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Effect of Vancomycin, tetracycline, *Persia americana* leaf extract and combinations thereof on antibacterial activity against pathogenic organisms

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Abstract

Many pathogenic bacteria have emerged resistance to drugs leading to high cost of treatment. Alternatively, people simultaneously use herbal and conventional medicine to combat infections without scientific evidence on their effectiveness. In an attempt to find out effectiveness of vancomycin, tetracycline, *P. americana* leaf extract and their combinations against *S. aureus*, *S. pyogenes* and *P. aeruginosa* a laboratory experiment was conducted. MIC and antibacterial activity were determined by macrodilution and disc diffusion methods respectively. Lowest values of MIC and MBC of extract were observed against *S. aureus*. Zone of inhibition (ZI) ranged from 0 to 31.00mm for antibiotics. Extract was more effective against *S. aureus* with ZI of 13.00 mm. Combination of vancomycin with extract showed highest improvement of 36.12% against *S. aureus*. Potency reduction was observed for tetracycline against *P. aeruginosa*. Therefore, addition of plant extract to antibiotics is being recommended as a best way of improving their potency.

Keywords: Effectiveness, *Persia americana*, leaf extract, antibacterial, potency, combination

1. Introduction

The impact of antibiotics in treatment of infectious diseases has dramatically declined in various settings. Often it is pathogens that cause foodborne and nosocomial infections such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* which have emerged resistance to several drugs due to improper use [1-5]. In Malawi, several attempts and efforts have been made to contain antibacterial resistance such as surveillance, training on proper prescription and drug use. Previously, there had been a call by WHO to integrate traditional and biomedical health systems in order to prevent, control and treat infections in Sub-Saharan Africa and no country has yet implemented this collaborative healthcare system including Malawi [6-8]. Alternatively, people use herbal medicine to fight antibiotic resistance either as a stand-alone or complementary therapy due to high cost of effective drugs and lack of adequate health facilities [9, 10]. Complementing antibiotics with herbal remedies may either improve or reduce their potency. However, studies on synergistic effect of local *Persia americana* leaf extract with Vancomycin and tetracycline in treatment of *S. aureus*, *S. pyogenes* and *P. aeruginosa* have not been done on Malawian plants or elsewhere. *P. americana* leaf contains bioactive compounds such as tannins, flavonoids, saponins, terpenoids, steroids, alkaloids and glycosides [11-15]. These compounds are responsible for their antibiotic properties and are considered comparatively safe when used correctly [14, 11, 16-18]. But others argued that the use of herbal medicine is not safe as people tend to sell them without proper scientific validity, lack of suitable policies and the fact that they can be contaminated by biological and chemical contaminants [19-23]. Until recently, there is no national policy, registration system laws and regulation of herbal medicine in Malawi [24]. One of the most used medicinal plants in Malawi is *P. americana* which is used to treat diarrhoea, dysentery, toothache, chronic gastritis and diabetes mellitus despite lack of recommended dosage. Dissatisfaction with conventional medicine has prompted Malawians to take conventional medicine (CM) and traditional medicine (TM) simultaneously in order to enhance recovery rate and alleviate sufferings associated with various diseases. The prevalence of simultaneous use of CM and TM is yet to be assessed, however, anecdotal evidence suggests that utilization of medical pluralism is common in Malawi and other Sub-Saharan Africa. Scientifically, it is safe to combine bioactive compounds from medicinal plants and conventional medicine [25]. Growing evidence suggests that herbal medicine enhances the effectiveness,

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reduces undesirable effects, increases stability and bioavailability of conventional drugs when used correctly [26-30].

This study will therefore help to curb antibiotic resistance, reduce severity of infections and duration of illness by improving effectiveness and bioavailability of aforementioned conventional drugs which are already on the market. The main aim of the study was to determine combined antibacterial activity of methanolic *P. americana* leaf extract with vancomycin and tetracycline against *S. aureus*, *S. pyogenes* and *P. aeruginosa*.

2. Materials and Methods

2.1. Collection of plant materials and microorganisms

Fresh mature leaves of *P. americana* were collected from Malawi Adventist University, Malamulo Campus in Thyolo district, Malawi. The leaves were collected during rainy season of February to March 2018. The plant's identity was confirmed by a botanist. The organisms that were used in this study were gram positive *Staphylococcus aureus* (ATCC 29213), *Streptococcus pyogenes* (ATCC 19615) and *Pseudomonas aeruginosa* (PSA). These organisms were obtained from Malawi Liverpool Welcome Trust.

2.2. Culture media, chemicals and antibiotics

Culture media, antibiotics and chemicals used for the present work were purchased from Lapken Suppliers in Malawi. All the laboratory materials used for extraction were suitable for laboratory use and were used as received without any further purification or treatment. Muller Hinton agar culture media was used for *S. aureus* and *P. aeruginosa* and blood agar for *S. pyogenes*. Methanol was used for extraction and antibiotics that were used are vancomycin and tetracycline.

2.3. Preparation of *P. American* leaf extract

The leaves were washed with running distilled water to remove contaminants and then shed dried for 7 days. The dried leaves were ground into fine powder using a laboratory mill. About 20g of powder were weighed and soaked in 100ml of 80% methanol for a period of 48 hours. The mixture was agitated to ensure proper mixing after addition of the solvent. They were then filtered using Whatman filter paper No.1 to obtain 100% concentration of the extract. The extract was stored in a refrigerator at 4 °C until ready for experiment.

2.4. Standardization of test isolates

The 24 hour old five colonies of test organisms from pure culture were transferred into 4 ml sterile saline solution which was adjusted to obtain a turbidity visually comparable to 0.5 McFarland standard at a well illuminated place with a white background [31]. The prepared inocula was used within 15 minutes from preparation time.

2.5. Determination of antibacterial activity of the antibiotics, leaf extract and their combinations

Antibacterial activity was done using disc diffusion method [32]. Filter paper discs (6 mm in diameter) were impregnated with leaf extract of 100% concentration and ready-made antibiotic discs were placed on the media that were previously inoculated with the standard inocula. For synergistic (combined) effect 10 µl of the extract was added to the antibiotic discs and then placed on the surface of inoculated media with *S. aureus*, *S. pyogenes* and *P. aeruginosa* separately. They were incubated overnight at 37 °C together with the controls. Antibacterial activity was assessed by

measuring the diameter of the zone of inhibition (clear area around the disc) in mm.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract

Two-fold serial broth macro dilution method was used to determine MIC [32]. The 100% concentration of the extract was serially diluted to achieve 1.5 mg/ml, 3.13 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml and 50 mg/ml and then standardized inocula of 0.1 ml of the test organism and extract were poured into 2 ml peptone broth tubes. Broth plus inoculum and uninoculated tubes containing broth plus extract were included as controls to check viability of inoculum and sterility respectively. Then the tubes were incubated at 37 °C for 24 hours. MIC was determined as the lowest concentration that completely inhibited visual growth of organisms. The tubes that did not show growth were streaked on Muller Hinton agar plates. The plates were incubated for 24 hours at 37 °C. The lowest concentration of the extract that did not show any growth of organisms was considered to be MBC.

2.7. Data analysis

Laboratory analysis was performed in triplicates. The data was analyzed using Statistical Package for Social Science version 20.0.

3. Results

3.1. MIC and MBC of methanolic *P. americana* leaf extract against *S. aureus* and *S. pyogenes*

Data on the MIC and MBC of methanolic leaf extract of *P. americana* against *S. aureus* and *S. pyogenes* are provided in Table 1. The preliminary tests showed that the extract had highest MIC of 12.5 mg/ml and MBC of 25 mg/ml against *S. aureus* while lowest MIC and MBC were both observed for *S. pyogenes*. The observable growth was recorded for the leaf extract concentrations below 6.25 mg/ml while concentrations above 12.5 mg/ml showed no observable growth for both aforementioned bacteria. Nearly all the concentrations of the extract did not inhibit growth of *P. aeruginosa*.

Table 1: MIC and MBC of methanolic leaf extracts of *P. americana* against test organisms

Organism	MIC/MBC	Concentration of <i>P. americana</i> leaf extract (mg/ml)					
		1.5	3.125	6.25	12.5	25	50
<i>S. aureus</i>	MIC	+	+	+	-	-	-
	MBC	+	+	+	+	-	-
<i>S. pyogenes</i>	MIC	+	+	-	-	-	-
	MBC	+	+	+	-	-	-
<i>P. aeruginosa</i>	MIC	+	+	+	+	+	+

Key: + Observable growth (Turbidity/ colonies), - No observable growth (No Turbidity/ No colonies).

3.2. Antibacterial activity of the antibiotics, leaf extract and combinations thereof

The results of antibacterial activity of Vancomycin, tetracycline, methanolic *P. americana* leaf extract and their combinations against test organisms are summarized in Table 2. Vancomycin showed greatest potency against *S. pyogenes* but not against *P. aeruginosa*. Similarly, *S. pyogenes* was the most susceptible organism against tetracycline while *P. aeruginosa* was the least susceptible to tetracycline. Antibacterial activity test showed that extract alone was more effective against *S. aureus* but did not show inhibiting effect

on *P. aeruginosa*. Individually, it was observed that only tetracycline had antibacterial activity against *P. aeruginosa*. Although vancomycin and extract were combined, their effect was not enough to inhibit growth of *P. aeruginosa*. However,

combined effect of vancomycin and extract showed greater potency against *S. pyogenes* just like in tetracycline plus extract.

Table 2: Antibacterial activity of antibiotics, extract and their combinations against test organisms

Organisms	Zone of inhibition (mm)				
	Vancomycin	Tetracycline	Extract	Treatment Combination	
				Vancomycin + extract	Tetracycline + extract
<i>S. aureus</i>	15.67 ± 1.53	28.00 ± 2.00	13.00 ± 0.00	21.33 ± 2.31	31.00 ± 2.00
<i>S. pyogenes</i>	19.33 ± 0.58	31.00 ± 1.41	10.00 ± 0.00	23.33 ± 1.15	35.33 ± 0.57
<i>P. aeruginosa</i>	0.00 ± 0.00	12.33 ± 1.15	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 0.00

Values are means ± standard deviations of triplicate determinations.

Of all the two combinations, addition of the extract to vancomycin showed the greatest improvement against *S. aureus* while the least improvement was recorded for tetracycline against aforementioned organism (Figure 1). It was noted that when the extract was added to tetracycline a reduction effect of -35.12% was observed while treating vancomycin with the same concentration of the extract no observable effect against *P. aeruginosa* was displayed. The percent improvement of the antibiotics ranged from 10.71% to 36.12%. Nearly all the combined formulations did not show any improvement against *P. aeruginosa*.

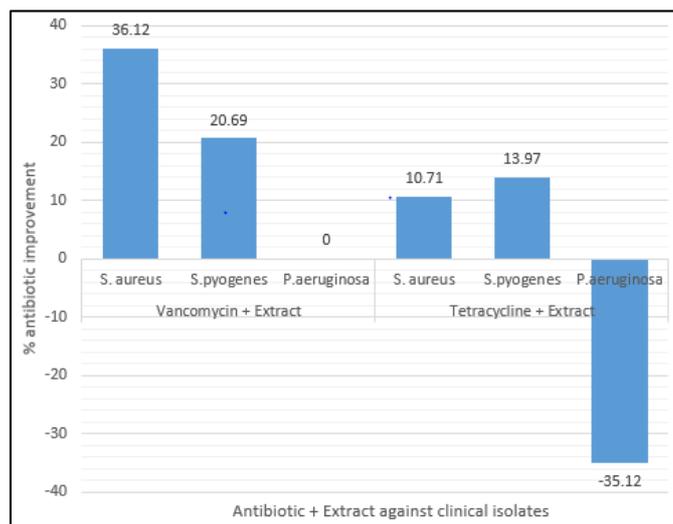


Fig 1: Percent improvement of antibiotics following addition of the extract.

4. Discussion

Medicinal plants produce various secondary metabolites in expression of self-defense against several stimulants. *P. americana* leaf extract contains bioactive compounds with antibacterial activity against selected organisms [11, 16]. The data from this study indicates that the extract was able to inhibit growth of *S. aureus* and *S. pyogenes* but not *P. aeruginosa* as evidenced by MIC. Low MIC and MBC observed for *P. pyogenes* means that it would be cost effective to use the extract alone against *S. pyogenes* because management and treatment of *S. pyogenes* infection would require little amount of plant materials hence conservation of plants and money unlike *S. aureus*. It is likely that these differences resulted from the variation in genetic makeup of these organisms that determines susceptibility of an organism to particular antibiotics. For instance, gram negative *P. aeruginosa* as expected showed resistance to the extract due to the nature of their cell wall [33, 34]. The MIC and MBC of the extract against *S. aureus* and *S. pyogenes* reported herein

are comparatively lower than those that had been previously published by others [11, 14]. Interestingly, others reported that *P. americana* leaf extract inhibited growth of *P. aeruginosa* and MIC had ranged from 30 mg/ml to 50 mg/ml [11, 14]. These differences could be attributed to the variation in solvents and their preparations, the strain of test organism and variety of *P. americana*. It is also well known that metabolism and accumulation of bioactive compounds in plants are highly dependent on environmental factors such as altitude, temperature, sunshine duration, light, soil, precipitation, humidity and moisture, hence variation in these findings [35-40]. An anecdotal evidence shows that level of maturity of the leaf also influences antibacterial activity although we could not find scientific evidence to support this claim.

There was clear indication that species type contributes to susceptibility to bioactive components, thus different species respond differently to the same drug due to genetic variation [41]. Data from this study clearly indicate that vancomycin, tetracycline and the extract were effective in inhibiting the growth of *S. aureus* and *P. aeruginosa* separately while *P. aeruginosa* was resistant to both vancomycin and the extract. It is worth mentioning that in the preliminary determination of MIC and MBC, the extract showed greatest potency against *S. pyogenes* but surprisingly, disc diffusion method showed that the extract greatly inhibited growth of *S. aureus* more than *S. pyogenes*. These results may suggest that the potency of the extract against *S. pyogenes* became compromised following incorporation of the extract into the disc. Perhaps change of physical nature of the extract from a solution state to solid form of the disc favored activity against *S. aureus*. The zone of inhibition for the extract against *S. aureus* was within the range of 6.0 mm to 15 mm reported by others [11, 14]. The contrast in these findings were due to various factors as indicated above. Resistance of *P. aeruginosa* to vancomycin was expected due to nature of the bacterial cell wall, endogenous inactivation of the drug, efflux pump action and possibility of acquired resistance therein [42-44]. Unlike in the present study, others reported presence of zone of inhibition for the extract against *P. aeruginosa* [11, 14]. The data on antibacterial activity of the extract constitute a rational evidence and scientific basis to justify and support the use of *P. americana* leaf extract for the treatment of *S. aureus* and *S. pyogenes*. This implies that the potency of these commercial drugs can be improved by combining them with the leaf extract.

In this study, the synergistic and additive interactions between bioactive compounds of the extract and the antibiotics were revealed. The presence of synergistic interaction implies that combination of vancomycin and tetracycline with the extract can be translated into useful clinical application in treatment and control of *S. aureus* and *S. pyogenes* infections. These

findings significantly contribute to development of effective antibiotics. On the other hand, the findings are also beneficial to those who practice medical pluralism by taking simultaneously herbal and conventional medicine. Considering that in Malawi and Sub-Saharan Africa, people simultaneously use herbal medicine and conventional medicine, the present study has added evidence that taking vancomycin or tetracycline together with *P. americana* leaf extract would be beneficial. This will help to curb antibiotic resistance, reduce severity of an infection and duration of illness. It is noteworthy that addition of the extract to tetracycline displayed antagonism (Figure 1). It is likely that the reduction effect resulted from inability of the extract to penetrate the outer membrane present in gram negative organisms, lipopolysaccharide layer that hinders access of most compounds. It is also assumed that some constituents of the extract interfered with active components of tetracycline resulting into inactive complexes^[45].

Based on our results, public awareness is needed to cost effectively use herbal remedies together with antibiotics. However, it should be stressed that it is not ideal to combine tetracycline with *P. americana* leaf extract against *P. aeruginosa* as it reduced the activity of the antibiotic with -35.12% reduction rate. However, combination of vancomycin and the extract would give the best results due to high improvement rate especially against *S. aureus* and *S. pyogenes*. Further investigations on bioavailability of combined treatments and their effects in the human body should be high priority as these were *in vitro* analyses.

5. Conclusion and Recommendations

The present study has provided evidence that the potency of the antibiotics can be improved by combining them with *P. americana* leaf extract. The results obtained have shown that the formulation of vancomycin with the extract greatly inhibited the growth of *S. aureus* and *P. aeruginosa*. These findings have potential implications in preventing antibiotic resistance, accelerating recovery rate and reducing cost of treatment. Based on our results, public awareness is needed to cost effectively use herbal remedies together with antibiotics. There is also a need to raise public's knowledge about health risks associated with combining tetracycline and the extract.

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