Peer reviewed **ORIGINAL ARTICLE**

EVALUATION OF ANTIMICROBIAL ACTIVITY USING *ARTEMISIA AFRA, PSIDIUM GUAJAVA AND ERYTHRINA LYSISTEMON* ON *STAPHYLOCOCCUS* SPECIES

NW Nsele *M Tech* | **S Moodley** *PhD (Medicine)*

Dept of Biomedical Sciences, Mangosuthu University of Technology, Umlazi, South Africa

Corresponding author: ***NW Nsele*** *| tel +27 31 907 7150 | nsele@mut.ac.za*

ABSTRACT

Background: Medicinal plants contain components of therapeutic value and provide an alternative form of available antibiotics to multiple infections. Development of microbial resistance to the available antibiotics has further enhanced the investigation of the role of antimicrobial activity of medicinal plants.

Method: Here we report the antimicrobial properties of *Artemisia afra, Psidium guajava and Erythrina lysistemon* extracts obtained by using two different extraction solvents. Extracts were subjected to tests using zones of inhibition, Thin Layer Chromatography and High Pressure Liquid Chromatography (HPLC).

Results: Pronounced antimicrobial activity was observed against *Staphylococcus aureus* using the ethanol extraction technique. However, no statistical significance was observed between the results produced using extracts from both methods A (according to dilution factor) and B (method 3a of the German Homeopathic Pharmacopoeia).

Conclusion: The potential for developing antimicrobials from plants *in vivo* provides a platform for phytomedicine and pharmacological studies.

KEYWORDS:

medicinal plants, extraction methods, *staphylococcus aureus*, antibiotics, antimicrobial sensitivity

INTRODUCTION

In bacteraemia, skin and wound infections, *Staphylococcus aureus (S aureus)* produces several virulent factors and toxins. This ability of *S aureus* increases the capacity of the bacteria to evade the human immune system.[1,2,3] Nevertheless the introduction of antibiotics has lowered the mortality rate of *S aureus* infections. However, the organism has rapidly developed resistance mechanisms against many microbial agents and antibiotic resistance has become a global concern.[4] The clinical efficacy of many existing antibiotics is threatened by the emergence of multidrug resistant pathogens.[5] Many infectious diseases have been known to be treated with natural products either as pure compounds or standardised plant extracts. As extracts, these provide unlimited opportunities for finding new drugs because of the unmatched availability of chemical diversity in herbal remedies. Therefore, researchers are increasingly turning their attention to natural medicine to develop drugs against microbial infections.[6,7,8] In view of these trends toward herbal medicine, many South Africans make use of plants as traditional healing remedies. Some of these plants include *Artemisia afra, Erythrina lysistemon* and *Psidium guajava*. Although use of natural medicine has not been scientifically validated, these plants are used to treat certain infections.[9]

Despite all these virtues, few studies have been conducted regarding indigenous medicine.[10] In developing strategies for treatment and prevention of disease, the medical and health community is assessing natural healing antibiotics for infectious organisms. Development of new treatments, are necessary, if these challenges are to be dealt with. In this study, the aim was to develop new treatment strategies which would encompass the development of new antimicrobials. The assessment of antimicrobial activity using both ethanol and water based extracts of three traditional medicinal plants (i.e. *Artemisia afra, Erythrina lysistemon* and *Psidium guajava*) on *S aureus* was evaluated to assist in the development of new antimicrobials. Here we report using a comparative study on the antimicrobial activity of these extracts. These extracts were obtained by two different methods, in order to choose which method gave the most efficient antimicrobial compound.

MATERIALS AND METHODS

Sample collection

Samples of leaves from *Artemisia afra* and *Erythrina lysistemon* and the bark from *Psidium guajava* were obtained and harvested early in the morning when the cells are more active.[11]

Methodology

Two methods were utilised, method A and method B as indicated below. Both methods utilised ethanol and water as solvents.

Extraction Method A

For all these two solvents (60% ethanol and water), the medicinal plants (*Artemisia afra, Erythrina lysistemon* and *Psidium*

guajava) were minced in an electrical mincer and weighed into a glass jar. Thereafter, one part of minced material was added to 3 parts of distilled water (1:3). The mixture was shaken for 5 minutes and left in a glass jar for 10 days. Mixtures were agitated once a day. At the end of the incubation period the mixtures were pressed through 100% cotton muslin cloth and filtered through a No 1 Whatman filter paper.[12] The resulting filtrates were aseptically transferred into labelled sterile bottles and stored at 4°C until use. All plants were left in the respective solvents for 10 days to ensure maximum extraction before filtration and dispensing.

Extraction Method B

Artemisia afra, Erythrina lysistemon and *Psidium guajava* extracts were prepared according to an adjusted method 3a of the German Homeopathic Pharmacopoeia.[13]

Preparation of the Water-Based Extraction of *Artemisia afra, Erythrina lysistemon* and *Psidium guajava*

Artemisia afra, Erythrina lysistemon (fresh plant part above ground) and *Psidium guajava* (bark) were harvested early in the morning. Plant material was immediately minced and one part was added to three parts of distilled water (1:3) according to calculation 1, 2 and 3 (Appendix A). The mixture was shaken for 5 minutes and left in a glass jar for 10 days at a temperature not exceeding 20°C. Mixtures were agitated once a day. At the end of the incubation period the mixtures were pressed through 100% cotton muslin cloth and filtered through a No 1 Whatman filter paper.[12] Volumes were made up by adding distilled water.

Preparation of the Ethanol Tincture of *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* extracts

The sample preparation was prepared as for the water based extraction. However, the dilutions were made using 86% ethanol according to calculation 4 (Appendix A). After the calculation and preparation the final ethanol tincture was at 60% made up to volume with distilled water.

Artemisiaafra, Erythrina lysistemon (fresh plant part above ground) and *Psidium guajava* (bark) were harvested early in the morning. Plant material was immediately minced in an electrical mincer and weighed into a glass jar. One part of minced plant material was added to three parts of 86% ethanol (1:3) according to calculation 4 (Appendix A). The mixture was shaken for five minutes and left in a glass jar for 10 days at temperature not exceeding 20°C, agitating the mixture once a day. Thereafter, it was pressed through 100% cotton muslin cloth and filtered through a No 1 Whatman filter paper to attain a clear filtrate.[12] The ethanol tincture was completed using 60% ethanol to make up the volume. Distilled water was used as a control preparation of 60% (v/v). The ethanol control was prepared using 65.90ml of 96% (v/v) ethanol diluted with sufficient distilled water to produce 100ml of 60% ethanol.[12]

Antibiotic Assay (AA) Discs

Antibiotic Assay discs purchased from Davies Diagnostics (Batch number 277653) were used as recommended by Invernizzi (2002).[14] Mueller Hinton agar, nutrient agar slopes and nutrient broth were prepared according to manufacturer's instructions. Blood Agar Base (BA) obtained from Oxiod was also prepared accordingly.

Microbial cultures and preparation of inoculums

The cultures of *S aureus* American Type Colony Collection (ATCC 29213) were maintained on nutrient agar slopes at 4°C and sub-cultured on to blood agar plates for 24 hours before use. These are known ATCC strains obtained from Davies Diagnostics (Pty) Ltd. A few colonies from the overnight cultures of *S aureus* were suspended in 5ml of nutrient in bijou bottles. This was swirled to allow even distribution of the culture. The suspension was then vortexed in a vortex mixer to enable adequate mixing. The suspension was made up to the equivalent of 0.5 McFarland turbidity standard as described by Thrupp (1980).[15]

Bacterial sensitivity testing (screening)

The methodology was in accordance with a modification of the Kirby-Bauer Antimicrobial Sensitivity Test Procedure.[16]

Inoculum containing 1 x 106 colony forming units (CFU) per millilitre (ml) was introduced on to the surface of Mueller Hinton Agar plates.

Inoculation of plates and Placement of Disc on the agar plate

Six Mueller Hinton agar plates were inoculated with *S aureus* using a sterile swab dipped into the well mixed overnight nutrient broth culture. The agar surface of each plate was inoculated in such a way that there was uniform growth. The plates were then divided into 5 quadrants and labelled as: water control, 60% ethanol control and vanillic acids control for *S aureus*, water-based extract of each plant and ethanol tincture of each plant. The antibiotic assay discs were soaked for 10 seconds in each solution, except for the amikacin and vancomycin discs which were purchased ready for use from Davies Diagnostics. Each disc was then gently placed on the surface of the agar plates and incubated at 37°C for 24 hours. After incubation, the plates were examined for the inhibition of growth around the discs. A positive result was indicated by a clear zone of inhibition surrounding the discs. Zone of inhibition was measured using a ruler and compared to those of the controls. Each extract testing was repeated six times to ensure consistency and to allow for statistical analysis. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by serially diluting the extracts using nutrient broth. Thin Layer Chromatography (TLC) was used to identify some active compounds. High Pressure Liquid Chromatography (HPLC) was utilised to confirm compounds identified by the TLC method.

Thin Layer Chromatography (TLC)

Thin Layer Chromatography plates were run using BAW: n-Butanol (80ml); Acetic acid (20ml); Water (50ml) as the mobile phase. Aliquots of prepared extracts of the active plant were spotted onto 20 × 20cm silica gel plates. The plates were allowed to run for ±4 hours in BAW and allowed to dry. Spots that were visible under normal light and ultraviolet light (254 and 366nm) were demarcated. Quercetin was used as the standard. The other aliquots of plant extracts were also run on the TLC plate using methanol (98ml): Water (2ml) as the mobile phase. The standard used on this plate was catechin. The bands formed were compared to the catechin standard. The spray used for quercetin identification was 0.5ml anisaldehyde, 10ml glacial acetic acid and 5ml concentrated sulphuric acid in 85ml methanol. The spray used for catechin identification was 1ml

concentrated sulphuric acid, 1ml acetic anhydride and 50ml chloroform. The retention factor (Rf) value was regarded as the distance the leading edge of component moves divided by the total distance the solvent front moves.

Developed TLC plates were first viewed under UV 254nm light, sprayed with anisaldehye reagent and sulphuric acid: acetic anhydride: chloroform reagent and again viewed using the same wavelength.

High Pressure Liquid Chromatography (HPLC)

The chromatographic system used was the Beckman HPLC system consisting of a double pump. Programmable solvent Module 126, Diode Array Detector module 168, with 32 Karat Gold software supplied with Beckman, Column C18 Bondapak 5µm dimension (250 x 4.6mm) was used. The chromatographic conditions were as follows: Mobile phase was Acetonitrile (80): water (20) for quercetin and methanol (4): 1 percent acetic acid in water (1) for catechin. Flowrate: 0.9ml/min; Reference standard: quercetin and catechin 2.0g dissolved in 10ml methanol. Sample size: initial extract in the assay was 25g/100ml. Injected volume was 5µl. The run time was 30min. The retention time for each standard was then compared to the retention time for the ethanolic extracts of plants.

Statistical Procedures

Water and ethanolic extracts from each plant were tested six times with *S aureus* organism. In total, the experiment was repeated 36 times for each plant extract. For each replication the zone of inhibition produced, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were recorded. The response of *S aureus* was also coded: growth or no growth. The number of replicates was determined in consultation with the statistician.

A Fisher's exact test was used to compare the number of replications that responded with the organism in each extract (ethanol and water) separately. The response was compared between the two extracts using Fisher's exact test to determine which extract was the more effective. If there were no differences between the extracts, the data was pooled. The analysis was repeated for each plant extract.

A second analysis compared the zones of inhibition measured in millimetres using a Kruskal Wallis test because of the small numbers of samples. The comparisons followed the same analysis pattern as the bacterial response.

RESULTS

The results obtained, indicated that the ethanol extracts of all three plants were the most active irrespective of the method of extraction. The activity was pronounced against *S aureus*. Water extracts of all three plants demonstrated no antimicrobial activity against the organism. There were no statistical significant differences between the results produced by extracts from method A and from method B. This experiment was able to produce zones of inhibition against *S aureus*, supporting the organism's antimicrobial sensitivity to the extracts of *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* (Table 1).

Interpretation of results for *S aureus*

The alternative hypothesis (H1) is accepted since $p \leq \alpha$ (0.05). Thus, there are significant differences in diameter of zones of inhibition between *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* tincture and 60% ethanol control on *S aureus*. This effect was also shown by the zones of inhibition produced in the Kirby Bauer Antimicrobial Sensitivity Test (Table 2).

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MICs and MBCs were performed on ethanolic extracts *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* since all demonstrated zones of inhibition against *S aureus*. The MIC and MBC for *Artemisia afra* indicated that the most effective dilution against *S aureus* was 1 in 2 with 83.33% effectiveness and the least effective dilution was 1 in 8 with 33.33% effectiveness. For *Erythrina lysistemon* the most effective dilution is 1 in 32 with 83.33% inhibition of growth and the least effective dilution being 1 in 64 with 16.67% inhibition of growth. Lastly the minimum inhibitory concentration and minimum bactericidal concentration for *Psidium guajava* demonstrated that the most effective dilution against *S aureus* was 1 in 32 with 100% no growth and the least effective dilution was 1 in 128 with 66.67% no growth (Table 3).

Identification of the active compound

An attempt was made to isolate two active compounds that are responsible for the inhibition of bacterial growth.

The presence of flavonoids was detected in the form of visible spots on the TLC plates. Spots similar to quercetin standard on one plate and catechin standard on another plate were observed. The RF value of the quercetin standard was 0.91 and that of the sample was also 0.91. After spraying the plate, the

Table 1: Zones of inhibition for *S aureus* using two extraction methods

	Extraction Method A	Extraction Method B
Extracts	*S aureus* zones of inhibition	*S aureus* zones of inhibition
Water control	0 mm	0 mm
Ethanol control	1 mm	1 mm
AA water extract	0 mm	0 mm
AAEthanol extract	5 mm	6 mm
El Water extract	0 mm	0 mm
El Ethanol extract	4 mm	4 mm
Ps Water extract	0 mm	0 mm
Ps Ethanol extract	4 mm	5 mm

AA – Artemisia afra; El – Erythrinalysistemon; Ps – Psidiumguajava

Table 2: Statistical analysis of results for extracts of *A afra*, *E lysistemon* and *P guajava*

Extracts for *S aureus*	P value	α value
A afra	0.003	0.05
E lysistemon	0.005	0.05
P guajava	0.003	0.05

P value = probability; α value = probability of rejection inhibition

Table 3: *S aureus* growth with MIC and MBC of various concentrations with *A afra, E lysistemon* and *P guajava* extracts

Dilutions	**1:2**		**1:4**		**1:8**		**1:16**		
Extracts	G%	NG%	G%	NG%	G%	NG%	G%	NG%	Total %
ethanol control (60%)	100	0	100	0	100	0	100	0	100
***A afra* ethanol (60%)**	16.67	83.33	33.33	66.67	66.67	33.33	100	0	100
Dilutions	**1:2 to 1:8**		**1:16 to 1:32**		**1:64**		**1:128 to 1:256**		
***Elysistemon* ethanol (60%)**	0	100	16.67	83.33	83.33	16.67	100	0	100
Dilutions	**1:2 to 1:8**		**1:16 to 1:32**		**1:64 to 1:128**		**1:256**		
***P guajava* ethanol (60%)**	0	100	0	100	33.33	66.67	100	0	100

G = growth; NG = no growth

Table 4: Results of thin layer chromatography and retention times of the compounds separated by HPLC

Compound	**Standard**	**A afra**	**E lysistemon**	**P guajava**
Quercetin	Rf value = 0.91	-	-	Rf value = 0.91
	Retention time = 2.33	-	-	Retention time = 2.47
Catechin	Rf value = 0.86	-	-	Rf value = 0.86
	Retention time = 2.43	-	-	Retention time = 2.42

band of quercetin standard was yellow and it was similar to one of the bands of *Psidium guajava* tincture test sample. On another plate, the RF value of catechin standard was 0.86 and that of the sample was also 0.86. After spraying the plate, the band of catechin standard was brown-yellow similar to one of the bands of *Psidium guajava* tincture test sample (Table 4). These spots were not observed for both *Artemisia afra* extracts and the *Erythrina lysistemon* extracts.

DISCUSSION

All three plants in this study, namely: *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* demonstrated the presence of antimicrobial activity. However, the method of extraction and the solvent used, influenced that antimicrobial activity. Experimentally, zones of inhibition were noted against *S aureus*. Traditionally, most plant extracts are prepared with water. In this particular study ethanol extracts were also prepared. Inhibitory zones were seen in plant extracts using ethanol, but were not seen in plant extracts using water. This is perhaps the reason why traditional healers may not sufficiently extract the compounds, which are responsible for antibacterial activity on organisms seen in numerous infections.[8,9] Alternatively, metabolic effects occurring *in vivo* may be responsible for activation of certain compounds in the human body irrespective of the type of solvent used for extraction. These metabolic processes depend on temperature, pH and other factors present *in vivo* that are absent *in vitro*.

Observation on the zones of inhibition differed for each plant extract. This indicates that the activity of the plant extracts have variable potency against *S aureus*. Some researchers have reported similar findings with various alternate plant extracts.[17,18,19] Using the caveat of processing, these findings were supported by the difference in the MICs and MBCs for *Artemisia afra, Erythrina lysistemon* and *Psidium guajava* against *S aureus*. The minimum inhibitory concentration and minimum bactericidal concentration for *Artemisia afra* indicated that the most effective dilution against *S aureus* is 1 in 4 with 83.30% inhibition of growth and the least effective dilution is 1 in 8 with 33.33% inhibition of growth. For *Erythrina lysistemon* the most effective dilution is 1 in 32 with 83.33% inhibition of growth and the least effective dilution was 1 in 64 with 16.67% inhibition of growth. When viewing the MIC and MBC for *Psidium guajava* it was demonstrated that the most effective dilution against *S aureus* was 1 in 32 with no growth and the least effective dilution was 1 in 128 with 66.70% inhibition of growth. The results revealed that all plant extracts were potentially effective in retarding and suppressing microbial activity against *S aureus*.

Techniques using TLC and HPLC identified the two active compounds as quercetin and catechin. Both quercetin and catechin are not completely dissolved in water and as a result, the controls were prepared using 60% ethanol. These compounds were isolated from the ethanolic tincture of *Psidium guajava*. However, active compounds were not present on *Erythrina lysistemon* and *Artemisia afra* plant extracts. The premise is that there are other compounds responsible for antibacterial activity in these particular plant extracts. A possible explanation could be that the water-based extract of these plants did not possess these two compounds. It is not surprising that there are differences in the antimicrobial effects of plant species, due to their phytochemical properties and differences among the various species. In this study it is quite possible that some of the plants were ineffective because they did not possess antibiotic constituents. Alternatively, the plant extracts may have contained antibacterial constituents, but not in sufficient concentrations to be effective. It is also possible that the active chemical constituents were not soluble in ethanol or water.[20] In conclusion, even although the microbial activity of certain plant extracts against *S aureus* is providing evidence of their biological activity, further investigations of these extracts is warranted.

According to the Kruskal Wallis test, the Chi-square was 0.001 for *Psidium guajava*, and 0.035 for both *Erythrina lysistemon* and *Artemisia afra*, against *S aureus*. This activity could possibly be due to the zones of inhibition, which were reduced in ethanol extracts of the dried plants. As a corollary of this, we observed that the extracts of dried plants were less active than that of the extracts of fresh plants. *S aureus* is one of the organisms that cause food poisoning with subsequent diarrhoea. By inference, the antibacterial activity of *Psidium guajava* extracts could be utilised in cases of diarrhoea infections caused by *S aureus*.

CONCLUSION

The development of antimicrobials from higher plants will potentially lead to the development of phytomedicine. Plant-based antimicrobials have enormous therapeutic possibilities, as the extracts can be used as antimicrobial inhibition against various microbes, with fewer side effects than those associated with synthetic antimicrobials.[21,22]

Continued research of plant-derived antimicrobials is required in order to determine the identity of the antibacterial compounds in plant extracts. As an example, this research could observe the effects of these extracts pertaining to the toxicity of cells. Recommendations from this study could serve as the catalyst for future developments of antimicrobials from various traditional plants.

APPENDIX A

Calculation 1: Water-based extraction of *Artemisia afra*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (50g) and D = % of drying (74.00%)
Start weight = 5g
End weight = 1.3g
Water lost weight = 3.7g
Therefore % weight loss = 74%
Substitution in the formula = 2x50x74÷100
= 74ml of distilled water was added to 50g of minced plant

Calculation 2: Water-based extraction of *Erythrina lysistemon*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (70g) and D = % of drying (74.60%)
Start weight = 5g
End weight = 1.27g
Water lost weight = 3.73g
Therefore % weight loss = 74.60%
Substitution in the formula = 2x70x74.6÷100
= 104.4ml of distilled water was added to 70g of minced plant

Calculation 3: Water-based extraction of *Psidium guajava*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (45g) and D = % of drying (67.50%)
Start weight = 4g
End weight = 1.35g
Water lost weight = 2.7g
Therefore % weight loss = 67.50%
Substitution in the formula = 2x45x67.50÷100
= 60.75ml of distilled water was added to 45g of minced plant

Calculation 4: Ethanol tincture preparation of *Artemisia afra*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (50g) and D = % of drying (74.00%)
Start weight = 5g
End weight = 1.3g
Water lost weight = 3.7g
Therefore % weight loss = 74.00%
Substitution in the formula = 2x50x74.00÷100
= 74ml of 86% ethanol was added to 50g of minced plant

Calculation 5: Ethanol tincture preparation of *Erythrina lysistemon*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (50g) and D = % of drying (74.00%)
Start weight = 5g
End weight = 1.3g
Water lost weight = 3.7g
Therefore % weight loss = 74.00%
Substitution in the formula = 2x50x74.00÷100
= 104.4ml of 86% ethanol was added to 70g of minced plant

Calculation 6: Ethanol tincture preparation of *Psidium guajava*

The weight of water from the plant was eliminated using the following calculation:
E3 = 2MD ÷ 100 where M = plant weight (50g) and D = % of drying (74.00%)
Start weight = 5g
End weight = 1.3g
Water lost weight = 3.7g
Therefore % weight loss = 74.00%
Substitution in the formula = 2x50x74.00÷100
= 60.75ml of 86% ethanol was added to 45g of minced plant

CONFLICT OF INTEREST STATEMENT

We declare that there is no conflict of interest in this manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank Mangosuthu University of Technology for their financial support. We would also like to acknowledge all the laboratory staff who contributed their time to assist in laboratory procedures.

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