chocked with smaller particles and/or higher flow rates for faster processing with extraordinary resolution and sensitivity (129). The UPLC is based on the premise of using a stationary phase with particles smaller than two (2) μm in diameter (129).

Liquid Chromatography-Mass Spectroscopy

LC–MS is an analytical chemistry approach that merges the actual dissociation potential of liquid separation (or HPLC) with the aggregate scrutiny potentials of mass spectrometry (MS). The advantages of LC-MS are similar to those of GC-MS: excellent selectivity, as well as the capacity to work with complex compounds (125). The ability to multiplex LC-MS tests, allowing for the measurement of numerous medicines and metabolites in a single run, is a beneficial feature of these assays. When compared to GC-MS screening, the use of LC-tandem MS for toxicology screening is appealing since it has the ability to provide more confidence in the identification process and also to identify a wider spectrum of medicines, toxins, and their metabolites, and to simplify sample preparation (130).

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a nuclei (nuclear)-specific spectroscopy with numerous applications in the physical sciences and industry. The inherent angular momentum characteristics of atomic nuclei are investigated using nuclear magnetic resonance, which employs a massive magnet (magnetic). Similar to other spectroscopic techniques, NMR relies on ionizing emission elements (audio waves) to ease transference between degrees of atomic powers (131). NMR is mostly used to check for the structure of fragments. The combination of Mass Spectrometry, NMR and Liquid Chromatography (LC–NMR–MS) provides metabolic organizational information about innovative pharmaceuticals in production, as well as their regular uses (125). The speed and sensitivity of NMR detection have recently been improved, and have been proven to be effective in pharmacokinetics, drug metabolism and development and toxicity research (132).

Gas Chromatography-Mass Spectroscopy

GC-MS is frequently used to test the purity and stability of organic compounds, as well as to distinguish, both qualitatively and quantitatively, between the constituents of a mixture. The preparation of the sample, loading, and dissociation on a GC apparatus are the first steps in a GC-MS experiment. MS is the GC detector and it generates a chromatogram that indicates the amount of each chemical in relation to its retention duration (133). Its application is employed in a variety of spheres inclusive of environmental chemistry for studies on water, soil and the atmosphere, as well as in forensic science for drug detection (or metabolites) well as in forensic science for drug (134).

Infrared Spectroscopy

Infrared spectroscopy (IR spectroscopy or vibrational spectroscopy) studies the interplay between infrared radiation and different materials through absorption, emission, or reflection. It evaluates mixtures in various forms; solid, liquid, or gaseous. A sample's infrared spectrum is recorded by passing an infrared light beam through it. Absorption occurs when the IR frequency is similar to the frequency of vibration of (a) bond(s). The amount of energy absorbed at each frequency can be determined by examining the transmitted light (or wavelength). A monochromator can be used to scan the wavelength range and to make this measurement. Alternatively, a Fourier transform (FTIR) device can be used to measure the complete wavelength range, and then a dedicated process can be used to build a transmittance or absorbance spectrum. Other methods in molecular vibrational spectroscopy include Raman spectroscopy, high resolution electron energy loss spectroscopy (HREELS), and electron energy loss spectroscopy (EELS) (135).

CONCLUSION AND FUTURE PERSPECTIVES

Ethno-veterinary practices have been identified and evaluated as an upshot in lowering the use of antibiotics and other veterinary medicines in dairy farming. The application of people's intellects, skills, techniques, traditions and faith is the domain of ethno-veterinary medicine. Thus, these skills need to be properly documented as they are shared or transferred from age to age only through the oral medium. Given the increasing interest in herbal medicines, sustainability programmes should be put in place to avoid the threats to these plants and their possible extinction. Proper investigations involving *in vivo* studies into the aforementioned are critical to the success of the war being waged against

antimicrobial resistance in the dairy industry. Hence, it is imperative that the ethno-pharmacology of medicinal plants should be exploited to optimize their valuable use as antibacterial, anti-inflammatory and immune-modulatory agents and antioxidants.

AUTHOR CONTRIBUTIONS

CA: conceptualization and resources. DA, BO, TA, JF, KM, and CA: writing–review and editing. DA: writing–original draft preparation. CA, AA, and OF: supervision and editing. All authors contributed to the article and approved the submitted version.

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Control of foot-and-mouth disease in a closed society: A case study of Soviet Estonia

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Foot-and-mouth disease (FMD) is a dangerous infectious disease of even-toed ungulates, however since 1991, the European Union has banned preventive vaccination. During the occupation of the USSR, there were two outbreaks in Estonia: the first started in 1952 (at which time the barns typically housed about 20 cows); and the second began in 1982 (a period when barns typically housed several 100 animals). Neither outbreak was reported to the international community. At that time, it was also forbidden to talk about the disease in the internal media, and speakers could be punished. This study sought to find answers as to how the disease was treated and eliminated in the Estonian SSR, how infected animals and milk were handled, and if some of the methods used can be applied today. Written archival sources and 29 interviews with specialists remembering the outbreaks were used. Preventive slaughter of animals in the USSR was prohibited during the outbreak. As a preventive measure vaccination was used, traveling out of their counties by people were restricted and disinfection mats were used on the roads. In sick animals, udder wounds were treated with various wound ointments, such as zinc ointment, but also ointment made from boiled spruce resin. Birch tar was also recommended in the literature for leg treatments. Mouth wounds were washed with potassium permanganate solution. Workers used rubber gloves when handling sick animals. The barns were disinfected with lime and ash water. The milk from the diseased cows was pasteurized and given to calves, pigs, or diseased animals. Animals that did not recover were transferred to a meat processing plant. The meat was kept in potassium permanganate solution before processing and canned or made into sausages. When the disease was discovered, farm workers were locked in barns and released only when the disease had been eliminated. Such inhumane treatment could only be practiced in a totalitarian society.

KEYWORDS

foot-and-mouth disease, collective farms, veterinary treatment of cattle, distortion of the truth, Soviet Union veterinary system

Introduction

Foot-and-mouth disease (FMD) is today considered to be one of the most problematic animal diseases, because it is very harmful to even-toed ungulate livestock farming. However, FMD has been endemic in Europe for a very long time, as early as the seventeenth century. The disease has become more common since the beginning of the twentieth century because of the widespread emergence of cattle breeding and increased trade in farm animals between regions (1). The biggest outbreak of FMD hit Europe in 1938–1939. The disease was detected at that time in all European countries.

The only country that was not affected by the disease at that time was the Republic of Estonia. This was achieved by imposing strict national preventive measures including import restrictions of animals from infected countries, disinfection of imported goods and hygiene requirements on migrant workers at border crossing points (2, 3). In 1939, the state railway company invested in the new washing unit for wagons, as livestock was transported by railway (4). The Veterinary Service in Estonia was aware of the preparation and use of foot-and-mouth disease vaccine used in Denmark and Sweden in the late 1930's (5). The first case of FMD was discovered in Estonia during World War II (6).

In early 1950's FMD was widespread in the Soviet Union (USSR), including in Estonia. Vaccination campaigns were conducted in parts of the country where the disease was spreading. In Estonia the disease was spreading mainly in the central and southern parts during these years (7). In the summer of 1956, a limited outbreak in smallholdings close to Tallinn was detected and successfully eradicated by killing susceptible animals in neighboring villages. This killing strategy was used for the first time here in the whole of the Soviet Union. Inspectors from Moscow were sent to check the local authorities and the farms to make sure it was implemented (8). In the 1960's, when there was a major FMD epidemic in other parts of the USSR, Estonia remained unaffected (9). The next time FMD was discovered in Estonia was 1982, when the epidemic that started in East-Germany reached Belarus and the Baltic Republics of the Soviet Union (10).

Sørensen et al. (11) proposed that in 1982 there was long-distance transmission of airborne virus FMD over the sea between Denmark and the former East-German, and that it was more likely transmission took place over water than over land due to the reduced surface turbulence over water (11). To prevent the disease, the first vaccinations were introduced in Europe as early as the 1920's (12). The European Union (EU) ceased preventive vaccination in 1991 after successful eradication of the disease in the member states. However, there is an ongoing debate if the vaccination should be more widely used, due to the animal welfare, economical, ethical and environmental consequences accompanying the killing strategy of FMD control (13, 14). Nevertheless, instead of vaccination, the main effort in disease prevention in the EU is based on bio-security measures and adapting animal husbandry to minimize the risk of spreading of infectious diseases (15, 16). Informing general public, involving people and the rapid availability of adequate information are also important during communicable disease outbreaks. In the case of a disease outbreak, it is very important to know where and how the infection was introduced to the farm. Thus, openness and transparency are very important in disease control (17).

Since the formation of the USSR in the 1920's, it began to shut itself down and create a "parallel society." One example of concealing the real situation was a secret order from 1976 which described a "list of information prohibited from publication in the open press, radio and television broadcasts" (18). Among other forbidden topics was: massive diseases of farm animals with botulism, brucellosis, anthrax, plague, and foot and mouth disease. Thus, the correct data on the actual extent of infectious diseases were not published domestically nor was information provided abroad (19). Compared to a market economy, the USSR was built on a planned economy and the top-down plans were strictly enforced. Both meat and milk production had to follow a state-set plan. Because, in case of FMD, animal performance is reduced, this also put pressure on the food industry to make use of sick animals' meat and milk. Dairy cattle farming has been historically one of the most successful sectors of agriculture in Estonia for more than a century (20). In the wake of FMD disease in cattle that hit Europe after World War II, comprehensive texts on the outbreaks can be found only from the Western Europe, such as the report on the 1982 outbreak in Denmark (21). Nevertheless, there are quite a few reviews and analyses of the outbreaks in the USSR in the scientific literature. At that time, only general teaching about the disease and its control was conducted in the Estonia SSR (22, 23), but the actual extent of the disease was not reflected in the media. It has also not been studied if ethnoveterinary medicine was practiced in cattle farms in the Estonian SSR. It has been studied from the 1940's and for recent decades (24) but not in the soviet era.

As there are limited studies (7, 25) covering control of enzootic cattle diseases in Soviet Estonia, this study takes the deeper look at the spread of FMD and its control in a closed political system. In this paper, authors have tried to answer the following questions: (a) how and by what means the control of FMD in cattle took place in a closed society; (b) whether the methods by which the disease was brought under control are still relevant today; (c) whether, and to what extent, public information was provided and whether this lack of information led to alternative methods of treatment of FMD; (d) how the meat and milk of FMD affected cattle were used in the situation of general food shortages in the USSR.

FIGURE 1
The map of Baltic Sea countries and Belarus.

Materials and methods

Description of the study area

Estonia is a country by the Baltic Sea (Figure 1). It borders Russia to the east and Latvia to the south. In 1710–1917, Estonia was part of the Russian Empire. From 1918 to 1940 the Republic of Estonia was independent (for a short period, in 1941–1944 it was under German occupation), and from 1940 to 1991 Estonia was annexed by the USSR. A public democracy movement peaked with the Singing Revolution in 1988, and in 1991 Estonia regained its independence (26).

Estonia is located in a geographical area suitable for milk production and this became an important industry in the twentieth century. The number of cattle and milk production increased from the end of the Second World War (Figure 1). Considering the conditions at the time, reliable milk production statistics for the 1940's and 1950's do not exist. It was quite common that the work of inspectors revealed shortcomings in both livestock and dairy production records. For example, in May 1952, it was established that the reporting of livestock in the collective farms of the Estonian SSR was true in 74% of the collective farms for cattle, 80% for pigs and 91% for sheep. In addition, there were collective farms that did not keep records of the animals at all (27). Until 1940, considerable amounts of meat and dairy products were exported to Western Europe, but later these were sent to the USSR's domestic market (28). With the collapse of the USSR, this market also disappeared and the number of cows decreased significantly, although milk production per cow increased (Figure 2).

As mentioned above, Estonia was the only country in Europe that did not have FMD in 1938/39. Later, two outbreaks of FMD spread in Estonia: in the summer of 1952 (which lasted for several years) and in the autumn of 1982 and winter of 1983, although the Estonian SSR had been officially declared free of FMD since 1963 (29). In this connection, preventive vaccination against FMD was discontinued in the Baltic States in the early 1970's (30).

At the time of the 1952 outbreak, most herds were small, up to 20 animals. However, by 1982, herds of several 100 had already emerged. Thus, in 1952 the disease incidences were relatively small, but during the 1982 outbreak, the disease spread mainly only in Southern and Central Estonia, but it affected a higher number of animals. According to Peterson (7) there were 29,081 cattle infected by FMD during outbreaks in 1951–1953.

Censorship

In order to understand the general atmosphere in the USSR in the early 1980's, a few aspects should be emphasized. The period from 1964 to 1987 was called a period of stagnation in the Soviet Union, which started with the coming to power of Leonid Brezhnev in 1964 and ended with the XXVII Congress of the Communist Party in February 1986. That period could be defined as an unprofitable economy managed by the administrative-command system, a sloppy attitude toward state property and a lack of motivation of employees. Aggressive russification in the form of language policy, mass immigration of migrant factory workers to the republics (such as the Baltic States) and extensive censorship. The last meant that the Communist Party controlled all sorts of media including television, radio broadcasting, and newspaper and books. Soviet censorship combined pre- and post-censorship into one, resulting in ubiquitous, all-seeing and controlling permanent censorship (31). The media was selective in terms of content of good and bad news, meaning that the content of the newspapers should present the Soviet citizen in a good way, highlighting his/her achievements to support the goals of the party. News about accidents, disasters, criminal behaviors and crop failures in agriculture in the USSR, had to be ignored or covered only briefly and superficially in the media. It should also be borne in mind that the KGB (Committee for State Security; in Russian *Komitet Gosudarstvennoy Bezopasnosti*) intercepted absolutely all telephones (32), including those of veterinary officials. Therefore, the security service had probably an overview of FMD in this way.

In addition to the media, tacit censorship also applied to professional communication. This is another example of how censorship had to be comprehensive in Soviet society. In a situation where the authorities sought to nurture the Soviet people with common loyal behavior and worldview, in addition to the abundance of pro-regime propaganda and the ideologization of almost every sphere of life, including agriculture, an attempt was made to establish total control over all self-expression (33).

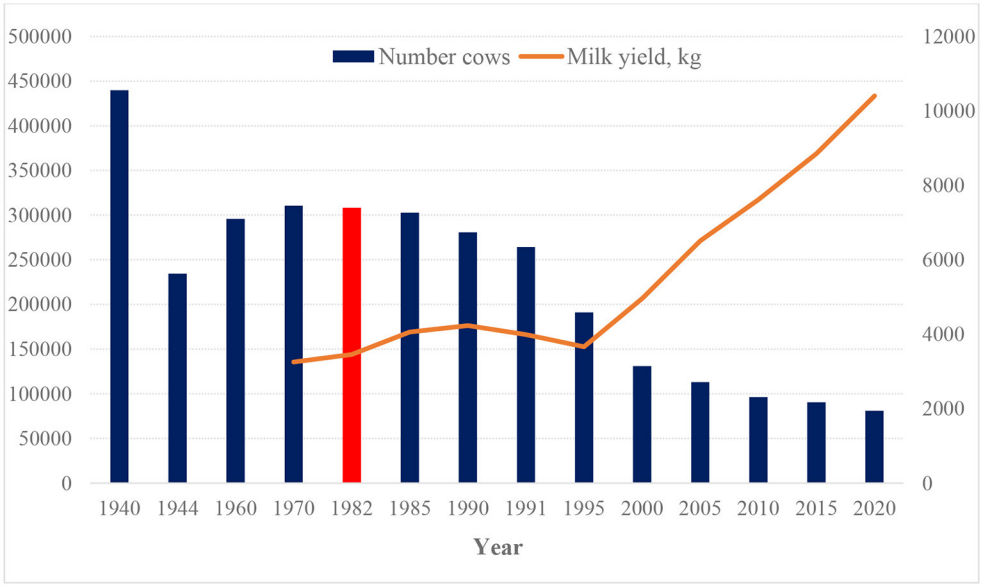

FIGURE 2
Number of cows and mean milk yield per cow between 1940 and 2020. There are no official data about milk production available from 1944 to 1960. Data from: www.epj.ee.

Data collection

Information on the control and spread of FMD in Estonia during the Soviet era was collected from the electronic databases of libraries (e.g., https://www.etera.ee/, https://dea.digar.ee/), from the National Archives (https://ais.ra.ee/), the National Broadcasting Archive (https://arhiiv.err.ee/) and a database of Estonian language articles (https://artiklid.elnet.ee/). Electronic searches of newspaper, magazine websites, research databases (e.g., https://scholar.google.com/, https://books.google.com/, https://www.biodiversitylibrary.org/) were also conducted using keywords related to FMD and the Soviet Union. There is a lack of information about FMD outbreaks in neighboring soviet republics or in other parts of the USSR during the studied period. In those few cases the papers are either published by foreign researchers (34) or published in Russian (35) limiting wider conclusions.

The prevention and action of during the livestock disease outbreaks in USSR was established by the Veterinary Act, which was amended over the years. According to the veterinary legislation of the USSR, in 1968, the FMD was mentioned in the list of contagious diseases, in the occurrence of which a threatened zone—quarantine around the sick object (territory) was established. Also, following the developed instructions, animals affected by the foot-and-mouth disease were to be destroyed (without using meat, skins and other slaughter waste) (36). In 1965 (changed in 1971), the instruction on measures to prevent and eradicate the FMD was adopted. This document described the protocol for the essential measures to eradicate FMD in a diseased area, measures in an epizootic area, as well as measures to control FMD during the transportation of animals. The instructions also described measures for removing quarantine and subsequent temporary restrictions. The document also draws attention to efforts to protect free farms from the introduction of FMD. In 1968, guideline on how to collect, preserve, and ship FMD virus to determine its type was developed. In 1969 (changed in 1970), a temporary guideline for using a special foot-and-mouth disease vaccine was developed (36).

In 1974, the current Soviet veterinary legislation was amended to require dairies to clean and pasteurize milk from FMD-threatened farms. Finally, in 1974, the Chief Veterinary Administration of the USSR Ministry of Agriculture adopted regulations on vaccination to prevent foot-and-mouth disease (37).

The digital archive of the Soviet central newspaper Pravda (https://www.eastview.com/resources/gpa/pravda/) was searched in order to find public information. The analysis focused on one of the most influential of the print media—the broadsheet newspaper "Pravda" (in English *truth*), the official newspaper of the Communist Party of the Soviet Union from 1918 to 1991. This newspaper was chosen because of its relatively wide circulation (11 million copies) and the regularity and duration of its publications in the Russian language. Selected narratives exploited by Pravda in portraying some aspects of FMD were traced. Rough content analysis was performed on the

newspaper, searching for texts dealing with the chosen theme. A total of 34 articles were selected. The small number of articles from one of the influential newspapers is clear. Nevertheless, we consider this a result in itself, an illustration of how little attention the Soviet media paid to FMD disease issues.

As there were very few written and archival sources, and the written sources of the USSR were often used in order to distort the truth, oral history was added as an important source. People who worked in kolkhozs and sovkhozs were interviewed (Supplementary material). The sovkhozs were under the state control, while the kolkhozs were a collective farm owned by the local people. In total, in February-April 2021, 26 people (17 males and nine females) were interviewed. The oldest respondent was born in 1928 and the youngest in 1964, mean age of male respondents was 71.2 and for females 70.9 years. The largest interviewed group based on (higher) education were veterinarians ($n = 15$) and zootechnicians/stockpersons ($n = 9$). One person was an agronomist and one was a biologist. Eight respondents were working as practicing veterinarians, and there was one farm manager, and one as stockperson at the farm. Six were university teachers (veterinary medicine or animal science), three researchers, three civil servants (state veterinary service), three (veterinary medicine) students, and one as zoo keeper during the outbreak of FMD in 1982. Most of the interviewees were retired or were working part time. The surveys were conducted in a semi-structured manner, based on a survey design. As the disease spread in Southern Estonia in 1982, most of the interviewers came from this region. In addition, four respondents had memories about the outbreak of the disease in the years 1952–1953. Respondents were informed that their data would be used for a scientific publication and that the data would be presented anonymously.

Results

General narrative of FMD in the Soviet Union: based on a survey of the newspaper "Pravda"

The first mention of FMD in Pravda was in 1924. Correspondents reported that FMD had been detected in the suburbs of Leningrad (currently Saint Petersburg). This led to the issue of a decree on the control against FMD, and preventive sanitary supervision over milk brought to Leningrad was strengthened (38). Furthermore, in 1925, FMD was involved in an international incident between Finland and USSR (39). In response to outbreaks of the disease in the Soviet Union, Finland closed the border to the movement of grain from the USSR to prevent the spread of FMD, thus stopping the export of bread. The article noted that the Soviets expressed indignation and checked the present state in the areas bordering with Finland. As a result, they put forward indisputable evidence of the absence of an FMD epidemic on the territory of USSR. They also emphasized the high level of development of veterinary medicine and guarantees for the non-spread of the disease. At the same time, continuing to deny the FMD epidemic on the territory of the Soviet Union, in 1926, Pravda urged the owners of sick animals to report the disease to the nearest doctor (veterinarian) immediately and not to consume raw milk or dairy products from diseased animals (40).

In the 1930's, the rhetoric of FMD narratives changed slightly. Correspondents noted specific outbreaks of the disease in different parts of USSR. Nevertheless, they assured their readers the disease passes quickly and without mortality (41, 42). But at the end of the 1930's and the beginning of the 1940's, the focus of attention of Pravda shifted to the countries of Western Europe. FMD was mentioned exclusively in world news chronicles (43–57). In the narratives, FMD was described as "a rampant disease that afflicts Western capitalist countries" with enormous negative consequences for their economies (44).

In the post-WWII period, Soviet correspondents referred to FMD as a bacteriological weapon used in the fight against USSR (58). FMD continued to feature only in the news section of the international press as an example of a disease spreading beyond the borders of the Soviet Union (59–67).

As for the territory of the Soviet Union, FMD was mentioned in the context of mandatory vaccination against the disease to prevent outbreaks and enormous economic damage (68). Also, Soviet correspondents noted the growth of international veterinary cooperation in the 1980's. So, for example, in 1983, within the framework of the Soviet-Afghan agreements, thanks to Soviet assistance in the creation of veterinary clinics and laboratories, Afghanistan was able to practically eliminate FMD on its territory (69).

In general, FMD was mostly mentioned in the pages of Pravda in the context of international news. No mention was found of FMD in the peak years of its spread in the Soviet Republic of Estonia.

Reflection of the disease that began in 1952 in the Estonian media literature and interviews

The first report of the disease did not appear in a national newspaper until July 1st 1952 (70). This was published by the young veterinarian Heino Mikk. He was one of the best-known speakers, and he published several articles later on this topic (71–73). It was forbidden to write in newspapers that the disease was spreading in Estonia, only how to recognize the disease and what control methods must be applied. It remained the only nationwide public announcement. Recommendations were also shared later in dozens of agricultural publications, so-called propaganda newspapers (74–76). Although thousands of

copies of these newspapers were published, they had virtually no readership at all, people were not interested in empty, large-typed propaganda, and therefore these newspapers were not bought or subscribed to (77).

The first documentary in which the real extent of the disease that struck Estonia in 1952 was discussed, was not broadcast on television until March 7th, 1988 (77). This was made possible by the innovation (*perestroika*) and disclosure (*glasnost*) reforms initiated by Mikhail Gorbachev (became the leader of the USSR in 1985). In the TV programme, veterinarian Heino Mikk (1924–2001) talked about his experiences in controlling the disease in 1952. He had just graduated from university as a veterinarian and had little experience at that time. He later became the chief veterinarian of the entire Estonian SSR in the field of disease control. He reported that the first outbreaks of the disease appeared in Estonia in the winter (January-February) of 1952. There were very few veterinarians at that time and they had no knowledge of the disease or its control. Although Mikk claimed that veterinarians did not have previously experienced with the disease, it can be assumed that they could have studied it at university. It was also forbidden for people involved in disease control to talk about it in the media. These were possibly the reasons why the disease began to spread widely; the damage caused by the disease at that time was colossal. The measures initially proposed were disproportionately stringent: for example, all movement in rural areas was banned, even on major roads. Farm personnel had to stay in the barns for a month, all gatherings and entertainment events were prohibited for more than half a year. However, the killing of infected animals was considered unthinkable.

Jaan Roos (1888–1965), an internally displaced person (protester against the Soviet regime), also described the real situation at that time in his diary (78). His diary of these years (1951–1952) was published only in 2004. By March 1952, the FMD situation in some regions of Southern Estonia had become very serious. In the quarantine area, the roads were closed and guarded, and any movement inside the area was punishable with 2 years in prison. However, people still moved in some places along secret paths during the quarantine. Even in areas where there was no disease, collective farms workers were forbidden to communicate with strangers. There were rumors that the disease had been brought to Estonia during the winter from Lithuania with sugar beet (78). The disease was reported to have been contracted by 14 people (77, 79).

Mikk and Endel Aaver (1927–2009), a senior researcher (virologist) at the Estonian Institute of Animal Husbandry and Veterinary Research, and a former adviser to the Ministry of Agriculture, Ants Laansalu (1938–2011) recalled that the disease which began in 1952 was very serious (77). However, more animals died from massive feed shortages than from the disease. In 1952, the number of cows decreased by 6%, of which about half died directly from the disease. There was practically no feed for cattle in the kolkhozes during the spring-winter period. Weakened cows were lifted up by rope every morning. Some animals stayed on the ropes for the whole day because they could not stand on their own.

Only four persons interviewed had personal memories of the outbreak that occurred in the early 1950's. Two of them had been involved as veterinarians in the 1950's. Now 82 years old a zootechnician (who worked in the 1950's) recalled that people wondered at the time how the disease suddenly came to Estonia and why. There had never been such a disease in Estonia before according their knowledge. Since most of the respondents had directly or indirectly experienced the outbreak, in general, their recollections were similar in several respects, such as the procedure for quarantine, farm lockdowns, disinfection mats on roads, militia guards at farm gates and so on. A zootechnician (aged 82) recalled the control of the disease as follows: "When I studied to be a zootechnic at vocational school in the 1950's, all the students were taken to the dairy farms. There we had to wash the mouths of the animals with a solution of potassium permanganate. The animals were kept for as little time as possible in the barns during the summer. The reason was because the cows' hooves become more damaged inside in the wet manure. The barns were disinfected with lime and ash water. Ash was gathered from the people by the village."

Estonian media coverage of the 1982/1983 disease and written sources

There are no reports or news stories in the newspapers about the FMD outbreak in 1982/1983. The first announcement about the FMD appeared in a national newspaper in November (80) and December (81). Again, it was not written that there was any disease in Estonia at all, but information was given as to how to recognize and control the disease. People were warned that the disease could also be transmitted to humans, yet it was said that processed meat (canned and sausages) from sick animals could be eaten and pasteurized milk could be drunk.

Due to the FMD epidemic in England in 2001, the topic of FMD became very popular in the media. In 2001, detailed recollections of the 1982 outbreak also appeared in the press. In one of them, a worker of the Vändra Kolkhoz recalled his experiences and comments were taken by Endel Aaver and an interview with Chief Inspector Mati Loit at the Veterinary Government of the Estonian SSR (79, 82). The topic was also covered a few years later where the manager of the cattle unit of the Laatre Sovkhoz (83) recalled his experiences. The harsh control techniques in the UK were similar to those in Estonia in 1982. People were locked in barns such in Laatre dairy farm (South Estonia), nearly 30 workers were enclosed for 42 days. Also, in Vändra cattle farm 10 workers were isolated for more than 30 days. Employees were not told they had to stay in the barn for weeks so at first, they did not take their personal

belongings with them. The gate doors of the cattle farms were guarded by militia 24-h a day. There were also strict rules inside the farm, men and women slept in separate rooms and drinking alcohol was forbidden. Also, vaccination of the animals started immediately. The milk from diseased cows was first added to dung for disposal, later it was pasteurized on site and fed to calves and vaccinated cows (in some cases to pigs). Hundreds of sick animals were moved to a meat processing plant after the end of the quarantine period. The abattoir was then subsequently disinfected. Everything had to be kept secret. For example, a veterinarian who accidentally mentioned the disease on the radio was punished (79, 82, 83). Rumors spread that it was an experiment and that the animals were intentionally infected. The reasons for the rumors were: the disease spread among the most advanced and hygienic farms (disinfection mats at the farm entrance, warning signs at the gates), previous secret searches on the farm, unknown people with large sums of money. The manager of the cattle unit of the Laatre Sovkhoz remembered that the situation on the disease had to be reported every day to unknown anonymous Russian-speaking officials. Disease control specialists were also brought to Estonia from other parts of USSR (83).

Aaver and Mati Loit said that in 1982 a total of eight outbreaks occurred in Estonia. According to Aaver, the FMD arrived in Estonia from Belarus through Lithuania and Latvia and reached the sea in the Western part of Estonia. However, there is not much information about the outbreaks in Lithuanian and Latvian SSR in 1982 (84), but in years 1986 and 1987 (85). In addition, Aaver pointed out that more than 30 people from Moscow, who had seen FMD in USSR almost every year, came to aid Estonian specialists at that time. Analysing the wind directions at that time, it has been concluded by researchers that the disease could be spread by the wind. It was found that when the direction of the prevailing wind changed, the infection also decreased (79, 82, 83).

Foreign media coverage of the disease that hit Estonia SSR

The 1982 outbreak reached the foreign media very quickly. During World War II, almost 80,000 Estonians fled abroad for fear of repression by the Soviet authorities. The largest diaspora was formed in Sweden. Estonians abroad maintained active communication with relatives who remained in their homeland. Thus, the first warnings of the disease in November in the national newspaper of the Estonian SSR reached numerous foreign Estonian newspapers in December 4: in Sweden (86), Canada (87), the USA (88), and Australia (89). However, in addition to official sources, foreign Estonian newspapers also relied on direct sources, which were letters from Estonia. Thus, the actual extent of the disease and the control measures were known abroad quite quickly and in detail. The disease spread from Russia to Lithuania and Latvia in the spring, and from there to Estonia in the autumn (90). The letters from Estonia SSR, also show that there was a great shortage of food in Estonia and ideological pressure was increasing (88). It is noted that, due to FMD, foodstuffs were no longer transported (exported) from Estonia SSR to the domestic market of the USSR and therefore more goods were available in the shops in Estonia (91). The few tourists that visited the Estonian SSR also reported on the control and spread of the disease (91). It was repeatedly emphasized that the Soviet Union denied any outbreak of the disease. However, Sweden suspended imports of reindeer, elk and bear meat from the USSR (92) and Finland suspended the ferry service (91). However, the foreign press did not know the whole truth at the beginning. It was initially thought that infected animals would be killed in Estonia and then burned or buried. However, this was not done.

As early as March 1983, a report appeared in US infectious disease reports mentioning an outbreak in the Baltic Soviet republics. However, there was no overview of the actual situation as reported. "However, detailed information is not forthcoming from this region" (84) but the June issue states that "The USSR has denied any outbreaks occurring in its Baltic regions / — /" (19).

Analysis of interviews on the 1982/1983 outbreak

The problem of secrecy and how the information was exchanged and rumors

Two respondents (a zootechnian aged 69 and a veterinarian aged 71) who worked at the country's first large farm in South Estonia highlighted that the dairy farm had been closed for 43 days, from November to the end of December, including Christmas. One of them believes that the disease was brought to the farm intentionally, in other words for training purposes. Respondents added that the disease was noticed when two cows began to exhibit the symptoms of the disease: bite their mouths, producing foam in mouth and intensive saliva flow.

One reason for intentional outbreak was that the farm was the production farm of the state animal science research institute, which had modern furnishings and living rooms for the employees (sauna, kitchen, rest rooms), which were not available on other collective farms. This played an important role in combatting FMD on the farm, and about 14–16 people were trapped in the barn. In addition to treating and feeding the animals, staff and clothing and other supplies had to be disinfected on a regular basis. The workload was several times higher than usual because the staff could not leave the farm. After the closure of the farm, beds and mattresses were brought to the staff. Food was provided on a daily basis, and one milking lady began to work as a chef. At that time there was a very strict order on the territory of the farm, there was no access for strangers either. There was a 24-h militia guard at the gate. Those

trapped on the farm did not see their family members, children or other relatives. This was very difficult psychologically for all the staff in addition to the physical load.

Another reason of intentional outbreak given by respondents was that somebody called every day at 9 o'clock and asked in Russian about the number of animals that were sick and if there were any dead animals. This raised doubts as they were not told who it was who called every morning and asked for these details. It is important to highlight that it was very common in the Soviet Union to conduct training exercises to practice actions for major accidents, including outbreaks of animal diseases, as part of civil protection exercises. Although there are several indications in the respondents' answers that the animals were intentionally infected with FMD, it should not be forgotten that the disease had been detected in the neighboring republic a few months earlier. In the north of Latvia SSR; Tallinn Zoo was closed in 1982 for about 6 months. As the zoo was visited by a significant number of Latvian visitors each year, the zoo had to be closed to avoid FMD. One of the interviewees mentioned that Tallinn Zoo had one of the most representative collections of Bovines and mountain goats in the world. The 1982 FMD outbreak also meant for the zoo that animal feed (such as hay) had to be purchased from Western Estonia instead of the usual southern regions, as there was no imminent threat of FMD from the west.

The effect of the restrictions on daily life of the farm employees

Another major issue that emerges from the survey is the various restrictions, such as road closures and the restrictions on people in the farm and the resulting trauma. One of the respondents (agronomist, aged 76) "people had fear because everyone had animals in their household." A former veterinarian (aged 84) reported: "People knew about the outbreak in southern Estonia. People were very afraid. The authorities also vaccinated one-cow farmers (the authors—cows were kept in virtually every household in rural areas)." A zootechnician (aged 74) narrated: "Cabbages and turnips remained in the field in the affected area because movement in that area was prohibited." A former farm manager (aged 69), remembered: "14–16 people were locked in on the farm. There was a lot of work. There were militia at the gate. The children could only be seen through the fence, as many as the spouse could hold." Several respondents also stated: "The restrictions were very strict at the time. In some places, roads were even dug up to distract traffic." "At the border of the districts there were disinfection mats, the driver had to come out of the cab, and walk across the mats. The shoes smelled afterwards. There was constant guarding of those mats. Almost half a year, but it varied by region; in general, restrictions lasted almost half a year" (agronomist, aged 76). A veterinarian (aged 66) stated that "veterinary students were taught at the university model-based disease control: blocking of roads, bridges and farms during potential animal disease outbreaks. Control instructions were described in detail. Informative posters near farms were common. Barriers at farms and on roads too."

It was also mentioned on a few occasions that the manure of the sick cows had to be stored separately, which could not be used as organic fertilizer for years. In some places, dead calves were also burned on bonfires near the cattle unit.

Treatment of sick animals with symptoms or preventive measures

When the udders had sores, the milkers protected their hands with rubber gloves while milking. Udder wounds were smeared with zinc ointment, but also udders smeared with ointments. Outside the kolkhoz, people were only allowed to have one cow at home, so the animals were kept in barns at home very carefully. During the period of FMD the milker was detained on the farm, and food was brought into the barn. Some cleaned the barns during the outbreak with juniper smoke and the Russian Orthodox believers near Lake Peipus also brought water consecrated from the church and watered the animals with this. Folk medicine was no longer used in the kolkhozes, but there are some indications of this being used by single cow owners. A former veterinary scientist (aged 74) said: "The cow was still being treated by a veterinarian, folk medicine was not used at the time." FMD vaccine was thought to have been available in the early 1980's as the disease spread across the Soviet Union. This was confirmed by several respondents, one (zootechnician, aged 69) stated that: "Vaccination started immediately in cases of the disease. No animals were killed as a result." This involved a great deal of effort. The cows that were caught were heavily blistered and could not eat normally and it was necessary to prepare a mixture of boiled milk (which could not be sold for human consumption) and grain meal, as it was not possible to feed hay because of the sharp stems irritating the blistered mouths of the cow. A zootechnician (aged 69): "It was painful for the cow to eat hay. They ate a little silage as it was softer." This meant that sick animals had to be fed several times a day to avoid loss of body condition. A veterinarian (aged 71) added: "The cows' mouths were rinsed with a copper preparation. Blisters and sores were also present on the hooves, which were smeared with ointments." It was also mentioned on several occasions that, because the animal was in pain, it led to thinness. It also meant a significant decline in milk yield, even after the cows had recovered. A zootechnician (aged 69) said: "The cows' milk yields did not recover after the end of the quarantine. However, the animals themselves recovered, their body weight returned to normal and the cows were alive." Interviews revealed that one large farm in South Estonia was able to prevent deaths due to huge efforts by the farm personnel, but another farm in the same region had more serious cases, resulting even in dead animals.

One veterinarian described the activities during high risk of FMD at a dairy farm in Central Estonia. The story of one

veterinarian (aged 85) was as follows: One day in year 1982, at 4 pm, the head of the Tartu District Veterinary Service convened the chief veterinarians of all the farms in Tartu district and distributed vaccines. By the evening of the same day at 11.30 pm, more than 3,000 cattle from our state farm, including 980 cows, had been vaccinated. Everything happened at lightning speed. This prevented the greatest danger. Tartu district is one of the few where there was no foot-and-mouth disease. In any case, the Chief Veterinary Officer of Tartu District later received a letter of honor and/or a letter of thanks from the Veterinary Government of the Ministry of Agriculture. The veterinarian added: "We were given information about foot-and-mouth disease at the monthly meetings of the chief veterinarians, and this was discussed at several meetings. As mentioned above, there were 3,000 cattle in the state farm, and about 12,000 piglets were produced each year. I had a total of 110 livestock workers, including three veterinarians and one assistant veterinarian. Stockpersons, together with the respective regional department heads, made every effort to prevent the spread of the disease. There were disinfection mats in front of both the large farm and each of the smaller barns, as well as a very large and long disinfection bath, where the long semi-trailers bringing concentrate feed could completely wet the wheel tires with disinfectant. These lorries were also disinfected by a trained specialist. A separate topic was the protective clothing of stockpersons and others, to which we paid more attention than before. The fact that many workers had cattle and pigs at home was alarming. The infection could potentially have started there as well. The FMD of 1982 passed our state farm, but the older milking ladies and workers remembered the beginning of the collective farm foot-and-mouth disease in 1952, when the children could not see their parents and the livestock workers had to live and sleep in the stable for weeks."

Use of milk from sick animals or meat from recovered animals

A zootechnician (aged 69) mentioned that "many animals suffered but recovered, while milk yields declined remarkably." The milk was not allowed to be transported out of the farm, it had to be fed either to youngstock or to cows. For this reason, a boiler was brought to the farm to pasteurize the milk. However, the affected animals sooner or later had to be culled. The interviewee added: A total of 92 animals were taken to the slaughterhouse, but in fact worse and thinner cows were sent there at first. However, a year after the disease outbreak, a large number of the animals had to be taken to a meat processing plant, a total of 1,400 animals. This was required by the veterinary regulations. The meat of the affected animals was made into canned food, i.e., the meat had to be heat-treated. And a stockperson (aged 82) remembered: "Animals who were weakened by the disease were transferred to a meat processing plant. The meat was kept there for a day in a bath with a solution of potassium permanganate before it was processed."

The outbreak of the disease meant that foodstuffs of animal origin could no longer be placed on the domestic market of the USSR and more of these goods were sold in the shops of the Estonian SSR. For example, one respondent (researcher, aged 83) mentioned: "Foot-and-mouth disease closed the borders of Soviet Estonia. The butter came back for sale in food stores because it was no longer allowed to send parcels with food products to other Soviet republics. At that time, it was common that people of Russian speaking nationalities (e.g., workers at large factories) sent a monthly package of food products to their relatives to other republics."

Discussion

Even the most prestigious Soviet magazine "Socialist Agriculture" (in Estonian *Sotsialistlik Põllumajandus*) did not address the topic of FMD, only good news topics were written about the exceeding of 5-year plans, increasing production in kolkhozes, effective application of research results in practice etc. However, it is known that due to the well-established system of the veterinary service, the information flowed quickly and the veterinarians of the whole country were aware of the seriousness of the situation. However, as can be seen from the respondents' answers, the population was also aware of the situation, although FMD was not covered in the newspapers or on television (all controlled by the state). The severity of the situation was also signaled by roadblocks at the borders of the district, disinfection baths and even militia guards to make sure that all vehicles and passengers were disinfected to prevent the spread of the disease. However, as mentioned by the interviewees on several occasions, there was minimal public talk about the disease, the epidemics were strictly regulated and there was no need to speak on the subject. Disease control was strictly regulated by the state, with its own chain of command and action plans.

The first teachings on the control of FMD to the general public, especially farm workers, were published in 1952. Thus, at the end of 1952, veterinarian Šolom Špungen (1908–1964) published a 32-page brochure describing the disease and control techniques. It had a print run of 5,000 copies (23). He also edited an informative color poster (size of 58 × 87.5 cm) and had a circulation of 3,000 copies. It was freely distributed (93).

We also looked at whether there was any attempt to replace the shortage of synthetic medicament in the USSR. Alternative remedies were recommended to veterinarians in the 1980's. For example, veterinarian-pharmacologist Richard Lumi (1905–2000) advised veterinarians that for animals with FMD, the claw guards and the interstitial skin of the claws should be lubricated with birch tar (94, 95). At that time, birch tar was also widely recommended for the treatment of human skin diseases (96). Lumi also recommended disinfecting barns with Sodium hydroxide, formaldehyde solution or chlorinated lime solution during a disease outbreak (94, 95).

Those respondents who remembered the outbreak of FMD in the early 1950's in the Estonian SSR said that animals were generally suffered little with the virus. Techniques were even used in which healthy animals were infected with saliva from a diseased animal. Similar treatment has been practiced by Fulani herdsmen in Northern Nigeria, who sometimes move their cows upwind of infected animals to prevent the FMD from spreading, and sometimes they move them downwind to expose the cattle to the disease, knowing that a mild case of the FMD will not be fatal and will confer immunity (97).

It seems that results of collectivisation in the 1940's also meant that folk medicine began to disappear from local livestock production. For example, plants such as *Crataegus oxyacantha* L., *Inula helenium* L., which had been used to treat cow's foot diseases in previous times (24), had already been replaced by ointments from veterinary pharmacies by the early 1950's. However, scientific experiments with folk medicine have been successfully conducted in India and Kenya. The treatment of FMD wounds with honey ointment is particularly promising (98, 99). Herbal treatment of FMD in domestic animals is a common practice in Africa, where the disease is more prevalent today. In Kenya, for example, *Ricinus communis* L. leaf is chopped and mixed with water, then used for topical application (100); *Allium cepa* L. bulb are chopped and given with salt for 2 days, for ruminants by mouth (101); *Stephania abyssinica* (Quart.-Dill. and A.Rich.) Walp. whole part plant is pounded, water is added, then drenced (102).

Another aspect is the fact that the Soviet authorities used agriculture as part of the propaganda. In the years 1949–1950, the elimination of family farms and farmer unions took place, and rural people were forced to participate in the formation of collective farms. However, in the late 1940's and 1950's, agriculture was characterized by low productivity as the state farms were plagued by a chronic shortage of labor and agricultural machinery (103). As the Soviet authorities used all means and resources to legitimize their power and to prove the uniqueness of Soviet agriculture, agricultural exhibitions gained unprecedented ideological significance in Soviet society (104). Due to the widespread outbreak of foot-and-mouth disease and poor weather conditions in 1952 (7, 70), agricultural exhibitions were banned in half of the districts in republic, but there was not the slightest indication in the newspapers about any decline in production in collective farms (104). Another reason for the abandonment of agricultural exhibitions was the massive mortality of livestock and the foreseeable consequence of the spread of animal diseases in such conditions. Between 1948 and 1950, eight to nine piglets died in the state farms of the Estonian SSR. And it was not surprising that illegal deaths of cattle often occurred in this situation (103).

As the USSR dealt with control centrally during the FMD outbreak of 1982, the Estonian archives today do not contain any reports or reports on the actual situation since then. They may be in Russian archives. However, for the 1952 disease outbreak, the Estonian archives contain many regional reports. Why was it necessary to address the 1982 outbreak at the highest level? The USSR delegation reported at the FAO 25th Foot-and-Mouth Disease Session in Rome, Italy, on April 12–15, 1983 that:

> *In the USSR, there have been rumors of FMD outbreaks in the Baltic States which have been thought to be free of FMD. The USSR Veterinary Authorities informed the Secretariat that an extensive prophylactic vaccination programme had been carried out in November 1982 and that no cases of FMD had occurred in this area. Five million cattle and four million sheep have been vaccinated with OAC vaccine in the Baltic provinces* [(105), p. 16].

The same report showed that in 1981 there were 14 outbreaks in the USSR and in 1982 there were nine outbreaks. They were said to be caused by 2-O, 1-O, 2-O1, and 1-O1 viruses (105). According to United States Department of Agriculture (19) report USSR denied any outbreaks occurring in its Baltic regions and cattle were vaccinated in fall 1982 to decrease the risk of infection. It is known that there were at least eight outbreaks in Estonia in 1982 alone, and before that the disease spread in Latvia and Lithuania (79). However, the veterinary officials from the central veterinary office of the Estonian SSR at a time of 1982/83 epidemic report in 1992 that this epidemic was caused by the serotype A22 (9). From the FAO report (105), it appears that this type of virus spread at that time only in Turkey. It is not clear what was the source of information for the Estonian veterinary officials regarding the type of the virus spreading in Estonia during the 1982/83 epidemic. In the FAO report (105), the representatives of USSR claimed that animals were vaccinated with a trivalent vaccine in which A22 was only one component. The others were O and C. Only O was needed for protection. Given the secrecy of the time, Estonian officials might have not known the virus type that was actually circulating in Estonia. Maybe they were able to draw this conclusion from the label on the vaccine bottle, where A22 was listed first. The second possibility is indeed that the virus that spread from East Germany (serotype O) never reached the Baltic republics and the epidemic was caused by a strain circulating in USSR without being reported to international organizations (A22).

As early as in the beginning of 1960's, USSR researchers under the Ministry of Agriculture were instructed to develop bio-weapons against livestock and crops. These bioweapons with pathogens had to be able to be attached to the bombers and be sprayed over large areas. One potential bioweapon virus was FMD (106). FMD has been considered the ideal source of bioweapons, also outside the USSR. It is thought that a number of outbreaks in Asia, as well as in England in 2001, may have arisen from the use of biological weapons (107). It cannot be

ruled out that a possible leak of the virus from the laboratory was concealed. There have been several cases of laboratory leakage in the European Union (108). It can be assumed that such things happened in the USSR as well. In addition, it has been assumed that the development of biological weapons related to livestock production continued after the collapse of the USSR (109).

Respondents stated that such thorough closures, with staff locked in at the farms, i.e., restrictions on human freedoms, could only be implemented in a totalitarian regime. As one respondent (veterinarian, 81 years old) put it: "Only in a totalitarian state can animal diseases be effectively controlled." In addition, it is clear from the respondents' statements that "in 1952 restrictions were wider, everything was locked. There were militia guards at the cattle farms." The situation was often very critical, as "Already in the 1950's, people at risk of death were kept away from stables and children could not see their mothers for weeks."

One important aspect to point out in the example of the Estonian SSR is that the meat of the affected animals was used for human consumption as there was already a great shortage of the most common food products at that time, there was no question of burying or burning cows infected with FMD. Besides, in May 1982 the Central Committee plenum of the Communist Party set a goal for the new Food Programme which was to promote and improve the productivity and output of agriculture in the Soviet Union.

By the early 1980's folk medicine was no longer practiced on cattle, at least not in the collective and state farms in Soviet Estonia, as was described by several respondents. There were one or more veterinarians working in larger dairy farms, and there was a chief veterinarian in each Kolkhoz. There were creams or solutions prescribed by a veterinarian which were used to treat blistering and hoof problems in dairy cattle in cases of FMD.

There is no clear evidence if there the vaccines before the 1982 outbreak. One of the respondents mentioned that, when the FMD outbreak took place, the vaccines were delivered by the authorities from Moscow. Due to the good functioning of the veterinary service at the time, and the very strict chain of command, the vaccine reached the regions fairly quickly.

As the outbreak of the disease in Estonia in 1950 showed, when the herds were small, the impact of the disease was smaller. The most important factor was economic instead: the transition from a free market economy to a planned economy and the resulting difficulties. Before World War II, Estonia had a market economy and it was only during the occupation by the USSR that the planned economy came into being. Our study found that newly opened large farms performed relatively well in controlling FMD, largely due to existing infrastructure. However, we do not have information on how farms with fewer cattle (e.g., <100 individuals) would have been able to control the disease. As D'souza and Ikerd (110) acknowledge, smaller farms could be more sustainable. To change the system, it is necessary to change the narratives [see also (111)]. One of the narratives that needs to be changed is that the milk of sick animals is not suitable for drinking and meat is not to be eaten. As the experience of the USSR has shown, it is possible to produce both meat and milk sustainably during an outbreak. The solution would be smaller slaughterhouses and smaller dairies. Smaller ones are more flexible and can also adapt better to special conditions.

Conclusion

The paper describes the events of 40 years ago in the Estonian SSR, dealing with the outbreak of foot-and-mouth disease that began in southern Estonia. The focus was coverage of the outbreak of FMD in the autumn of 1982 in the local media. As agriculture was a priority for the entire Soviet Union, the Communist Party's narrative was to treat agriculture only for good. The latter meant that the party watched very closely how agriculture was presented in the public media.

Due to the growth in demand for animal origin products in the Soviet Union, dairy farming in the Estonian SSR developed significantly in the 1970's and 1980's. In the first half of the 1970's, the first large cattle barns with a capacity of up to 1,000 animals were built, which also included the corresponding infrastructure for personnel. When interviewing farm managers and animal husbandry specialists at the time, we tried to get an adequate overview of what happened in the autumn 1982. Unfortunately, this paper failed to explain why FMD broke out on large farms. However, some of the interviewees hypothesized that dairy farms in the Estonian SSR, as the most advanced in the entire Soviet Union, were used as a testing ground to monitor the development of the disease and its control mechanisms. The main argument to support this claim was that large dairy farms had the appropriate infrastructure to allow staff to remain on the farm during the quarantine period.

Based on the newspapers and scientific publications of that time, it might be assumed that there was no FMD in the Estonian SSR in 1982. However, most of the republic knew that the traffic restrictions on the roads were related to the disease outbreak in South Estonia. At the same time, the outbreak was covered in the media of neighboring countries, which described them in some detail. Thus, the present study is another example of the narrative of the system in the Soviets, where troubles and problems had to be silenced and only progress and victories could be publicly affirmed, which the Communist Party largely wrote at its own expense.

Methods used in a totalitarian system cannot be used in a free society. The trauma that people were forced into the barns (more than a month in some cases) also affected the next generation. Preventive actions are the most important in the

control of FMD. It appears from the interviews that at that time people were generally more accustomed to the restrictions of the state, because that was the (Soviet) time.

Data availability statement

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

Author contributions

MK, AV, and RK conceived the study. MK, AV, JP, and RK gathered the data. RS supervised the study. MK and RK analyzed the data with the help of the co-authors. MK drafted the first version of the manuscript which was then reviewed, edited, and approved by all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.828583/full#supplementary-material

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