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In vitro Antioxidant and Anti-staphylococcal Activity of *Bixa orellana* Linn. and *Milicia excelsa* Welw

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOA designed the study and approved the final draft. Author OOE managed the analyses of the study and prepared the final draft of manuscript. Author AFS performed the assays. All authors read and approved the final manuscript.

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ABSTRACT

An estimated 80% of persons in the world depend on medicinal plants for their healthcare needs. It is essential to study these plants in a bid to evaluate their therapeutic properties, adding credence to their applications in folklore. In this study, we report the antioxidant and antibiotic properties of *Bixa Orellana* (BO) and *Milicia excelsa* (ME) against *Staphylococcus aureus* isolates. Antioxidant profile, Total phenol content and total flavonoid content of both plants were performed using standard methods. Also, methanolic extracts of *Bixa orellana* and *Milicia excelsa* prepared in concentrations ranging from 3.125 mg/ml to 100 mg/ml were assessed for their antimicrobial activity using the agar well diffusion method. Total phenol content was 1.039 mg/g and 1.032 mg/g while total flavonoid content was 0.76mg/g and 0.5mg/g for *Milicia excelsa* and *Bixa Orellana* respectively. Both plant extracts also showed good free radical scavenging ability. The *S. aureus* isolates showed a dose-dependent sensitivity to the test extracts with sensitivity reducing at lower concentrations. *Bixa Orellana* showed better activity than *Milicia excelsa*. However, *Milicia excelsa* inhibited more organisms at lower concentrations (≤25 mg/ml). This study has shown the efficacy of both plants as alternative herbal treatments.

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1. INTRODUCTION

The Sub-Saharan African region is estimated to have one of the highest burdens of human staphylococcal infections [1]. This is due in part to such factors as the high prevalence of HIV infections [2,3], poor hygiene practices [4] densely populated living conditions [4] and other factors not yet fully understood. Accompanying the high burden of Staphylococcal infection in the region is a high prevalence of drug resistance species with studies reporting high resistance to Penicillin and Tetracycline [5,6] which happen to be two of the most utilised antibiotic variants. *Staphylococcus aureus* is an important human pathogen causing infections ranging from relatively mild skin and soft tissue infections to life-threatening infections [7]. Antibiotic resistance in *S. aureus* contributes to morbidity and mortality. Many medicinal plants have been shown to possess activity against *Staphylococcus spp.* Hence their suitability as a palliative to the current antibiotic crisis.

Bixa Orellana (BO) is found in many tropical countries where it is most frequently used as a dye in food products and textile[8]. Previous studies have reported the presence of phytochemicals such as terpenoids, flavonoids and carotenoids [9]. Studies have also shown that BO possesses antidiarrheal [10], antigonorrhoeal [11] and antifungal properties [12]. It has been reported to possess anti-inflammatory activity for bruises and wounds and has been used for the treatment of bronchitis and wounds. In addition, it is applied to the skin as a makeup and as a sunscreen [8]. BO is used traditionally as a laxative and in the treatment of malaria [13].

Milicia excelsa (ME), commonly known as 'Iroko', is an African tropical tree characterised by its ability to grow as high 50 meters. It is used traditionally to treat coughs, cure heart problems and clear gastric obstructions [14]. The plant has been reported to be active against *S. aureus* and to promote wound healing [15].

Many phytochemicals have been found to possess both antioxidant and antimicrobial properties. This study was conducted to assess the antioxidant and anti-staphylococcal potentials of *Bixa orellana* and *Milicia excelsa*.

2. METHODOLOGY

2.1 Plant Material

Fresh leaves of *B. orellana* and *M. excelsa* were obtained from the grounds of Covenant University and were authenticated at the Forestry Research Institute of Nigeria, Ibadan where voucher specimens were deposited. The collected leaves were air dried and blended into fine powder.

2.2 Extraction

Extraction was performed as previously described by Ayepola and Adeniyi [16]. 150mg of each plant was weighed and continuously refluxed with 700 ml methanol in a Soxhlet's apparatus. Resulting extracts were then concentrated at 40-50°C in a rotary evaporator. The concentrated extracts were kept in airtight bottles and stored in the refrigerator until needed.

2.3 Microorganisms

Twenty-Five *S. aureus* isolates were obtained from the microbiology laboratory of Covenant University and used in this study. Isolates were confirmed to be *S. aureus* using standard microbiological techniques.

2.4 Antioxidant Screening of Plant Extracts

The methods used for the antioxidant screening of the plants were adapted from Harborne [17] and Edeoga et al. [18], with slight modifications. The tests carried out were for the following:

2.5 Total Phenol Count (TPC)

In the total phenol count, 600 µl of distilled water was added to 10µl of leaf extracts followed by 50 µl of 10% FC (Folio-Ciocalteu) reagent. 150 µl of 7% Na_2CO_3 was added and vortexed. The mixture was then incubated for 8 minutes at room temperature. 190µl of distilled water was then added and the mixture was allowed to stand for 2 hours and the absorbance read at 765 nm using a spectrophotometer. The blank was prepared by replacing 10 µl of leaf extracts with water. A standard calibration plot was generated at 765 nm using known concentrations of gallic acid. The phenol content in the test samples

were calculated from the calibration plot and expressed as mg Gallic acid equivalent/ml of sample.

2.6 Total Flavonoid Count (TFC)

The total flavonoid count was carried out by adding 400 µl of distilled water to 10 µl of leaf extracts, followed by 30 µl of 10% aluminium chloride. The mixture was incubated at room temperature for 5 minutes. 200 µl of 1 molar NaOH followed by 240 µl of water were added to the mixture and vortexed thoroughly. The absorbance was read at 570 nm using a spectrophotometer. A standard calibration plot was generated at 570 nm using known concentrations of pyrocathecol. The concentrations of flavonoids in the test samples were calculated from the calibration plot and expressed as mg pyrocathecol equivalent/g of sample.

2.7 DPPH Assay

The free radical scavenging activity of the extracts was measured *in vitro* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay in which 100 µl of extract was added to 1ml 0.004% methanolic solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl). The mixture was then incubated for 30 minutes in the dark and the absorbance was read at 517nm using a spectrophotometer.

2.8 Hydrogen Peroxide Scavenging Activity

For this assay, 100 µl of extracts were incubated with 0.6 ml of H_2O_2 (40 mM in a phosphate buffer, 0.1 M, pH 7.4) in the dark for 10 minutes. A negative control was set up in parallel with entire reagent except extract. Afterwards, 1ml of dichromate reagent (5% potassium dichromate and glacial acetic acid in ratio 1:3) was added and boiled for 10 minutes. The absorbance was read at 620 nm.

2.9 Nitric Oxide Radical Scavenging Ability

For this assay, 63 µl of 10 mM Sodium Nitroprusside was added to 63 µl plant extract. The mixture was then incubated for 2.5 hours. 250 µl of Sulfanilamide reagent was then added to the mixture and incubated for 30 minutes. 250 µl of 0.1% NED (Naphthylethylene diamine dihydrochloride) was also added to the mixture and incubated for 30 minutes at room temperature. The absorbance was read at 540 nm.

2.10 Evaluation of Antimicrobial Activity of Plant Extracts

Antimicrobial activity of plant extracts was determined using methods previously described by Ayepola and Adeniyi [16]. 0.1 ml of test isolates, cultured overnight, was seeded onto already prepared Mueller Hinton agar plates. A sterile 7mm cork borer was used to make wells into which 0.1 ml of extract at different concentrations (3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml, 100 mg/ml) was dispensed. The plates were incubated at 37°C for 18-24 hours and after that observed for zones of inhibition.

3. RESULTS

3.1 Antioxidant Activity

Both plants had similar TPC and TFC (Table 2). TPC in this study was 1.039 and 1.033 mg Gallic acid equivalent/ml extract for BO and ME respectively while TFC was 0.582 and 0.767 mg pyrocathecol equivalent/g extract for BO and ME respectively. Antioxidant activity was observed in both plant extracts using three methods. ME had a higher DPPH and H_2O_2 scavenging ability than BO while BO was better than at inhibiting NO (Table 1).

3.2 Anti-Staphylococcal Activity

Both plant extracts showed dose-dependent activity against the *S. aureus* isolates with higher concentrations showing higher activity (Table 3 and Fig 1). BO showed better activity at all concentrations than ME. Considerable antibacterial activity was observed in both plants; 9-20 mm for BO and 9-16mm for ME. No antibacterial activity was observed in both plants at concentrations below 12.5 mg/ml.

Table 1. Antioxidant activity of *Bixa orellana* and *Milicia excels*

	% DPPH inhibition	% No inhibition	% H_2O_2
BO	57.13	79.39	61.65
ME	70.94	66.53	69.87

Antioxidant activity measured at 1mg/ml concentration of extract.

Table 2. Total phenol content & total flavonoid content of *Bixa orellana* and *Milicia excels*

	TPC (mg Gallic acid equivalent/ml)	TFC (mg pyrocathecol equivalent/g)
BO	1.039	0.582
ME	1.033	0.767

Table 3. Antimicrobial activity of plant extracts against *S. aureus* isolates

Organisms	*Bixa orellana*				*Milicia excels*			
	100 mg mL-1	50 mg mL-1	25 mg mL-1	12.5 mg mL-1	100 mg mL-1	50 mg mL-1	25 mg mL-1	12.5 mg mL-1
SA001	17	15		-	12	11	8	
SA002	18	14		-	13	11		-
SA003	17	14	13	11	12	11		-
SA004	18	16		-	14	11		-
SA005	16	13		-	13	11		-
SA006	16	15	12	-	13	12	12	-
SA007	19	16		-	13	10		-
SA008	15	12	9	-	14	13	11	9
SA009	17	14		-	13	11	9	8
SA010	16	14		-	16	9		-
SA011	15	13		-	11	10		-
SA012	15	13		-	13	11	10	9
SA013	19	14	12	-	13	10	12	-
SA014	17	15		-	12	11		-
SA015	19	14		-	14	12		-
SA016	16	15	11	-	13	11	11	-
SA017	14	13	11	9	13	12	10	9
SA018	18	15		-	14	12	11	9
SA019	16	13	11	9	11	9	11	-
SA020	14	12		-	12	11		-
SA021	17	14	12	10	13	10		-
SA022	20	14		-	14	12		-
SA023	15	12		-	13	11		-
SA024	16	14		-	16	11		-
SA025	18	14		-	13	11	9	-

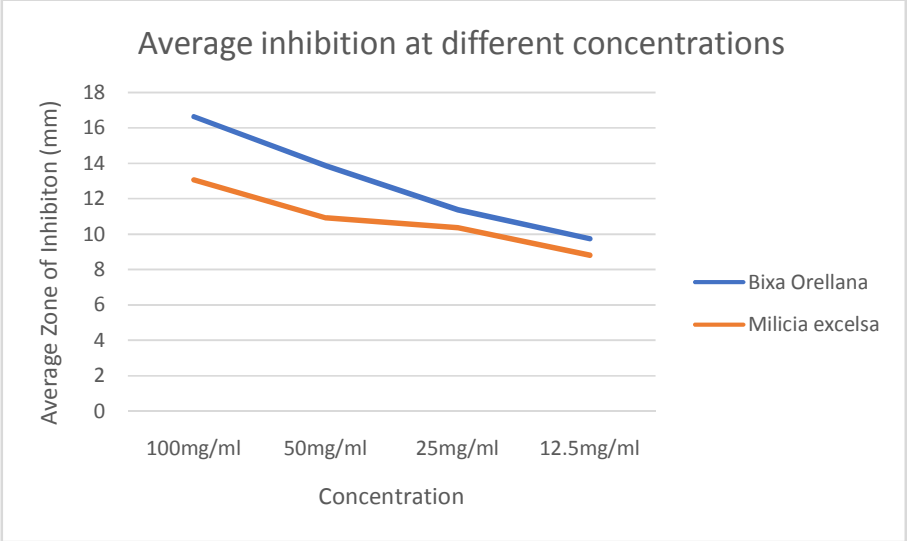

Fig. 1. Average inhibition of plant extracts at different concentrations

4. DISCUSSION

The TPC reported in this study support those found in previous studies where a range of 0.30-1.84 mg gallic acid equivalent/mg was reported for BO [19]. The phenol content of a plant extract is dependent on such factors as method of extraction and the environment hence the variations in measurements from different studies. Flavonoids were also detected in both plants as previously reported [15,19]. The presence of flavonoids and phenols in plant extract is usually an indicator of its antioxidant activity which is further confirmed using such assays as DPPH and NO inhibition assays. Both plants were able to reduce DPPH at all concentrations, however, ME was a better scavenger then BO. Furthermore, the percentage inhibitions recorded in this study is lower than that reported in similar studies [9,20]. Again, procedural differences could be the reason for the observed disparity. Similar patterns were observed in the NO and H_2O_2 scavenging assay where dose-dependent scavenging activities were observed. The good NO and H_2O_2 scavenging activity of both plants is consistent with the high phenol and flavonoid content recorded. The data from the antioxidant screening suggests that both plants effectively scavenge reactive oxygen species and could protect against oxidative damage.

The antibacterial activity of BO and ME has been previously documented against gram-negative and gram-positive bacterial species [8–11,15]. It is especially interesting that both plant extracts possess antibacterial properties against *S. aureus* as demonstrated in this study. However, BO exhibited a higher activity. The presence of flavonoids and phenols in both plants may also be responsible for the antibacterial activity. Flavonoids are a group of polyphenolic substances which possess biochemical and antimicrobial activities. They are known to exert their antioxidant activity via radical scavenging, metal ion chelation, and membrane protective efficacy [21].

5. CONCLUSION

The present study has demonstrated the antioxidant and anti-staphylococcal activity of *Bixa Orellana* and *Milicia excelsa*. Both plants may be exploited as a ready source of antioxidant and antibacterial for the treatment of infections caused by *S. aureus*. This is important because of the increasing prevalence of multidrug-resistant *S. aureus* strains currently threatening public health globally. More research will need to be done to identify the bioactive compounds in both plants as well as their toxicity and pharmacological activities.

CONSENT

It is not applicable.

ETHICAL APPROVFAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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