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Assessment of synergistic antibacterial activity of combined ethanolic leaf extracts of Azadirachta indica and *Persea americana* against Staphylococcus aureus

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ABSTRACT

Staphylococcus aureus (S. aureus) is one of the most dangerous bacteria because of the fatal infections that result from its colonization and multiplication in susceptible host organisms like human beings. This has led to the misuse of antibiotics, causing resistance of S. aureus against commonly used antibiotics. This continued use and misuse of antibiotics has resulted in the emergence of multidrug-resistant microorganisms, which is becoming a huge problem in animal farms, clinical human healthcare settings, and the community. *P. americana* and *A. indica* have both been used by locals in the management of numerous conditions ranging from infectious to non-infectious diseases. The main aim of this research is to assess the synergistic antibacterial activity of extracts of *P. americana* and *A. indica* against *S. aureus* in comparison to individual plant extracts. The study was experimental, involving the extraction of the plant leaves by maceration of dry powder in 70% ethanol for 3 days. The extracts were tested for the presence of phytochemicals, and the antibacterial activity was demonstrated using the agar well diffusion method. Results from phytochemical screening showed the presence of phenols, tannins, saponins, flavonoids, and terpenes in both plant extracts. The yields from the extracts of P. americana and A. indica were 16.77% and 12.59%, respectively. The MIC of P. americana was 6.25 mg/ml, and for A. indica, it was 25 mg/ml against S. aureus ATCC 25953. All extracts showed significant activity in comparison to vancomycin. The combination of the extracts was found to have a significant effect due to synergism with an FIC of 0.438 at a ratio of 1:3 and an FIC of 0.143 at a ratio of 3:1 of P. americana to A. indica. The extracts at a ratio of 1:1 showed indifference with an FIC of 1.25.

Keywords: Synergistic antibacterial activity; Combined ethanolic leaf extracts; Azadirachta indica; Persea Americana; Staphylococcus aureus

INTRODUCTION

Staphylococcus aureus is a dangerous bacterium due to the fatal infections that result from its colonization and multiplication in susceptible host organisms, including humans [1, 2]. Susceptibility to S. aureus infections increases with reduced host immunity caused by HIV/AIDS, organ transplants, immune-suppressants like corticosteroids, and cancer therapy[3–5]. Mild skin and soft tissue infections caused by S. aureus can be self-limiting, but moderate to severe infections can progress to life-threatening conditions such as endocarditis, septic arthritis, osteomyelitis, or bacteremia if not treated appropriately. Globally, the prevalence of MRSA (Methicillin-resistant Staphylococcus aureus) is progressively increasing, with significant regional variation [6]. The high prevalence of MRSA in Uganda is attributed to the empirical treatment of S. aureus infections without laboratory confirmation to guide therapy in health and veterinary care settings. The continued use and misuse of antibiotics have resulted in the emergence of multidrug-resistant microorganisms, becoming a significant problem in animal farms, clinical human health care settings, and the community [7]. Over the past two decades, this has necessitated the review of natural sources of drugs to develop herbal remedies for these resistant strains of microorganisms globally [8]. Alternative and complementary medicines have been used in the treatment of numerous diseases since early humankind [9]. The WHO estimates that about 80% of the population in developing countries, including Uganda, rely on traditional medicine for their primary healthcare needs [10] due to its wide availability and accessibility to communities. Plants have been a major source of many herbal therapeutic agents for many years, and their medicinal properties are due to the phytochemicals they produce. *Persea americana*, locally known as "focado" in Lugisu, is endowed with many secondary metabolites such as tannins, saponins, flavonoids, alkaloids, anthraquinones, phenols, terpenes, and phlobatannins [11]. It has been used in managing diabetes and inflammation, among other conditions, and has reported antimicrobial activity, including against S. aureus [12]. *Azadirachta indica* produces isoprenoid and non-isoprenoid phytochemicals such as diterpenoids, triterpenoids, azadirone and its derivatives, tannins,

glycosides, saponins, among others [13]. These chemical substances are responsible for most of the medicinal uses of this plant. Originating from India, it has been used to alleviate many diseases such as malaria, ulcers, skin diseases, boils, diabetes, and respiratory infections, among others [14]. It has also been shown to inhibit several microorganisms, including S. aureus [15]. This research, therefore, aims to demonstrate the synergistic antibacterial properties of A. indica and P. americana against S. aureus to develop local remedies that are cost-effective and potential *S. aureus* is a prominent cause of bacterial infections worldwide, with MRSA becoming increasingly common as a source of nosocomial infections [16]. These infections increase patients' hospital stays and the economic burden on families and the health sector in general. Antibacterial resistance is an increasingly serious threat to global public health, hindering the effective control, prevention, and therapeutic management of a rising range of infections caused by bacterial and other microorganisms. The high prevalence of S. aureus resistance to antimicrobials leads to increasing hospital admissions, management costs, and even death from bacterial infections. This situation also forces patients to seek more expensive drugs, often less available in developing countries. Despite the increasing bacterial infections caused by S. aureus and the rising resistance to available antibacterials, pharmaceutical companies are producing very few or no new antibiotics to replace those that are no longer effective for many bacterial infections [17]. Although the antibacterial activities of *P. americana* and A. indica individually have been shown against S. aureus, their combined antibacterial activity has not been assessed. The combination of *P. americana* and A. indica could potentially have better potency and a better toxicity profile due to the lower doses required.

METHODOLOGY

Study Design

This was an experimental study to assess the synergistic antibacterial activity of ethanolic leaf extracts of neem tree (*A. indica*) and avocado tree (*P. americana*) against S. aureus.

Study Area and Study Population

The extraction and demonstration of the antibacterial activity of the extracts of P. americana and A. indica against S. aureus were conducted at Kampala International University in the Pharmacognosy Laboratory and the Department of Microbiology Laboratory, respectively. The plants used were obtained from well-maintained gardens in Kitagatta. The study microorganisms were cultures of S. aureus (ATCC25953) attained from the Kampala International University Microbiology Laboratory.

Variables

Independent Variables

- Concentrations of P. americana ethanolic leaf extracts used.
- Concentrations of A. indica ethanolic leaf extracts used.

Concentrations of the combined ethanolic leaf extracts of A. indica and P. americana used.

Dependent Variables

Bacterial zones of inhibition.

Selection Criteria

Only healthy, pest-free, and disease-free plant leaves were collected and used in the analysis.

Extraction of *A. indica* and *P. americana*

A. indica

The leaves were washed with distilled water, air-dried in a shade for ten days, and ground into powder using a blender in the Pharmacy Chemistry Laboratory. About 300 g of the fine powder was macerated with 70% ethanol for 72 hours with frequent shaking. The extract was then filtered using Whatman No. 1 filter paper, dried using a hot air oven at 55°C, and stored in a refrigerator at 4°C until use.

P. americana

The leaves were washed with distilled water, air-dried for ten days, and ground into a fine powder using a mortar and pestle. A total of 100 g of the fine powder was macerated for 72 hours in 70% ethanol with frequent shaking. After maceration, the crude extract was filtered using Whatman No. 1 filter paper, dried using a hot air oven at 55°C, and stored at 4°C in a refrigerator.

The percentage yield of dried extracts of A. indica and P. americana was calculated using the formula:

$$\text{Percentage yield of extracts} = \frac{\text{Mass of the dried crude extract}}{\text{Mass of powdered plant material macerated}} \times 100\%$$

Preparation of the Agar

Mueller-Hinton agar (38 g) was suspended in 1 liter of distilled water, heated to boil to dissolve completely, and autoclaved at 121°C for 15 minutes to sterilize. The sterilized mixture was allowed to cool to 25°C before being poured into sterile petri dishes.

Inoculation of S. aureus on Mueller-Hinton Agar

A sterile swab was used to inoculate S. aureus onto the agar by dipping the swab into the stock of S. aureus and passing it over the surface of the dried agar three times to ensure even distribution. The plates were incubated at 37°C for 18-24 hours.

Phytochemical Screening

The extracts were qualitatively tested for the presence of saponins, flavonoids, tannins, phenols, and terpenes using standard methods described by[14]

Table 1: Phytochemical screening.

phytochemical	Procedure
Saponins	To 2ml of the extract was added 6 ml of distilled water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam which indicated the presence of saponins
Flavonoids	To 1 ml oftest extract in a test tube, was added a few drops of 2% NaOH, an intense yellow colour which turns colorless on addition of few drops of HCI confirmed the presence of flavonoids
Tannins	Extract (O. lg) was dissolved in 5ml of water. To 2ml of extract solution in a test-tube, was added 10% alcoholic FeCh solution (Braymer's test.). The fonnation of deep green colored solution indicated the presence oftannins.
Phenols	To 1ml of test extract in a test tube, was added few drops of 5% FeCh solution.The appearance of green-blue or deep blue (black) color indicated the presenceof phenolic compounds.
Terpenes	5ml of extract was mixed with 2ml of chloroform followed by careful addition of 3ml of concentrated sulphuric acid to form a reddish layer which confirmed the presence of terpenes.

Validation of Antibacterial Activity of *A. indica* and *P. americana* Extracts against S. aureus

The agar well diffusion method was used. Sterile nutrient agar was poured into sterile petri dishes at 45°C and allowed to solidify. S. aureus cultured in nutrient agar for 18 hours at 37°C was adjusted to 0.5 McFarland standard with sterile distilled water. The S. aureus suspension was inoculated onto Mueller-Hinton agar plates to make a lawn. Wells of about 5 mm diameter were made in the media using a sterile cork borer and filled with 100, 50, and 25 mg/ml of P. americana and A. indica extracts separately. Vancomycin (30 µg/ml) served as the positive control, and DMSO (10%) as the negative control. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured in mm and recorded. The experiment was done in duplicates.

Determination of the Effect of Combined Extracts of *A. indica* and *P. americana* Against S. aureus

Combinations of A. indica/P. americana were made in ratios of 25:75, 50:50, and 75:25 (v/v) and serially diluted. Minimum inhibitory concentrations (MICs) were obtained by agar dilution method. The functional inhibitory concentrations (FIC) of each extract in combination were determined using:

Determination of the effect of combined extracts of A. *indica and P.americana* against *S. aureus.*

The combinations of *A.indica/P.americana* were made in the ratios of 25:75, 50:50 and 75:25 (v/v). The prepared concentrations were diluted serially with dilution factor of2.

MICs of the extracts were obtained by agar dilution method (the initial concentration was 100mg/ml in the first test tube) by observing the tube that has minimum growth. The functionalinhibitory concentrations (FIC) of each of the extract in combination were determined using the equation below;

FIC (extract in combination) = MIC of the extract in combination/MIC of the extract a lone.

The sum of the FIC *(A. indica)* and FIC *(P. americana)* gave the FIC index from where an inferencewas drawn, thus the effect of combination was classified as synergism, additive ,indifference and antagonistic, if the FIC(index) was<l, =I, >1 S2, and >2 respectively [18].

Data Management and Analysis

Data were collected by direct measurement of the zones of inhibition using a calibrated meter ruler and tabulated. GraphPad Prism version 7 was used to obtain means and standard deviations. A one-way ANOVA test was run to compare the diameters of the zones of inhibition for different extracts. A P-value ≤ 0.05 was considered statistically significant.

Quality Assurance

Good laboratory practices (GLP) were ensured at all study levels, including sterilization of all instruments and glassware, proper labeling of all reagents, and sourcing culture media from certified suppliers. All study plants and microorganisms were identified by experienced taxonomists, and calibrated weighing balances and instruments were used.

Biohazardous Material Handling

All petri dishes contaminated by microorganisms were sterilized in an autoclave at 121°C for 15 minutes. Biohazardous and contaminated wastes were placed in red biohazard waste bags in the laboratory and disposed of accordingly.

Ethical Considerations

Permission to conduct the study was sought from the Institutional Review Board, School of Pharmacy, Kampala International University. Permission was also obtained from the Microbiology Department, School of Health Sciences, to use the Microbiology Laboratory and strains of S. aureus organisms.

RESULTS

Table 2: The percentage yield of the plant extracts

Plant	Amount of powder used(g)	Extraction yield(g)	Percentage yield (%)
P. americana	100.0	16.770	16.77
A.indica	300.0	37.777	12.59

Table 3: The results of phytochemical screening

Test	Observation [+=present, - =absent, +++= high concentration]	
	P. Americana	**A. indica**
Saponins	+++	+
Flavonoids	+	+
Tannins	+	+
Phenols	+	+
Terpenes	+	+

The antibacterial activity of the extracts of *P. americana* and *A. indica* at different concentrations.

The antibacterial activity of *P. americana* and *A. indica* extracts at lO0mg/ml and 50mg/ml is notstatistically significant, the p value at lO0mg/ml is 0.0740 and at 50mg/ml is 0.6402 which are greater than 0.05, and at 25mg/ml of the extracts, the antibacterial activity of *P. americana* and *A. indica* is significant because the p value is <0.0001. The antibacterial activity of lO0mg/ml of *P. americana* and *A. indica* extracts as compared to thepositive control(vancomycin) is statistically significant with the same p value of< 0.001 as shown in the table below.

Table 4: Mean and standard deviation of diameters of zones of inhibition at differentconcentrations of plant extracts and the controls.

Concentrations of the extracts used (mg/ml)	Mean inhibition zone diameter ±SEM (mm)	
	P. Americana	*A. indica*
100	13.5(±1.5)	10.0(±0.0)
50	11.0(±1.0)	9.5(±0.5)
25	10.50(±0.5)	0.0
Vancomycin 0.03µg/l	30.50(±0.5)	30.50(±0.5)
DMSO 10% V/V	0.0	0.0

The antibacterial activity of the combined extracts of *P. americana* and *A. indica* at different concentrations

The antibacterial activities of the extracts at lO0mg/ml of the ratios 1:1 vs 1:3, and l: 1 vs 3: 1 arenot statistically significant and comparing the concentrations of *P. americana* and *A. indica* of lO0mg/ml with vancomycin 30µg/ml,

the antibacterial activity is significant due to the p value being <0.0001.
The antibacterial activities at 50mg/ml of the extracts of *P. americana* vs the ratios of 1: 1, 1:3 and 3: 1 are not statistically different. And at 25mg/ml of the extracts, the antibacterial property of *P. americana* vs 1;1 and 1:3 is statistically significant with the p values of 1:1(0.0331) and 1:3(<0.0001) compared to *P. americana* vs 3:1 which is not statistically significant with the p valueof 0.9160 as shown in the table below.

Table 5: Mean and standard deviation of diameters of zones of inhibition at different concentrations of combined plant extracts.

Concentration of the extracts used (mg/ml)	P: A(1:1)	P: A(1:3)	P: A (3:1)
100	11.5(±0.5)	12.5(±0.5)	14.5(±0.5)
50	11.0(±1.0)	10.0(±0.0)	12.5(±0.5)
25	8.5(±0.5)	0.0	11.0(±0.0)

Note: *P = P. americana, A= A indica.*

Determination of MICs and FICs of the plant extracts at different ratios againststaphylococcus aureus.
The MICs of *P. americana* and *A. indica* extracts are 6.25mg/ml and 25mg/ml respectively and theMICs obtained from ratios 1:1, 1:3 and 3:1 include; 6.25mg/ml, 6.25mg/ml and 0.39lmg/ml respectively. FIC of the extracts was obtained as a ratio of MIC of plant extract in combination to MIC of theindividual plant extract as shown in the table below.

Table 6: MIC and FIC of *P. americana* and *A. indica* against staphylococcus aureus

Plant extracts	MIC (mg/ml)	FIC (mg/ml) based on MIC of*P. americana*	FIC (mg/ml) based on MIC of*A. indica*	FIC index
P. Americana	6.25			
A. lndica	25.0			
Combinations of P: A				
1:1	6.25	1.0	0.25	**1.25**
:3	6.25	0.25	0.188	**0.438**
3:1	**0.391**	**0.046**	**0.097**	**0.143**

DISCUSSION

The percentage yield of 70% v/v ethanolic leaf extracts of P. americana and A. indica were 16.77% and 12.59% w/w, respectively. In comparison to other studies, hexane extracts of A. indica yielded 28.4% w/w, and water extracts yielded 16.1% (El-Mahmood et al., 2013). Another study by Semantic Scholar on P. americana extract yielded 4.21% using 98% methanol and 13.7% using chloroform. Both dry P. americana and A. indica ethanolic extracts were found to have significant amounts of tannins, saponins, terpenes, phenols, and flavonoids. In a previous study by Idris[19], all these phytochemicals were present. The MIC of ethanolic leaf extract of P. americana obtained was 6.25 mg/ml against S. aureus ATCC 25953. This is lower than the reported MIC of 25 mg/ml [20]. This discrepancy could be due to the botanical fact that trees from different locations may demonstrate different properties since they are grown in different soils and varying climates, and are exposed to different chemical and biological flora [21]. The MIC of ethanolic leaf extract of A. indica was 25 mg/ml against S. aureus ATCC 25953. This is higher than the reported MIC of 6.25 mg/ml [22], where water was the solvent. This difference could be due to environmental variability leading to low concentration of phytochemicals, high polarity of ethanol at 70% used in this experiment consequently leading to low extraction yield of less polar phytochemicals, the temperature used in drying the extract, storage conditions affecting the

stability of the phytochemicals, and the method used in MIC determination (agar dilution method). Comparing vancomycin, whose MIC is 30 µg/ml, to the MICs of both plant extracts (6.25 mg/ml and 25 mg/ml for P. americana and A. indica, respectively), it shows that vancomycin is more potent and effective against S. aureus ATCC 25953 than the individual extracts with a p-value of <0.001. The combination of ethanolic leaf extracts of P. americana and A. indica at a ratio of 1:1 with the MIC of 6.25 mg/ml were found to be indifferent, as shown by an FIC index of 1.25. At the ratio of 1:3 with the MIC of 6.25 mg/ml, they were found to be synergistic, as shown by the FIC index of 0.438. The combination of ethanolic leaf extracts of P. americana and A. indica at a ratio of 3:1 with the MIC of 0.391 mg/ml were found to possess significantly higher activity against S. aureus ATCC 25953 compared to ratios 1:1, 1:3, and the individual extracts of P. americana and A. indica. This combination was found to be synergistic, as shown by the calculated FIC of 0.143. This synergism is probably due to the action of phytochemicals produced by both plants, such as terpenes that act by destroying cellular integrity and subsequently inhibiting the respiration and ion transport process [23], and saponins that interact with cell membrane cholesterol, affecting the cell membrane proteins' availability to bacteria and opening calcium ion-dependent conductance channels, leading to improper functioning of targeted cell membrane proteins, thus preventing bacteria from initiating an infection [23].

CONCLUSION

Data from this study strongly suggest that the ethanol extract of P. americana leaves exhibits more in vitro antibacterial activity against S. aureus ATCC 25953 strains compared to A. indica leaf extract. It appears that the antibacterial activity follows a dose-dependent pattern, with the greatest zones of inhibition noted at 100% concentration. There is much potential for the development of these extracts, especially for use against S. aureus strains, with ideal extraction methods. There is significant activity of the ethanolic leaf extracts of P. americana and A. indica against S. aureus ATCC 25953. The study showed that the combination of the plants is more potent than the individual plant extracts alone. Therefore, the combination should be considered in the management of infections caused by S. aureus.

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