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Antibacterial activities of *Zanthoxylum zanthoxyloides* and *Anogeissus leiocarpus* against some oral pathogens

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Abstract

The extracts of stem and leaves of selected chewing sticks, Zanthoxylum zanthoxyloides (candle wood) and Anogeissus leiocarpus (African birch) were assayed for their antibacterial activities. The chewing sticks were obtained in Kakun-Kabba metropolis, Nigeria, The extracts were tested against Pseudomonas aeruginosa and Staphylococcus aureus isolated from swabs collected from 2 patients with oral problems attending the Dental section of General Hospital Minna, Nigeria. Phytochemical screening of the extracts revealed the presence of alkaloids, steroids, tannins, phenols, flavonoids, terpenes, anthraquinones and saponnins. Aqueous, methanol and acetone extractions were carried out to obtain the active ingredients of the chewing sticks. The agar diffusion method was used to assay the antibacterial activity of the extracts at different concentrations (120 mg/ml, 160 mg/ml and 200 mg/ml). The aqueous, methanol and acetone extracts of the chewing sticks showed inhibitory activity on the growth of P. aeruginosa and S. aureus. The minimum inhibitory concentration (MIC) of the extracts ranged between 120mg/ml and 160mg/ml and minimum bactericidal concentration (MBC) ranged between 160mg/ml and 200mg/ml. Out of the nine fractions purified by thin layer chromatograph (TLC), six showed inhibitory activity against P. aeruginosa with zones of inhibition ranging from 10mm to 12mm while seven showed activity against S. aureus with zone of inhibition ranging from 10mm to 22mm. The TLC fraction competed well with the activity of commercial antibiotic (ampicillin) and data from the present study have shown that chewing sticks is a potential drug for oral hygiene and treatment of oral pathogens.

Keywords: *Zanthoxylum zanthoxyloides, Anogeissus leiocarpus,* Minimum inhibitory concentration, Minimum bactericidal concentration, Phytochemical screening.

Introduction

Zanthoxylum zanthoxyloides is an indigenous plant used widely as chewing sticks for teeth cleansing in West Africa. It is well known for its varied uses in trado-medical practice. The root, root-bark and other parts of the plant are used in treating dental diseases, various medical problems[1]. *Anogeissus leiocarpus* (African birch) is a tall deciduous tree native to savannas of tropical Africa. It is the sole West African species of the genus *Anogeissus*, a genus otherwise distributed from tropical central and east Africa through tropical Southeast Asia and a major plant commonly used as chewing stick in Nigeria. *A. leiocarpus* is also another plant species used in traditional medicine as a remedy for many ailments of livestock[2].

Some African chewing sticks are also reported to contain fluoride ions, silicon, tannic acid, sodium bicarbonate and other natural plaque inhibiting substances that can reduce bacterial colonization and plaque formation. Sodium bicarbonate has mild abrasive properties and is, thus, used as a dentifrice in addition to having a mild germicidal action[3]. The high concentrations of chloride inhibit calculus formation and help in removing stains from the teeth. Calcium saturation of saliva inhibits demineralization and promotes re- mineralization of tooth enamel. A great number of these plant species have related medicinal properties that may be antibacterial[4].

This study was aimed at determining the antibacterial activity of the *Z. zanthoxyloides* and *A. leiocarpus* against oral pathogens microorganisms as well as compare the antibacterial activities of the different extracts: aqueous, methanol and acetone extract which are the common solvents used by traditional medicine practitioners.

Materials and methods

Collection and identification of plant materials: Fresh leaf and stem of *A. leiocarpus* and *Z. zanthoxyloides* were collected from a forest around Kakun-Kabba metropolis in Kogi State, Nigeria. The plants were washed off dirt in running water and were air-dried for four weeks. The samples were then pounded and grounded into powder using milling machine. The powdered samples were collected into sterile nylon bag and store in a cool dried place.

Preparation of plant extract: The cold maceration method was used in the extraction of the active materials in the plant[5]. The 100 g of each dried powdered stems and leaves were weighed into three containers and extracted in 400ml each of methanol, acetone and aqueous for 72 hours with constant shaking. The mixtures were then filtered using muslin cloth and the filtrate was collected into separate beakers. These were then placed on a water bath to allow the solvent to evaporate to get the stems and leaves extracts. The extracts were transferred to sterile bottles and stored at 4^{o}C.

Collection and screening of bacterial isolates: The test isolates*: Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from clinical sample at General Hospital Minna, Niger State and inoculated into fresh nutrient agar then incubated at 37^{o}C for 24hours. A mixed culture was obtained; it was sub-cultured unto nutrient agar to get a pure culture. Further confirmation of the isolate was done by carrying out Gram staining and biochemical tests.

Standardization of test microorganisms: The isolated organisms was standardized[6], the organism was picked using wire loop and inoculated in nutrient broth for 24 hours at 37^{0}C. The 0.2ml of 24 hours culture was placed into 20ml of sterile nutrient broth and incubated for 3-5 hours to standardize the test organisms to 10^{6}cfu/ml.

Screening of crude extracts for antimicrobial activity: The agar- well diffusion was the method used to determine the antimicrobial activity of the extract using concentration of 120mg/ml, 160mg/ml and 200mg/ml. Sterile nutrient agar that has cooled to 45°C was poured into sterile Petri dish and allowed to solidify. The sterile swab sticks were used to spread the standardized test organisms on the surface of the solidified agar. In each plate, four wells were created using 4mm sterile cork-borer; 0.2ml of the extract was introduced into each of the wells using a syringe. These were allowed to diffuse into the medium by living the agar at the point of inoculation without shaking for at least 1 hour. The plates were incubated at 37°C for 24 hours. The zones of inhibition were observed using a meter rule and recorded in millimeters. Ampicillin was used as positive control and the zone of inhibition were recorded and compared with those of the extracts.

Determination of minimum inhibitory concentration and minimum bactericidal concentration: The minimum inhibitory concentration (MIC) was determined[7] and the minimum bactericidal concentration (MBC) was also determined[8].

Phytochemical screening of the extracts: Phytochemical analysis were carried out on the extract samples gotten from the stems and leaves of *Z. zanthoxyloides* and *A. leiocarpus* using standard procedures. The extracts were screened for alkaloids**,** saponins, tannins, steroids, flavonoids, anthraquinones and phlobatannins[9].

Purification of extracts using thin layer chromatography: The thin layer chromatography (TLC) was prepared using aluminum coated plate. The 0.2g of the extract was dissolved in 1ml of distilled water. A concentrated band of the extract solution was made on the aluminum coated TLC plate using capillary tube about 2cm from the base of the TLC plate. Several solvent were used as mobile phase as to determine the most suitable solvent for the extracts purification. The solvent used were 100% chloroform, 100% ethyl acetate, chloroform/ ethyl acetate ratio 1:4, chloroform/ethyl acetate ratio 4:1 and chloroform/ethyl acetate ratio 1:1.

The solvent was poured into the development glass tank, covered, shake and allowed to stay for 20 minutes so that the tank will be saturated with the solvents; the aluminum plate was inserted into the development tank with the sported area toward the base of the tanks but higher than the level of the solvent to avoid sagging. The tanks were covered properly, the solvent was allowed to move a little beat close to the top, plate was removed and the solvent front was marked so as to calculate the retardation factor. It was dried and placed in an iodine tank to make the sport visible and later viewed with UV lamp for proper result. The solvent system which separated the extract the best was used as the preparative thin layer chromatography[6].

Determination of antibacterial activity of preparative thin layer chromatography fractions: The extracts fractions gotten from preparative TLC bands was reconstituted to a concentration of 200mg/ml and was introduced into the well agar containing the test organisms, the plate were incubated for 18-24 hours at 37^{o}C and observed for zone of inhibition.

Results and discussion

Phytochemical screening of the crude extracts: The phytochemical screening of the crude extracts showed that the acetone, methanol and aqueous extracts contain alkaloid, tannins, flavonoids, steroids, Anthtraquinones, phlobatannin and terpenes. The result also showed they lacked saponins (Table-1).

Antimicrobial activities of crude extracts: The extract gotten from *Z. zanthoxyloides* and *A. leocarpus* showed various antimicrobial activities on the test organisms. All the extract from acetone, methanol and aqueous showed activity against the bacteria isolates. The diameter of zone of inhibition of varying concentration of the crude acetone, methanol and aqueous against the selected organisms is presented in Table-2.

The susceptibility testing of the crude extracts at 120mg/ml, the crude methanol and acetone extracts of *Z. zanthoxyloides* were the most active on *P. aeruginosa* with zone of inhibition of 23 mm while the crude methanol extract of *A. leiocarpus* was most active on *S. aureus* with zone of inhibition of 23mm. Almost all the *Z. zanthoxyloides* extract show no activity on *S. aureus,* except methanol extract (leaf) of *Z. zanthoxyloides* with zone of inhibition of 18mm (Table-2).

The susceptibility testing of the crude extracts at 160mg/ml, the crude methanol, acetone and aqueous extract were all active on *P. aeruginosa* with zone of inhibition ranging from 20mm - 28mm and *S. aureus* with zone of inhibition ranging from 20mm - 30mm except water extract (leaf) of *Z. zanthoxyloides* which did not inhibit *S. auerus* (Table-2).

The susceptibility testing of the crude extracts at 200 mg/ml, the crude methanol, acetone and aqueous extract were all active on *P. aeruginosa* with zone of inhibition ranging from 20mm - 29mm and *S. aureus* with zone of inhibition ranging from 24mm – 34mm except water extract (leaf) of *Z. zanthoxyloides* which did not still show inhibit *S. aureus* (Table-2).

The minimum inhibitory concentration and minimum bactericidal concentration of *Z. zanthoxyloides* and *A. leiocarpus* for *P. aeruginosa:* The result show the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for acetone, methanol and aqueous of *Z.* zanthoxyloides and *A. leiocarpus* for *P. aeruginosa* are shown above. The minimum inhibitory concentration of all the extract on *P. aeruginosa* was 120mg/ml except acetone extract of *Z. zanthoxyloides* stem and aqueous extract of *Z. zanthoxyloides* leaf while the minimum bactericidal concentration ranged from 160mg/ml-200mg/ml for all the extracts (Table-3).

The minimum bactericidal concentration of *Z. zanthoxyloides* and *A. leiocarpus* for *S. aureus:* The result for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for acetone, methanol and aqueous of *Z. zanthoxyloides* and *A. leiocarpus* for *S. aureus* are shown above, the minimum inhibitory concentration of all the extract on *S. aureus* ranged from 120mg/ml – 160mg/ml except water extract of *Z. zanthoxyloides* leaf, which show no activity at the range of the concentration used while the minimum bactericidal concentration ranged from 120mg/ml -200mg/ml (Table-4).

Result of fractions obtained from the preparative thin layer chromatography: The fractions obtained from the preparative thin layer chromatography were used for antimicrobial activity against the test organisms at a concentration of 200 mg/ml. Only two were not inhibited by the purified fractions on *S. aureus* and on *P. aeruginosa,* only three were not inhibited. The diameter of zone of inhibitions for the fractions obtained from thin layer chromatography against the test organisms is shown in Table-5.

Table-1: Phytochemical constituents of crude extracts of *Z. zanthoxyloides* and *A. leiocarpus.*

Extract	Alkaloid	Saponins	Tannins	Phenol	Terpenes	Steroids	Flavonoids	Anthraquinones	Phlobatannin
ZzLW	+	-	-	+	+	+	-	+	-
ZzSW	+	-	+	-	+	+	-	-	+
ZzLA	+	-	-	-	+	-	+	-	-
ZzSA	+	-	-	-	+	+	+	+	-
ZzLM	+	-	+	+	+	+	-	-	+
ZzSM	+	-	-	-	+	+	+	+	-
AlLW	+	-	+	+	+	+	-	+	+
AlSW	+	-	+	+	+	+	-	+	+
AlLA	+	-	+	+	+	+	+	+	+
AlSA	-	-	+	+	+	+	-	+	+
AlLM	+	-	+	+	+	+	-	-	+
AlSM	+	-	+	+	+	+	-	+	+

Key:+=Present, -=Absent, ZzLW-*Z. zanthoxyloides* leaf (water), ZzSW-*Z. zanthoxyloides* stem (water), ZzLA-*Z. zanthoxyloides* leaf (acetone), ZzSA-*Z. zanthoxyloides* stem (acetone), ZzLM-*Z. zanthoxyloides* leaf (methanol), ZzSM-*Z. zanthoxyloides* stem (methanol), AlLW- *A. leiocarpus* leaf (water), AlSW- *A. leiocarpus* stem (water), AlLA- *A. leiocarpus* leaf (acetone), AlSA- *A. leiocarpus* stem (acetone), AlLM- *A. leiocarpus* leaf (methanol), AlSM- *A. leiocarpus* stem (methanol).

Table-2: The zone of inhibition of *Z. zanthoxyloides* and *A. leiocarpus* against the test organisms.

Extracts	*P. aeruginosa* Concentration (mg/ml)			*S. aureus* Concentration (mg/ml)		
	120	160	200	120	160	200
ZzLW	NA	26	28	NA	NA	NA
ZzSW	20	22	27	NA	21	25
ZzLA	21	24	28	18	24	25
ZzSA	NA	20	24	NA	29	30
ZzLM	23	28	29	NA	28	32
ZzSM	20	20	26	NA	30	34
AlLW	19	26	28	16	23	25
AlSW	20	20	22	18	20	24
AlLA	22	24	25	21	22	24
AlSA	23	26	28	21	22	24
AlLM	16	20	21	23	24	25
AlSM	17	19	20	23	26	28
Control	28	34	39	26	32	36

Key: NA- Not applicable, ZzLW- *Z.zanthoxyloides* leaf (water), ZzSW-*Z. zanthoxyloides* stem (water), ZzLA- *Z. zanthoxyloides* leaf (acetone), ZzSA- *Z. zanthoxyloides* stem (acetone), ZzLM-*Z. zanthoxyloides* leaf (methanol), ZzSM-*Z. zanthoxyloides* stem (methanol), AlLW- *A. leiocarpus* leaf (water), AlSW- *A. leiocarpus* stem (water), AlLA- *A. leiocarpus* leaf (acetone), AlSA- *A. leiocarpus* stem (acetone), AlLM- *A. leiocarpus* leaf (methanol), AlSM- *A. leiocarpus* stem(methanol).

Table-3: Minimum inhibitory concentration and minimum bactericidal concentration of *Z. zanthoxyloides* and *A. leiocarpus* for *P. aeruginosa.*

Extract	MIC (mg/ml)	MBC (mg/ml)
ZzLW	160	160
ZzSW	120	160
ZzLA	120	160
ZzSA	160	200
ZzLM	120	200
ZzSM	120	200
AlLW	120	160
AlSW	120	200
AlLA	120	160
AlSA	120	160
AlLM	120	160
AlSM	120	200

KEY: ZzLW-*Z.zanthoxyloides* leaf (water), ZzSW-*Z.* zanthoxyloides stem (water), ZzLA-*Z.zanthoxyloides* leaf (acetone), ZzSA-*Z.zanthoxyloides* stem (acetone), ZzLM-*Z.* zanthoxyloides leaf (methanol), ZzSM-*Z.* zanthoxyloides stem (methanol), AlLW-*A. leiocarpus* leaf (water), AlSW- *A. leiocarpus* stem (water), AlLA- *A. leiocarpus* leaf (acetone), AlSA-*A. leiocarpus* stem (acetone), AlLM-*A. leiocarpus* leaf (methanol), AlSM-*A. leiocarpus* stem(methanol).

Table-4: The minimum bactericidal concentration of *Z. zanthoxyloides* and *A. leiocarpus* for *S. aureus.*

Extract	MIC (mg/ml)	MBC (mg/ml)
ZzLW	NA	NA
ZzSW	160	160
ZzLA	120	160
ZzSA	160	160
ZzLM	160	160
ZzSM	160	160
AlLW	120	160
AlSW	120	160
AlLA	120	200
AlSA	120	200
AlLM	120	120
AlSM	120	160

Key: NA-Not applicable, ZzLW-*Z.zanthoxyloides* leaf (water), ZzSW-*Z. zanthoxyloides* stem (water), ZzLA-*Z.zanthoxyloides* leaf (acetone), ZzSA-*Z. zanthoxyloides* stem (acetone), ZzLM-*Z. zanthoxyloides* leaf (methanol), ZzSM-*Z. zanthoxyloides* stem (methanol), AlLW-*A. leiocarpus* leaf (water), AlSW-*A. leiocarpus* stem (water), AlLA-*A. leiocarpus* leaf (acetone), AlSA-*A. leiocarpus* stem (acetone), AlLM-*A. leiocarpus* leaf (methanol), AlSM- *A. leiocarpus* stem(methanol).

Table-5: The zones of inhibition of TLC fractions obtained against selected organisms.

Extracts	*P. aeruginosa*	*S. aureus*
ZzLW	12	NA
ZzSW	12	19
ZzSA	12	10
ZzLM	10	15
ZzSM	12	17
AlLW	NA	NA
AlSW	NA	15
AlSA	10	22
AlSM	NA	12
Control	30	25

Discussion: The results for the phytochemical analysis of the extracts of *Z. zanthoxyloides* and *A. leiocarpus* (Table-1) indicated the presence of alkaloids, tannins, terpenes, steroids, anthraquinones and phlobatannnins. This is in agreement with previous findings[10]. The human oral cavity harbors diverse range of bacteria, fungi and protozoa as normal flora but may cause dental diseases in poor oral hygiene and in immune suppressed individuals[11]. Alkaloids have a wide range of pharmacological activities including antimalarial (e.g. quinine), antiasthma (e.g. ephedrine), anticancer (e.g. homoharringtonine), antibacterial (e.g. chelerythrine)[12]. The repeated process of using chewing sticks releases fresh sap containing fluoride, which seems to wet the tooth enamel and adequately reach caries susceptible sites and contribute towards caries prevention[13].

Tannin exerts an astringent effect on the mucous membrane, thus reducing the clinically detectable gingivitis[14]. Tannins also inhibit the action of glucosyltransferase thus reducing plaque and gingivitis[5]. Resins form a layer over the enamel and thus protects against caries. Alkaloids exert bactericidal effect in the oral cavity. Essential (volatile) oils possess characteristic aroma and exert antiseptic action. The presence of compounds with phenolic groups gives oils acidic properties and could possibly be responsible for its antimicrobial activities. The presence of higher terpenoids that have carboxylic acid groups could also be responsible for the activity of the organic extracts as well as the anti-inflammatory and anti-bacterial properties of tannins[15]. These classes of compounds are known to show curative activity against several bacteria and it is not surprising that these plant extracts are used traditionally by herbalist to cure bacteria related ill-health. The microorganisms isolated in this study are *S. aureus* and *P. aeruginosa* which is not a known normal flora of the mouth and were also reported as causing dental diseases[16].

The result obtained from the susceptibility testing of crude extract (Table-2) shows the varying antimicrobial activity of the crude extracts, against the test organisms. This is in contrast to previous report that an insignificant antibacterial activity for root extracts of *Z. zanthoxyloides* and *A. leiocarpus*, using agar well diffusion method[10]. This may be due to different in plant part used. The extract gotten from *Z. zanthoxyloides* and *A. leocapa* showed various antimicrobial activities on the test organisms. All the extracts from acetone, methanol and aqueous showed activity against both organisms.

The diameter of zones of inhibition of the acetone extract of *Z. zanthoxyloides* ranged from 18-30mm and that of *A. leiocarpus* ranges from 21-28mm. the diameter of zone of inhibition of methanol extract on *Z. zanthoxyloides* ranges from 20mm-34mm, *A.leiocarpus* ranges from 16-28mm. The diameter of zones of inhibition of the aqueous extract on *Z. zanthoxyloides* ranged from 20-28mm, on *A. leiocarpus*, it ranges from 16-28mm. The control (Ampicillin) showed zones of inhibition ranging from 26-39mm at the same concentration used for the extract.

The MIC is shown in Table-3, which is the lowest concentration of the extract that inhibited the growth of the test organisms after an overnight incubation in a broth medium ranges from 120mg/ml to 200mg/ml and on plating out the concentration which showed no turbidity to determine the minimum bactericidal concentration (MBC) some of the test organism grew at the same concentration that they were been inhibited while some did not grow and at an increase concentration, the test organisms did not grow. These imply that, the extracts are bactericidal.

Nine fractions with different retardation factor were obtained on carrying out thin layer chromatography (TLC) of methanol, acetone and aqueous extract, which shows that methanol, acetone and aqueous extract of *Z. zanthoxyloides* and *A. leiocarpus* contain several compounds. On testing the microbial activity of the fractions, both acetone, methanol and aqueous extract of *Z. zanthoxyloides* were active on *P. aeruginosa* and *S. aureus* with zone of inhibition ranging from 10- 19mm except aqueous (leaf) extract that was not active against *S. aureus* while only acetone extract *of A. leiocarpus* was active on *P. aeruginosa* with a zone of inhibition of 10mm while methanol, acetone and aqueous extract (stem) were active against *S. aureus* with the zone of inhibitions of 15mm, 22 mm and 12mm respectively, this competed well with the control (ampicillin) (Table-5).

Conclusion

The results reveal the presence of medicinally active constituents in the two plants studied *(Z. zanthoxyloides* and *A.leiocarpus*). The phytochemical compounds identified in this study have earlier been proved to be bioactive. The presence of some of these compounds have been confirmed by previous workers to have medicinal as well as physiological activity and therefore could be said to be responsible for the efficacy of the leaves of the plants studied in treatment of different ailments. The plant extracts could therefore be seen as a potential source for useful drug. However, the medicinal use of these plants should be encouraged while it is suggested that further work should be carried out to elucidate the possible mechanism of action of these extracts.

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