Journal of Pharmaceutical Research International

23(2): 1-12, 2018; Article no.JPRI.41205
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Qualitative Determination of the Secondary Metabolites and Evaluation of the Antimicrobial Activity of Leaf Extracts from Different Plant Families (Boraginaceae, Fabaceae, Lamiaceae and Lauraceae) against Microorganisms of Clinical Importance

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Authors' contributions

This work was carried out in collaboration among all authors. Author RAY designed the study, did literature searches, researched and modified protocols accordingly, wrote the first draft of the manuscript and edited the manuscript to its final version. Author KLC did literature searches, performed all experiments with statistical analysis and wrote the first draft of the manuscript. Author AAM helped out in the phytochemical screening and analysis. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2018/41205
Editor(s):
(1) Dr. Emmanuel Christy Jeyaseelan, Department of Botany, University of Jaffna, Sri Lanka.
Reviewers:
(1) Maciej Tadeusz Strzemski, Medical University of Lublin, Poland.
(2) P. Rameshthangam, Alagappa University, India.
(3) Daohong Chen, China.
Complete Peer review History: http://www.sciencedomain.org/review-history/25634

Original Research Article

Received 31st March 2018
Accepted 15th July 2018
Published 21st July 2018

ABSTRACT

Aims: This study was conducted to investigate the antimicrobial activities of *Sassafras albidum* (Nutt.)*, Ehretia anacua* **(**Terán & Berl.)*, Melissa officinalis* (Linn.)*, Eysenhardtia texana* (Scheele)*,* and *Melissa odorata.* Specifically, this study aims to evaluate the antimicrobial potential and to qualitatively determine presence of secondary metabolites in the different leaf extracts.
Place and Duration of Study: Plant leaves were collected from the San Antonio Botanical Garden in Texas. The microbial assays and chemical analysis were done at the Department of Biology and

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Chemistry, Texas A&M International University, Laredo Texas. This study was done from October 2013 to May 2016.

Methodology: Leaves were collected and aqueous, acetone, diethyl ether, and ethanol leaf extracts were prepared. Antimicrobial activity against bacteria and fungi were investigated via disc diffusion assay. Phytochemical screening was done to qualitatively determine secondary metabolites.

Results and Conclusion: The ethanol and diethyl *E. anacua* (Boraginaceae) extracts showed a statistically significant antimicrobial activity against *S. aureus.* Although the values, 7.4 mm and 7.5 mm for the ethanol and diethyl ether extracts could be low values for zone of inhibitions, the potential for *E. anacua* for anti-*S. aureus* activity cannot be undermined. Phytochemical analysis showed detectable presence of alkaloids, diterpenes, and phenols in the ethanol and diethyl *E. anacua* extracts. Results of this study, although preliminary, demonstrated the potential of *E. anacua* as a new source of bioactive metabolites. Further investigations are needed in order to specifically identify, quantify, and isolate the bioactive compounds that might act against *S. aureus* associated skin infections.

Keywords: Antimicrobial; Ehretia anacua; leaf extracts; S. aureus; secondary metabolites.

1. INTRODUCTION

Traditional medicinal plants serve as potential sources of new antimicrobial agents, since they possess natural products, including secondary metabolites and their derivatives [1,2,3]. Secondary metabolites could include alkaloids, flavonoids, tannins, terpenes, quinones, and resins. These plant secondary metabolites serve as defense mechanisms against many microorganisms, insects, and herbivores [4]. Secondary metabolites are the subject for many research studies since these compounds exhibit many biological activities. These include antibacterial, antifungal, anticancer, and anti-inflammatory.

Antimicrobial properties are important for future pharmacological uses. There has been a continuous interest in the therapeutic use of natural products because of availability, less side effects, and less abusive potential [5,6,7]. Over the years, many scientists have performed research on different plant families to be able to identify antimicrobial activities and secondary metabolites [2,8,9,10,11]. Phytochemical screening of different plants has revealed numerous bioactive compounds including alkaloids, tannins, flavonoids, glycosides, and saponins.

Previous studies reported plants belonging to Boraginaceae, Fabaceae, Lamiaceae, and Lauraceae to have medicinal properties. In this regard, representative plants from these families were used in this study. The Boraginaceae family has been found to have biological activities that include antioxidant, wound healing, anti-inflammatory, and antimicrobial properties; these properties are attributed to the presence of naphthoquinones, alkaloids, flavonoids, polyphenols, phytosterols, terpenoids, and/or allantoin [10,12,13,14,15,16]. Likewise, several plants from the family Fabaceae have been reported to possess potential of antimicrobial activity [2,17]. For example, methanol and ethyl acetate bark extracts of *Adenanthera pavoina* L showed antimicrobial activity against gram-positive, i.e. *S. aureus,* and gram-negative strains, i.e. *E. coli*. The antimicrobial activity could be attributed to the reported presence of saponins, alkaloids, tannins, flavonoids, and steroids in both methanol and ethyl acetate extracts [18]. *Sideritis* species, which belongs to the family Lamiaceae, has been used for centuries for their anti-inflammatory and antimicrobial properties [2,8]. Some of the chemical constituents in *Sideritis* identified to be responsible for pharmacological activity are: terpenes, flavonoids, essential oil, iridoids, coumarins, lignanes, and sterols [8]. Under the Lauraceae family, the leaves of *Cinnamomum* species are used in traditional medicine for pains, while the leaves of *Laurus nobilis* L. have been known to contain medicinal compounds (tannins, acidic materials, and volatile oil) [11].

Drug-resistant pathogens' infections are reported at 700,000 annual deaths and are estimated to increase to 10 million by 2050. Included in these numbers is the contribution of antibiotic resistance in addition to failure of anti-malarial drugs and antiviral therapy [19,20]. It is widely known that there is an increasing and continuous prevalence of antibiotic-resistant bacteria emerging from the extensive use of antibiotics.

This situation may render the current antimicrobial agents insufficient to control at least some bacterial infections [21]. According to the 2012 World Health Organization (WHO) report, antibiotic resistance should be regarded as a global threat comparable to climate change and terrorism [22]. The use of traditional medicinal plants to search for new antimicrobial agents is an important line of research because of the resistance acquired by several pathogenic microorganisms.

The microorganisms used in this study namely, *Staphylococcus aureus,* Methicillin-resistant *S. aureus* (MRSA)*, Pseudomonas aeruginosa, Escherichia coli, Yersinia enterocolitica, Serratia marcescens* and Vancomycin-resistant *Enterococci faecium* (VREF) are of clinical importance. *S. aureus* is a major component of the normal microflora of animals and humans, colonizing the nasal cavity, naso-pharynx, skin, and mucous membranes. However, staphylococci can occasionally cause opportunistic infections in many animal species, including mastitis and suppurative conditions [23]. *S. aureus* is also a common pyogenic agent in humans and several animal species, such as abscess formation [24].

P. aeruginosa is an opportunistic microorganism commonly found in wound infections in animal species e.g. canine otitis externa and cystitis, the uteri of mares, and the eyes of horses with corneal ulcers. In humans, *P. aeruginosa* can cause bacteremia in people with burns, leukemia, or cystic fibrosis [23,24]. Most strains are resistant to common antimicrobial agents and are therefore difficult to eliminate. Another organism used in this study is *E. coli*. It is an opportunistic pathogen in many animal species and humans, causing urinary tract disease, abscess, and pneumonia. *E. coli* can also cause septicemic disease in foals, calves, piglets, puppies, and lambs; enterotoxigenic diarrhea in newborn farm animals; and of edema disease in pigs [24].

Y. enterocolitica is commonly found in animal products (i.e., milk and pork) and may be a source of human infection. It can cause acute gastroenteritis, terminal ileitis (inflammations of the small intestine, specifically the ileum), and septicemia in other primates [24]. This microorganism is responsible for a zoonotic infection known as yersiniosis, which causes food poisoning; pigs in particular are carriers of *Y. enterocolitica* strains [23]. On the other hand, *S. marcescens* is an environmental organism that can cause bovine mastitis and septicemia in immunosuppressed mammals [23]. Vancomycin-resistant *E. faecium* (VREF) are linked with infections of the urinary tract, wound, and bloodstream; enterococci are the second most common nosocomial pathogen and the pathogenic cause of bloodstream infections in patients in intensive care units. Bloodstream infections due to vancomycin-resistant *Enterococcus faecium* (VREF) are associated with substantial morbidity [25]. There has been a significant increase in resistance to antibiotics, specifically vancomycin, thus the management of enterococcal infections has been challenging [26].

The fungal microorganisms used for this study were *Candida albicans*, *Aspergillus niger,* and *Trichophyton mentagrophytes*. *C. albicans* (candidiasis), inhabits the mucous membranes of most mammal and bird species. The disease is related to immune and hormonal inadequacies, and reduced colonization resistance; these conditions account for susceptibility of infants, diabetics, subjects on antibiotics and steroids, patients with catheters, and mammary glands of lactating cows [24]. *A. niger* is a ubiquitous mold with opportunistic pathogenic patterns. It is present in the soil, vegetation, feed, and secondarily in air, water, and exposed items.

Aspergillus spp. can cause disease in several ways, including mycotoxicoses and can be involved in allergic reactions to humans; it can also cause aspergillosis, which is acquired from environmental sources, generally by inhalation or ingestion [23,24]. *T. mentagrophytes*, a dermatophyte, is a mold capable of parasitizing only keratinized epidermal structures (i.e., skin, hair, nails). Dermatophyte infection, also known as ringworm, is a zoonotic disease; the zoophilic dermatophytes are obligate pathogens, primarily parasitizing animals but also capable of infecting humans [24].

This study aims to evaluate the antimicrobial potential of different leaf extracts and to identify their secondary metabolites. In this study, gram-positive and gram-negative bacteria were used to examine the broad-spectrum antibiotic potential of the leaf extracts understudy. Secondary metabolites are known to be more active against gram-positive bacteria and less active against gram-negative bacteria [27]. In addition, the leaf extracts were used to determine their antifungal activities. The rising frequency of fatal mycoses associated with the increasing use of immunosuppressed medical therapies have

stimulated research directed towards the discovery of novel antifungal agents [28]. Due to the complexity of the fungal cell the pace of antifungal activity discovery and development is exceedingly slow [29], thus the determination of antifungal activity of a leaf extract would be noteworthy.

2. MATERIALS AND METHODS

2.1 Plant Collection

Plant leaves were collected from labeled plants/trees growing in the San Antonio Botanical Garden in Texas. Table 1 presents the plant species collected for this study. The plant material was ground to a fine powder.

2.2 Plant Extracts

In the preliminary study, aqueous and ethanol extracts of *Sassafras albidum* (Nutt.), *Ehretia anacua* (Terán & Berl.), *Melissa officinalis* (Linn.), *Eysenhardtia texana* (Scheele), and *Melissa odorata* were prepared by mixing the powdered samples in sterile distilled water or 70% ethanol, respectively. They were then allowed to incubate in a heating water bath shaker for 18 h at 30°C. Following filtration and centrifugation, the supernatant was collected, and lyophilized using a freeze dry system (Labconco™). Thereafter, each stock was diluted using dimethyl sulfoxide (DMSO) to 66.7, 50, and 25 mg/mL.

Preliminary results of the antimicrobial assay only showed *E. anacua* extract to have antimicrobial activity. In this regard, only *E. anacua* leaves were used in soxhlet extraction (Chemglass™) procedure. *Ehretia anacua's* diethyl ether, acetone, and ethanol extracts were prepared separately. Each extract was further concentrated by a rotary evaporator (Heidolph North America) and a freeze-drier system (Labconco). The extracts were prepared to 25 mg/mL.

2.3 Microorganisms

The microorganisms used in this study were isolates and strains provided by American Type Culture Collection (ATCC) and Presque Isle Culture: *Staphylococcus aureus* ATCC 700699 (MRSA), vancomycin-resistant *Enterococci faecium* ATCC 700221 (VREF), *Staphylococcus aureus* 4651, *Pseudomonas aeruginosa* 99, methicillin-resistant *Escherichia coli* strain B 337, *Serratia marcescens* 4651, *Yersinia enterocolitica* 330, *Candida albicans* 925, *Aspergillus niger* 922 and *Trichophyton mentagrophytes* 1047.

2.4 Antibacterial Susceptibility Testing

The turbidity of an overnight broth culture was adjusted to a level of A= 0.132 ± 0.005 at 625 nm. This level is optically comparable to the 0.5 McFarland standards. A spectrophotometer (Bausch and Lomb, Model Spectronic 20) was used to adjust the absorbance of the suspension. This yields a bacterial suspension of approximately 0.5-1.0 x 10^8 CFU/mL. Agar plates were inoculated with 100 µL of the respective adjusted bacterial suspension. The disk diffusion method [30] with modifications, was used to determine zone of inhibition values for the plant extracts. Mueller Hinton agar was prepared according to the manufacturer's instructions. Sterile Whatman® antibiotic assay discs were impregnated with 20 µL of the plant extracts or DMSO. The following antibiotic discs were used as antibiotic positive controls: (1-2) *S. aureus* and *P. aeruginosa*- 10 µg of streptomycin; (3) MRSA- 30 µg of novobiocin and 10 units of penicillin; (4) VREF- 30 µg of chloramphenicol and 30 µg of vancomycin; (5-6) *E. coli* and *S. marcescens*- 5 µg of ciprofloxacin; and (7) *Y. enterocolitica*- 30 µg of chloramphenicol. The plates were incubated at 37°C for 18-20 h. After incubation, zone of inhibition (largest and smallest) measurements were taken using a vernier caliper. The test was done in three replications and each replication was carried out in triplicate.

2.5 Antifungal Susceptibility Testing

Two methods of antifungal susceptibility testing were employed, depending on the morphology of the fungi being tested. *C. albicans*' morphology includes convex colonies which were similar to bacterial colonies, thus the same antimicrobial susceptibility testing was used, with a few modifications. The modifications were: PDA media, 100 units of Nystatin, and incubation for 72 h at 28°C. The test was done in three replications and each replication was carried out in triplicate.

For filamentous fungi producing septate hyphae, PDA and SDA media was used for *A. niger* and *T. mentagrophytes*, respectively. The bottom of each agar plate was marked 1.5 cm from the perimeter to create a new circular circumference. A loop-full of fungal sample was transferred from

Table 1. Plant species used in this study

Scientific Name	Common Name	Family
Ehretia anacua (Terán & Berl.)	Anaqua, Knockway	Boraginaceae (borage)
Eysenhardtia texana (Scheele)	Texas kidneywood	Fabaceae (pea)
Melissa odorata	Lemon Balm	Lamiaceae (mint)
Melissa officinalis (Linn)	Common balm	Lamiaceae (mint)
Sassafras albidum (Nutt.)	Sassafras, Cinnamon Wood, Ague Tree	Lauraceae (laurel)

**This table was created with the information provided by the United States Department of Agriculture: Natural Resources Conservation Service and Lady Bird Johnson Wildflower center at the University of Texas at Austin.*

the culture to the respective media plate, within the boundaries of the newly drawn boundary. Sterile water was pipetted onto the center of the media plate and was mixed with the fungal hyphae to spread it evenly within the new drawn boundary. Plates were incubated at 28°C for 24 h (*A. niger*) or 7-8 days at room temperature (*T. mentagrophytes*). Assay discs were impregnated with 20 µL of the plant extracts or DMSO; they were then placed on the perimeter of the newly drawn boundary. The negative control was DMSO, while positive was 100 units of Nystatin [31]. The plates were then incubated, for 48 h at 28°C and 9 days at room temperature for *A. niger* and *T. mentagrophytes,* respectively. After the second incubation, each disc was observed for a zone of inhibition (length and height) using a vernier caliper. The test was done in three replications and each replication was carried out in duplicate.

2.6 Statistical Analysis

A single factor Analysis of Variance (ANOVA) and Post Hoc Bonferroni test were performed, in order to determine which treatment group's mean zone of inhibition was significantly different, in terms of zone of inhibition, from the respective negative and positive controls. All analyses were carried out using IBM SPSS Statistics Version 20™ software, provided by Texas A&M International University.

2.7 Phytochemical Screening

Phytochemical analysis was performed on the plant extracts found to have a potential for antimicrobial activities. The plant extracts were subjected to standard chemical tests to determine which secondary metabolites are present in the plant species [32,33,34,35, 36,37]. Chemical tests were done in three replications and each replication was carried out in duplicate.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Activity of Aqueous and Ethanol Extracts from Different Plant Families (Boraginaceae, Fabaceae, Lamiaceae, and Lauraceae)

Fig. 1 demonstrates the mean zone of inhibitions (ZOI) against *S. aureus* of all aqueous and ethanol leaf extracts from the five plant species understudy. It demonstrates that the ethanol *E. anacua* extracts were found to be significantly different ($p<0.05$), in terms of mean ZOI (9.3 mm, 13.0 mm, and 12.2 mm at 25 mg/mL, 50 mg/mL, and 67 mg/mL respectively), from the negative controls (6 mm, water, and DMSO), for the anti-*S. aureus* activity. Results showed evidence that the ethanol *E. anacua* extracts possess anti-*S. aureus* activity. This is the first report on the antimicrobial activity of *E. anacua leaf extract.*

E. anacua belongs to Boraginaceae family, results of this study is similar to a study on *C. gilletii* and *L. erythrorhizone,* also belonging to the Boraginacae family. *C. gilletii* and *L. erythrorhizone* extracts also exhibited anti-*S. aureus* activities [38, 39, 40, 41]. On the other hand, similar to *C. odontophyllum,* both extracts were specific only for anti-*S. aureus* activity [42]. The antibacterial activities of hexane, acetone, methanol, and aqueous leaf extracts from *C. odontophyllum* were tested against two-gram positive bacteria (*S. aureus* and *Bacillus cerus)* and two gram-negative bacteria (*P. aeruginosa* and *E.coli).* The acetone and methanol *C. odontophyllum* extracts exhibited anti-*S. aureus* activity*;* all plant extracts were found to not have antimicrobial potential for the other microorganisms tested. From phytochemical screening, they detected the presence of terpenoids, tannins, flavonoids, and phenols in *C. odontophyllum* extracts. The authors attributed the anti-*S. aureus* activity to the secondary metabolites present in both plant extracts [42].

In our study all 5 aqueous plant extracts and 4 out of the 5 plant species for the ethanol plant extracts were found to be not significantly different (P = 0.05), in terms of mean zone of inhibition, to the negative control, for anti-*S. aureus* activity (Fig. 1). In addition, all aqueous and ethanol plant extracts for the plant species tested, were found to not have a significant antimicrobial potential for *P. aeruginosa,* MRSA, or VREF activity (data not shown). All aqueous and ethanol plant extracts for the plant species tested, were found to be significantly different from the positive control, Streptomycin. This is not surprising since most secondary compounds with anti-microbial activities have been reported to be extracted by ethanol as compared to aqueous extraction. Ethanol extraction has been shown to be the most effective extraction method for isolating the bioactive phytocompound [43]. On the other hand, for the ethanol extracts of *E. texana, S. albidum, M. odorata,* and *M. officinalis*, it is possible that the antimicrobial secondary metabolites, or if at all present, are in concentrations that cannot exhibit antimicrobial activity detectable by the assays performed. In addition, MRSA and VREF bacteria have developed mechanisms to become resistant to antibiotics; the secondary metabolites extracted from the ethanol *E. anacua* extract could have not been present in high enough concentrations to have antimicrobial activity against MRSA nor VREF. It could also be likely that the secondary metabolites in *E. anacua* are not inhibitory to any of the biological processes that allow MRSA or VREF to survive. Some plant extracts have been found to have anti-*P. aeruginosa* and MRSA activity, but it could be attributed to the flavonoids present as secondary metabolites [44, 45]. Since there was no detectable flavonoids in the ethanol extract of *E. anacua,* this could be one of the possibilities why *E. anacua* extracts did not exhibit anti-*P. aeruginosa* and MRSA activity.

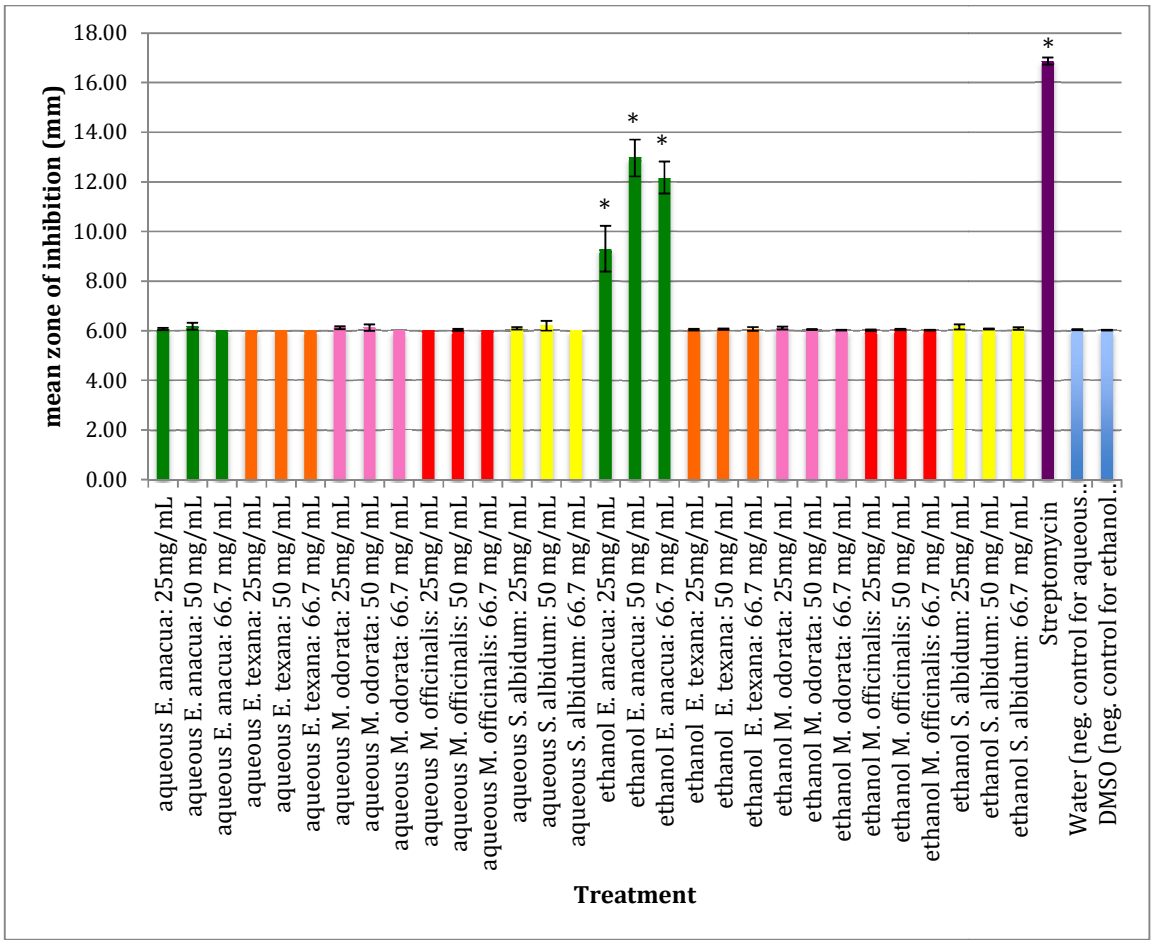

Fig. 1. Determination of anti-*Staphylococcus aureus* activity against all plant extracts tested
**denotes significance at 5% level*

3.2 Antimicrobial Activity of *E. anacua* Extracts

Since the ethanol *E. anacua* extract was found to have an anti-*S. aureus* activity, *E. anacua* leaves were subjected to soxhlet extractions using different solvents. As demonstrated in Fig. 2, both ethanol (7.4 mm) and diethyl ether (7.5 mm) *E. anacua* extracts, and streptomycin (17.3 mm) were found to be significantly different (P= 0.05), in terms of mean zone of inhibition, to the negative control (6.0 mm); therefore, compared to the acetone extract, the ethanol and diethyl *E. anacua* extracts showed a statistically significant antimicrobial activity against *S. aureus.* Although the values, 7.4 mm and 7.5 mm for the ethanol and diethyl ether extracts could be low values for ZOI, the potential for *E. anacua* for anti-*S. aureus* activity cannot be undermined. In this regard, our results will give rationale for the isolation, purification, and identification of the bioactive compound in the *E. anacua* extracts that is responsible for the observed anti-*S. aureus* activity. The isolation and purification of the bioactive compound in the *E. anacua* extracts will be an exciting and impacting research endeavor especially for scientists in search for and/or in need of a natural product against *S. aureus.* In addition, with the anti-*S. aureus* activity found in *E. anacua*, although not as promising compared to other plant extracts, the presence of such an activity will provide the basis for future research activities. This could include antimicrobial activity against other microorganisms; antimicrobial activity with extracts from other parts of *E. anacua* plant i.e. stem, bark, flower; and antimicrobial assays using different extracts, i.e. chloroform, hexane, and ethyl acetate.

The acetone, diethyl ether, and ethanol *E. anacua* extracts were found not to have a significant antimicrobial potential against *P. aeruginosa, Y. enterocolitica, E. coli, S. marcescens, C. albicans, A. niger,* and *T. mentagrophytes* (data not shown). It is not surprising that the ethanol and diethyl ether *E. anacua* extract possessed anti-*S. aureus* activity but did not exhibit antimicrobial activity against the gram-negative bacterium and fungi. *S. aureus* is a gram-positive bacterium, while *P. aeruginosa, S. marcescens, Y. enterocolitica* and *E. coli* are gram-negative bacteria. It has been shown that secondary metabolites are more active against gram-positive than gram-negative bacteria. This difference has been attributed to the different cell wall and membrane structure between the gram-positive and gram-negative bacteria. The secondary metabolites can penetrate the peptidoglycan envelope and reach the cell membrane of gram-positive bacteria easier than in gram-negative bacteria, which are protected by a lipopolysaccharide envelope [46].

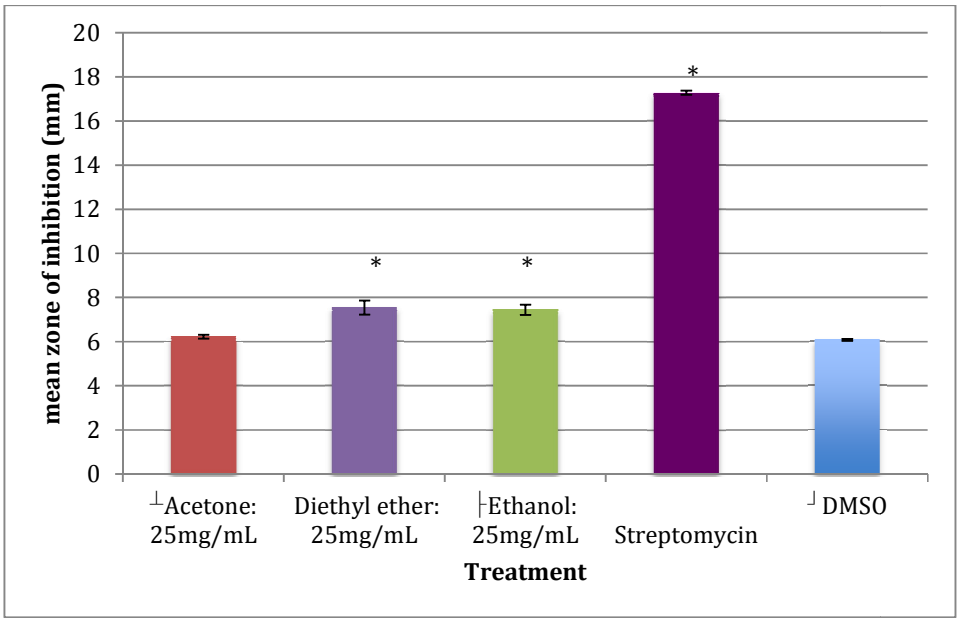

Fig. 2. Determination of anti-*Staphylococcus aureus* activity against *Ehretia anacua* leaf extracts

**denotes significance at 5% level*

Flavonoids and saponins have been studied to be able to disrupt cell membrane integrity [47]. Our phytochemical analysis results (Table 2) of the ethanol and diethyl ether *E. anacua* extracts did not possess detectable flavonoids and saponins. This could also be the reason that the *E. anacua* extracts did not exhibit antifungal activity against *C. albicans, A. niger,* and *T. mentagrophytes.*

Acetone, diethyl ether, and ethanol *E. anacua* extracts, were found to be significantly different (*P*= 0.05) from the positive control, Streptomycin. In addition, all crude plant extracts were found to be significantly different than the respective antibiotic used for each tested microorganism. The commercial antibiotics are expected to be more potent than the crude plant extract since the antibiotic is a pure compound. Plant crude extracts contain many compounds, and could include compounds that will act as antagonists to the compounds that are exhibiting the antimicrobial activity; this could by why the *E. anacua* extracts where found to be not as potent, compared to Streptomycin, for anti-*S. aureus* activity.

3.3 Phytochemical Analysis

Since the ethanol and diethyl ether *E. anacua* extracts were found to have anti-*S. aureus* activity, these extracts were subjected to chemical tests in order to determine the classes of secondary metabolites present. As shown in Table 2, both ethanol and diethyl ether *E. anacua* extracts were positive for several classes of secondary metabolites. For the ethanol *E. anacua* extract - - alkaloids, cardiac glycosides, diterpenes, and phenols were present, while for the diethyl ether *E. anacua* extract - - alkaloids, diterpenes, and phenols were present. These findings are novel as no other studies has yet reported the secondary metabolites present in *E. anacua* extracts.

Cardiac glycosides are naturally occurring compounds with a steroidal framework and are used for the treatment of congestive heart failure and as anti-arrhythmic agents. One example of a cardiac glycoside was extracted from the *Digitalis lanata* leaf. The cardiac glycoside is a digoxin, a moderately polar compound used for the treatment of different heart conditions and not reported for any anti-bacterial activity; therefore, this secondary metabolite present in the ethanol *E. anacua* extract is probably not the compound exhibiting the anti-*S. aureus* activity [48, 49].

Diterpenes are a class found in one of the major group of secondary metabolites, known as terpenoids; they contain 4 isoprene units creating a 20-carbon compound [50]. The terpenoids are diverse substances, ranging in polarity where most of the classes are insoluble in water. Nonpolar components would be extracted with the nonpolar solvent, diethyl ether. Several compounds in the *E. anacua* diethyl ether extract could be compounds of the class diterpenes. On the other hand, terpenes could also be present in the ethanol *E. anacua* extract. Terpenes are known to defend many plants against herbivores, by being toxins and feeding deterrents to many

Table 2. Determination of the presence of secondary metabolites for the ethanol and diethyl ether *Ehretia anacua* extracts through qualitative phytochemical tests

Chemical tests for secondary metabolites	Ethanol extract	Diethyl ether extract	Observations
Alkaloids	Positive	Positive	et: +++ (heavy flocculation with both reagents) de: ++ (turbidity with Wagner's reagent)
Diterpenes	Positive	Positive	emerald green solution
Sterols/ Triterpenes	Negative	Negative	et: beige in chloroform phase de: 4 phases present (beige to dark green)
Saponins	Negative	Negative	no foam present
Phenols	Positive	Positive	black solution
Flavonoids	Negative	Negative	dark olive green/ brown
Anthranol glycosides	Negative	Negative	Solution olive solution with white precipitate
Tannins	Negative	Negative	no precipitate present
Resins	Negative	Negative	no precipitate
Cardiac glycosides	Positive	Negative	et: dark emerald green solution de: light olive green solution
Cyanogenic glycosides	Negative	Negative	yellow picrate paper

**Observations: et=ethanol E. anacua extract; de=diethyl ether E. anacua extract*

herbivorous insects and mammals [50]. It is then possible, that diterpenes present in the ethanol and diethyl ether *E. anacua* extracts, could be the main secondary metabolites exhibiting the anti-*S. aureus* activity. As most terpenes are lipophilic, they readily interact with membrane proteins. Thus, terpenes can increase the fluidity and permeability of the membranes, which can lead to uncontrolled efflux of ions and metabolites and even to cell leakage, resulting in necrotic or apoptotic cell death [51]. The mechanism of action of terpenes for antimicrobial activity is not fully understood, but is thought to involve membrane disruption due to their lipophilic nature, consequently they can modulate the activity of membrane proteins and receptors or ion channels [51,52].

Plants produce a large variety of secondary compounds that contain a phenol, which are classified as phenolics. Plant phenolics are a chemically heterogeneous group of nearly 10,000 compounds. Some are soluble only in organic solvents, while others are water-soluble carboxylic acids. This explains the potential of phenol compounds to be extracted in both nonpolar diethyl ether and polar ethanol *E. anacua* extracts. Phenolics have been known to play a variety of roles in plants such as: serving as defenses against herbivores and pathogens; attracting pollinators; absorbing harmful ultraviolet radiation; or reducing the growth of nearby competing plants. Many simple phenolic compounds are important in plants as defenses against insect herbivores and fungi [50]. Consequently, phenols present in both ethanol and diethyl ether *E. anacua* extract could be contributing to the anti-*S. aureus* activity. The mechanism proposed for phenolics includes: phenolic toxicity to microorganisms via enzyme inhibition by the oxidized compounds; their reaction with sulfhydryl groups; or by building hydrogen, hydrophobic and ionic bonds, thus modulating their 3D structures and in consequence their bioactivities [51,52].

Alkaloids are organic heterocyclic nitrogen compounds, which make up 15,000 secondary metabolites [50]. They contain nitrogen, which is usually derived from an amino acid. Alkaloids are of considerable interest because of their medicinal properties. Since most alkaloids are alkaline, the nitrogen atom is protonated; they are positively charged and are generally water soluble [50]. As expected, both ethanol and diethyl ether *E. anacua* extracts extracted the alkaloid compounds. Many alkaloids are used as pharmaceuticals, including morphine, codeine, caffeine, and nicotine [50]. The role of chemical defense for plant alkaloids is supported by their wide range of physiological effects on animals and by the antibiotic activities that many alkaloids possess [53]. Several plant extracts, such as aqueous *Tamarindus indica* and ethanol *Morinda citifolia,* have exhibited antimicrobial activity, including *S. aureus*, where phytochemical screening finds that their major constituents are alkaloids [54,55]. Phytochemical analysis from the chemical tests showed that the ethanol and diethyl ether *E. anacua* extracts contain alkaloids. It is also possible that the alkaloids contributed to the observed anti-*S. aureus* activity. In particular, the pyrrolizidine alkaloids, frequently found in members in the Boraginaceae family, could be exhibiting this antimicrobial activity. However, these alkaloids render most plants toxic to mammals [51]. The mechanism of action of highly aromatic alkaloids is attributed to their ability to intercalate with DNA [56].

Therefore for both the ethanol and diethyl ether *E. anacua* extracts, we propose that alkaloids, diterpenes, and phenols contribute to the *S. aureus* activity. In this regard, our preliminary results will provide a basis for further investigations that will include quantitative phytochemical analysis of the secondary metabolites, HPLC (high performance liquid chromatography) for the isolation and purification of the bioactive compound in *E. anacua* and mass spectrometry for the identification of the specific phytochemical responsible for the anti-*S. aureus* activity in *E. anacua*. The isolation, purification, and identification of the bioactive compounds in the *E. anacua* extracts will be an exciting and impacting research endeavor especially for scientist in search for and/or in need of natural product against *S. aureus.*

4. CONCLUSIONS

Results showed evidence that the ethanol and diethyl *E. anacua* extracts possess anti *S. aureus* activity. Phytochemical analysis demonstrated that ethanol *E. anacua* extract was positive for alkaloids, cardiac glycosides, diterpenes, and phenols, while diethyl ether *E. anacua* extract was positive for alkaloids, diterpenes, and phenols. This is the first report on *E. anacua*'s antimicrobial activity and their secondary metabolites. Alkaloids, diterpenes, and phenols either individually or synergistically are likely responsible for the observed antimicrobial activity.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

ACKNOWLEDGMENTS

The authors would like to thank Ms. Sasha Kodet and the staff of the San Antonio Botanical Garden. The authors also would like to thank TAMIU GREAT Program (funded by Title V PPOHA Program United States Department of Education Award #P031M105048) for funding this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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