

***IN VITRO* ANTIMICROBIAL ACTIVITY OF MEXICAN PLANTS ON BOVINE MASTITIS BACTERIA: PRELIMINARY STUDIES**

ATIVIDADE ANTIMICROBIANA IN VITRO DE PLANTAS MEXICANAS SOBRE BACTERIAS DE MASTITE BOVINA: ESTUDOS PRELIMINARES

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ABSTRACT: The present study aimed to evaluate the efficacy of traditionally used antibacterial plants (*Euphorbia prostrata*, *Schinus molle*, *Brickellia veronicaefolia* and *Lepidium virginicum*) in Santa Rosa Range in Guanajuato against major mastitis-causing pathogens. The selected plants were phytochemically screened for alkaloids, flavonoids, polyphenols, saponins, anthraquinone, cardiac glycosides, steroids and terpenoids. Moreover, to achieve the main purpose of the present research, extracts of selected plants were tested for *in vitro* antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Shigella* spp., *Bacillus subtilis*, *Clavibacter* spp., *Proteus vulgaris* and *Salmonella* spp. All these bacterial were isolated from clinical mastitis in dairy cows. The results indicated that the ethanolic extract of the aerial part of *L. virginicum* exhibited the strongest antimicrobial activity, and it could be potential candidate specie for the development of novel veterinary drugs with low cost and fewer side effects.

KEYWORDS: Antibiotic. Ethnomedicines. Dairy animals. Infectious disease. Mastitis.

INTRODUCTION

Bovine mastitis, an inflammation of the mammary gland caused by bacterial infection, is a highly prevalent disease in dairy cattle characterized by physical, chemical and usually bacteriological changes in milk and by pathological changes in the glandular tissues (LEBLANC et al., 2006). It is considered to be one of the most important diseases affecting the world's dairy industry (WELLENBERG et al., 2002). Mastitis is a complex disease caused by a variety of bacterial pathogens, mainly *S. aureus* and *S. agalactiae* (GOMES; HENRIQUES, 2016).

Current mastitis treatment, based on different intramammary antibiotic treatments, is very inefficient, because many bacteria are resistant to them (ZAFALON et al., 2007). Moreover, drug residues in milk have a potential health hazard effects for the consumer and may cause allergic reactions, interference in the intestinal flora and the rapid emergence of resistant bacteria (SAINI et al., 2012). For these reasons, this problem continues to deserve the attention of researchers and general population.

If we consider that approximately two-thirds of clinically used antibacterial therapies are derived from natural products, plant extracts could be an

alternative for antibiotic agents (FARHA; BROWN, 2016). Alternative treatments to bovine mastitis with bacteriocins (PIETERSE et al., 2010) and plant derived compounds (BASKARAN et al., 2009; MUBARACK et al., 2011) have been described. For example, PHYTO-MAST® is a botanical preparation used for the intramammary treatment of clinical mastitis on organic dairy farm and demonstrated with good therapeutic effect (PINEDO et al., 2013).

Although ethnobotanical knowledge in human health have been widely reported in Mexico (SHARMA et al., 2017), the application of medicinal plants to veterinary medicine is poorly described. We believe that traditional medicine based on phytotherapy may complement and offer alternatives for animal disease control, especially in the search for new drugs antibiotics. For this reason, the main scope of the present study was to collect the knowledge of a rural community from Guanajuato State on the medicinal plants and herbal remedies used to treat infectious diseases, and evaluate the *in vitro* activity of extracts from selected plants against several gram positive and gram negative bacterial strains isolated from milk of cows with mastitis. To the authors' knowledge, this is the first study to establish the possible application of these plants into the veterinary

phytotherapy. Thus, this research will provide scientific validation to traditional knowledge helping to develop novel veterinary drugs. In this sense, the present study aimed to evaluate the efficacy of traditionally used antibacterial plants (*Euphorbia prostrata*, *Schinus molle*, *Brickellia veronicaefolia* and *Lepidium virginicum*) in Santa Rosa Range in Guanajuato against major mastitis-causing pathogens.

MATERIAL AND METHODS

Collection of information and plant samples

This study was conducted in local communities located in Santa Rosa Range in Guanajuato State, Mexico, situated between 20°45'–21°25' N latitude and between 100°53'–101°25' O longitude. Data collection was done using semi-structured interviews and discussions with selected informants according to Bieski et al. (2015). The interviews took place between the period of July and December 2016. A free list method was used, which consisted of asking the informants to list the medicinal plants they knew and/or had used, the mode of preparation, the part used, the dosage and duration of treatment. Among all the documented plants only four highly used plants (*Euphorbia prostrata*, *Schinus molle*, *Brickellia veronicaefolia* and *Lepidium virginicum*) were selected for further *in vitro* screening. Additional ethnomedicinal, phytochemical and pharmacological information of these species was collected from a systematic literature review using different databases such as EMBASE, MEDLINE, PubChem, PubMed, SciELO, Science Direct and Scopus, using the search terms “*Euphorbia prostrata*”, “*Schinus mole*”, “*Brickellia veronicaefolia*”, “*Lepidium virginicum*” “medicinal plant”, “traditional uses”, “praditional Medicine” and “ethnobotany”, independently or in combination.

To evaluate the relative importance of the plant species based on its relative use among informants we used the use value (UV) index:

$$UV_i = \sum U_i / ns$$

where U_i is the sum of the total number of use reports by all informants for a given species i , and ns is the total number of informants. A high UV indicates that the plant is important, and a low UV suggests few reports are related to its use.

Plant materials collected during the study were washed under running tap water, rinsed in distilled water, dried in shade for 2 days and grinded to fine powder. All powdered samples were stored in

airtight container at 4°C until used. Moreover, during field research specimens of each specie were collected, herborized and deposited for taxonomic identification in the National Herbarium, Mexico: *Euphorbia prostrata* (voucher: 1428601), *Schinus molle* L. (voucher: 1445827), *Brickellia veronicaefolia* (Kunth) A. Gray (voucher: 1464269) and *Lepidium virginicum* L. (voucher: 1218999)

Preliminary Phytochemical Analysis

Preliminary qualitative phytochemical analyses of the crude powder of the 4 plants were carried out for the detection of tannins, alkaloids, saponins, flavonoids, steroids, anthraquinone, cardiac glycosides and terpenoids following the methods reported by Parekh and Chanda (2007)

Preparation of the extracts

Plant material was, where possible, prepared in a way consistent with the traditional preparation methods (according to collected information: Decoction of the aerial part in “aguardiente”, a mixture ethanol-water 2:1). 100 g of finely powdered aerial parts of plant samples were put into beakers containing 250 mL of 70% ethanol, covered with parafilm and then heated at 80°C in oil bath for 1hr with continuous stirring. The extract were filtered using Whattman filter paper (No. 1) and the solvents were removed under reduced pressure at 40 °C. These crude extracts were stored in stock vials and kept at 4°C in refrigerator for further use.

Bacterial strains

The *in vitro* antibacterial activity of the extracts was tested against *S. aureus*, *E. coli*, *Shigella* spp., *B. subtilis*, *Clavibacter* spp., *P. vulgaris* and *Salmonella* spp. All these bacterial were isolated from clinical mastitis in dairy cows and obtained from the University of Guanajuato. The pathogenicity of these bacterial strains was confirmed in our laboratory.

Antimicrobial Activity of the extracts using disc diffusion method

All the bacteria were incubated at 37°C for 24 h into broth medium. The test microorganisms were diluted to a count of 10⁸ CFU/mL and then transferred from nutrient broth to sterile Muller Hinton agar plates (CLEIDSON et al., 2007). Subsequently, filter paper discs (5 mm in diameter) saturated with 10 µL of each hydroalcoholic extracts (15 mg/ml) were placed on surface of each inoculated

plate with Gentamicin (100 µg/ml) as positive control. The diffusion of extracts was allowed for 1 hr at room temperature on a sterile bench. The plates were then sealed with Parafilm X and the plates were incubated at 37°C for 24 h and 72h. The plates were observed for the presence of inhibition of bacterial growth and that was indicated by clear zone of inhibition of bacterial growth around the wells. Diameters of the inhibition zone were measured in millimeters (mm). Overall, cultured bacteria with halos equal to or greater than 7 mm were considered susceptible to the tested extract. Experiments were carried out in triplicates and average zone of inhibition and standard deviations were calculated. The results were expressed as mean ± SD.

Determination of Minimum Inhibitory Concentrations

All tested extracts exhibited antimicrobial activity at a concentration of 15 mg/ml. Therefore, this concentration was manipulated to determine their minimum inhibitory concentrations (MIC) using agar well diffusion method (MOSTAFA et al., 2018). Different concentrations 10, 5, 2.5, and 1.25 mg/mL were prepared by two-fold serial dilution. 1 ml of each prepared inoculum was pipetted into sterile Petri dishes followed by the addition of molten agar and mixed well. Then, four wells were made on each plate, and 100 µl of 10, 5, 2.5, and 1.25 mg/mL of each extract was transferred to the respective wells. Plates were kept in the refrigerator for 30 min and then incubated at 37°C for 24 h. Experiments were carried out in triplicates. The MIC was considered as the lowest concentration which inhibited the growth of the respective microorganisms. All assays were performed in triplicate. DMSO was served as a control for ethanolic extracts.

RESULTS

Ethnomedicinal plants used for the prevention and treatment of bacterial infections

It is fundamental to record and preserve the traditional knowledge on medicinal plants for future generations. In 2016 we started a project to collect

ethnobotanical information concerning medicinal species traditionally employed in the popular medicine in the Bajío region, Mexico.

Forty-five selected informants, including 3 traditional medicine practitioners were interviewed. The respondents confirmed that the best time to harvest the plants from the wild is before or during flowering. The ethnobotanical survey results revealed that different medicinal plants were used for recipe formulation for treating various diseases, among them *Ephorbia prostrata*, *Schinus molle*, *Brickellia veronicaefolia* and *Lepidium virginicum* were most commonly used to treat bacterial infections. The pharmacological preparations were mostly decoctions of aerial parts of vegetal species (i.e. leaves, fruit and stem), and usually the remedies are a mixture of several medicinal plants. Oral administration was the most frequently used route of administration, followed by topical applications, and for most of the remedies, the dose seemed to be a function of body size. The interviewees did not mention side effects for any of the listed plants (Table 1). The results showed that the most frequently reported diseases were bacterial infections and pain, and that the same part of the plant was used to treat different conditions. The use value for the selected plants were generally high (Table 1). *L. virginicum* was the most frequently used species in the local area according to its high use value (0.96). It was mentioned by 96% of all the informants.

Quantitative evaluation of phytochemicals extracted from antibacterial plants

Preliminary phytochemical analysis revealed the presence of flavonoids and polyphenols in large quantity in the selected antimastitis plants as compared to other secondary metabolites. It is remarkable that only in *L. virginicum* were observed alkaloids, while saponins were present in a very small quantity, as compared to alkaloids and flavonoids, only in *E. prostrata*. Steroids were present in a large quantity among the selected plants. Highest terpenoids contents were found in *S. molle* followed by *B. veronicaefolia* (Table 2).

Table 1. List of the plant species cited in the study: scientific name, family name, common name, part used, local medicinal uses, mode of preparation and use value (UV).

Plant species	Common name	Part used	Local medicinal uses	Mode of preparation	UVs
<i>Euphorbia prostrata</i> (Euphorbiaceae)	Golondrina	Aerial part	Eye Diseases Dysentery Diuretic Antifungal Antiprotozoal	Decoction of aerial part is given	0.86
<i>Schinus molle</i> (Anacardiaceae)	Pirul	Fruits, leaves, essential oils	Antibacterial Genito-urinary diseases Antispasmodic Healing Analgesic Anti-inflammatory Antiviral, Antifungal	Fruits are given a bath in the decoction of leaves	0.75
<i>Brickellia veronicaefolia</i> (Asteraceae)	Peistó	Aerial part	Dysentery Antibacterial Analgesic Gastritis	Decoction of aerial part is given	0.89
<i>Lepidium virginicum</i> (Brassicaceae, Cruciferae)	Lentejilla	Aerial part	Dysentery Antibacterial	Decoction of aerial part is given	0.96

Table 2. Phytochemical screening of the selected anti-bacterial plants.

Phytochemical constituents	Tests	Plant name			
		<i>E. prostrata</i>	<i>S. molle</i>	<i>B. veronicaefolia</i>	<i>L. virginicum</i>
Alkaloids	Mayer	-	-	-	++
	Wagner	-	-	-	++
	Dragendorff	-	-	-	++
	Hagers	-	-	-	+++
Flavonoids	Shinoda	+	++	++	-
	Basic test	+	++	++	-
Tannins/Phenol	Ferric chloride	+	++	+++	++
Saponins	Foam test	+	-	-	-
Anthraquinone	Borntrager	-	-	-	-
Cardiac glycosides	Keller-Kiliani	+	-	-	-
Steroids	Liebermann-Burchard	++	+	+	++
	Salkowski	++	+	+	++
Terpenoids	Knollar	-	++	++	-
	Tschugajen	-	++	++	-

+++ = Copiously present, ++ = Moderately present, + = Slightly present, - = Absent.

Table 3. Bacterial zone of inhibition (mm) by extracts of selected anti-mastitis plants at 15 mg/ml.

Microorganism	Diameter of inhibition zone at different concentration levels (mm)									
	<i>E. prostrata</i>		<i>S. molle</i>		<i>B. veronicaefolia</i>		<i>L. virginicum</i>		Gentamicin	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	
<i>S. aureus</i>	9.8±1.9	10.4±1.3	na	na	8.0±0.2	8.5±0.2	8.5±0.9	9.2±0.7	17	
<i>E. coli</i>	na	na	na	na	na	na	7.9±0.1	11.4±0.5	25	
<i>Shigella</i> spp.	na	na	7.9±0.1	12.2±1.5	8.7±0.3	9.3±0.5	na	na	18	
<i>B. subtilis</i>	8.4±0.5	8.9±0.1	8.4±0.2	11.9±4.5	na	na	8.6±0.6	11.5±1.3	16	
<i>Clavibacter</i> spp.	na	na	na	na	na	na	8.7±0.1	9.6±0.6	15	
<i>P. vulgaris</i>	na	na	8.9±0.2	11.7±0.4	na	na	8.8±0.1	15.0±1.7	27	
<i>Salmonella</i> spp.	na	na	na	11.5±2.4	9.5±0.7	10.1±0.3	8.2±0.3	14.7±0.3	22	

Antibacterial activities

In the present study a total of four plant species belonging to four different families were screened for antimicrobial activity. These plants are traditionally used to treat various ailments by local inhabitants, traditional healers and herbal practitioners

in Santa Rosa Range, Guanajuato. *L. virginicum* extracts had antimicrobial activity against test organisms (Table 3), with minimum inhibitory concentration (MIC) ranging from 6 to 10 mg/mL (Table 4), while the other three extracts had MIC range of 7 to 14 mg/mL each (Table 4).

Table 4. The Minimum Inhibitory Concentration (MIC) of plant extracts against the test microorganisms.

Microorganism	Minimum inhibitory concentration MIC (mg/mL)			
	<i>E. prostrata</i>	<i>S. molle</i>	<i>B. veronicaefolia</i>	<i>L. virginicum</i>
<i>S. aureus</i>	10.0	nd	12.0	10.0
<i>E. coli</i>	nd	nd	nd	8.0
<i>Shigella</i> spp.	nd	20.0	14.0	Nd
<i>B. subtilis</i>	12.0	8.0	nd	8.0
<i>Clavibacter</i> spp.	nd	nd	nd	10.0
<i>P. vulgaris</i>	nd	9.0	nd	6.0
<i>Salmonella</i> spp.	nd	8.0	10.0	6.0

DISCUSSION

The medicinal plants used in the traditional medicine may complement and offer alternatives to improve animal healthcare and hence enhanced living standards of rural communities (LANS et al., 2007). The use of phytomedicine is very common in Mexico, where medicinal herbal repertoire is one of the world's most diverse. Despite this great tradition and the potential use that these plants could have in human health in Mexico and the growing interest of veterinarians in herbal medicine, little research had been made on the application of ethnobotanical knowledge to veterinary medicine.

In the present study the efforts were made to investigate the antimicrobial activity of those plant species which are used to treat various bacterial

infectious diseases by the local population in Santa Rosa Range, Guanajuato. An ethnobotanical survey was undertaken to collect information from local communities from July to December 2016. Twenty-three plants were identified as of interest for their role in traditional health practices of the community. A total of four species (*E. prostrata*, *S. molle*, *B. veronicaefolia* and *L. virginicum*) belonging to four different families were found to be the most frequently used and mentioned in the case of infectious disease, with use value between 0.75-0.96 (Table 1).

For antimicrobial activity the hydroalcoholic extract of each plant (*E. prostrata*, *S. molle*, *B. veronicaefolia* and *L. virginicum*) was prepared and evaluated in concentration of 15 mg/ml by agar diffusion method, using Gentamicin (100µg/ml) as the

standard. The high zone of growth inhibition exhibited is in agreement with the results of the microbial growth inhibition (Table 4). It was found that although all extracts presented antimicrobial activity to at least one of the tested microorganisms, *L. virginicum* showed broad spectrum activity against 86 % of selected bacterial pathogens isolated from clinical mastitis in dairy cows. However, it did not show any activity against *Shigella* spp. at this concentration. In this point is important to indicate that the microorganism *E.coli*, which is already known to be multi-resistant to drugs, was susceptible only to the extract from *L. virginicum*. This result could correlate with the fact that this herbaceous plant is used widely in the area of study as a remedy to treat very intense gastro-intestinal infectious. This positive response of hydroalcoholic extract of *L. virginicum* on different strains of bacteria is in agreement with the findings of Avila Acevedo et al. (1993) who demonstrated that the aqueous extract of this specie was effective against *Escherichia coli*, *Salmonella typhi* and *Shigella boydii* with a MIC = 0.220 mg/ml. However, this plant has not received much attention. Significant antibacterial activity might be due to high concentration of alkaloids shown by this specie in the phytochemical analysis, which reduce the viability of gram positive and gram negative bacteria (CUSHNIE et al., 2014). Present study reveals that crude hydroalcoholic extract of *S. molle* showed similar inhibition zones against *Shigella* spp., *B. subtilis* and *P. vulgaris*. This result is according to studies conducted by Martins et al. (2014) the essential oil from this specie showed antimicrobial activity against gram positive, gram negative pathogenic bacteria and food spoilage fungi. The inhibitory activity of this plant could be due to presence of polyphenols and flavonoids in large quantity, which have been widely reported as antimicrobial agents (COPPO; MARCHESE, 2014). On the other hand, the crude extract of *B. veronicaefolia* showed sharp inhibition zones against *S. aureus* and *Salmonella* spp., as well as *Shigella* spp. *B. veronicaefolia* is available commercially in herbal stores and is presently and commonly used by diabetics as a cheaper alternative to insulin. The hypoglycaemic effect was confirmed and a bioactive compound has been isolated (ANDRADE-CETTO; HEINRICH, 2005). Finally, *E. prostrata* only showed activity against *S. aureus* and *B. subtilis*.

The results indicated that as for as antimicrobial activity is concerned, the ethanolic

extract of the aerial part of *L. virginicum* exhibited the strongest antimicrobial activity as compared to *E. prostrata*, *S. molle* and *B. veronicaefolia*, though all these plants are used for the same purposes in different localities. The Gentamicin was used as the reference antibiotic in the present study, and it have showed the antimicrobial activity against all the test microorganisms used in the present research.

The present study shows that the plants used in the traditional system of medicine in Santa Rosa Range, Guanajuato, possess antimicrobial activity which in the extracted form can be utilized successfully to treat infectious diseases as bovine mastitis. This ethnobotanical information offer a great reservoir for the discovery of antimicrobial drugs.

The results obtained in the present research have very promising antimicrobial activities against bacteria causing bovine mastitis, therefore supporting the ethnomedical uses of these species. Our study, which is still at a preliminary stage, justify the use of these medicinal plants to develop new veterinary drugs, with low cost and without side effects, avoiding the use of antibiotics. This will be a great contribution in improving animal's health. Furthermore, it is desirable to make in depth studies of these plants for the isolation and characterization of antimicrobial compounds responsible for some of the activity we have observed, prior to further bioassays. It is important to indicate that studies on in vivo trials should also be carried out to validate the present results.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by CONACYT and SIP-IPN. We are also grateful to Julio César Enrique López Salazar who voluntarily agreed share his rich knowledge on medicinal plants.

RESUMO: O presente estudo teve como objetivo investigar a eficácia de plantas antibacterianas tradicionalmente usadas (*Euphorbia prostrata*, *Schinus molle*, *Brickellia veronicaefolia* e *Lepidium virginicum*) na cordilheira Santa Rosa, em Guanajuato, contra os principais patógenos causadores de mastite. As plantas selecionadas foram rastreadas fitoquimicamente quanto a alcalóides, flavonóides, polifenóis, saponinas, antraquinona, glicosídeos cardíacos, esteróides e terpenóides. Além disso, para atingir o objetivo principal da presente pesquisa, extratos de plantas selecionadas foram testados quanto à atividade antibacteriana *in vitro* contra *Staphylococcus aureus*, *Escherichia coli*, *Shigella* spp., *Bacillus subtilis*, *Clavibacter* spp., *Proteus vulgaris* e *Salmonella* spp. Todas essas bactérias foram isoladas de mastite clínica em vacas leiteiras. Os resultados indicaram que o extrato etanólico da parte aérea de *L. virginicum* exibia a atividade antimicrobiana mais forte e poderia ser uma espécie potencial candidata ao desenvolvimento de novos medicamentos veterinários com baixo custo e menos efeitos colaterais.

PALAVRAS-CHAVE: Antibiótico. Etnomedicina. Mastite bovina. Doença infecciosa.

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