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ANTIMICROBIAL AND PHYTOCHEMICAL ATTRIBUTES OF *DENNETTIA TRIPETALA* F. BAKER ROOT AND BARK EXTRACTS

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ABSTRACT

The antimicrobial activities and phytochemical constituents of aqueous and hexane extracts of both the bark and roots of *Dennettia tripetala* F. Baker (*Annonaceae*) were determined using routine methods. Flavonoids, saponins, phenolic compounds, volatile oil, carbohydrate and reducing sugars were present in the aqueous extract of the root and bark, but absent in the hexane extracts. The antimicrobial activities of the extracts were tested against bacterial and fungal isolates using agar diffusion method.The commercial antibiotics used as positive reference standards to determine the sensitivity of the isolates were Gentamicin and Fluconazole.The aqueous extract of the root showed inhibitory activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa*, with zones of inhibition ranging from 0.01mm to 30mm. The aqueous root extract inhibited the growth of *Pseudomonas aeruginosa* and *Enterococcus faecalis* with MIC values at 50 mg/ml.The results showed that the aqueous root extract possesed antimicrobial attributes as indicated by its activity on test bactetrial isolates. Phytochemicals were not present in the hexane extracts and the extracts showed no activity against the test organisms.

Keywords: *Dennettia tripetala*, aqueous extract, hexane extract, bark, root, phytochemistry and antimicrobial

INTRODUCTION

Records of indigenous knowledge from various parts of the world illustrate an age long tradition of plants being a major bioresource base for health care (**Idu *et al*., 2000a**). It has been found that some drugs are synthesized from plants (**Idu *et al*., 2007**). A medicinal plant can be defined as any plant which in one or more of its organs contain substances that can be used for therapeutic purpose or which are precursors for the synthesis of useful drugs (**Sofowora, 1982)**. Plant secondary metabolites constitute one of the most numerous and widely distributed groups of substances in the plant kingdom and have been known to be responsible for the therapeutic activities of medicinal plants **(Ataman *et al*., 2002).** Phytomedicines derived from plants have shown great promises in the treatment of intractable infections **(Iwu *et* al., 1999)** and *in vitro* screening methods could provide the needed preliminary observations necessary to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations **(Ozumba, 2003; Pessini *et al*., 2003)**. *Dennettia tripetala* Baker F. (pepper fruit) belongs to the family Annonaceae. It is found in the tropical rainforest region of Nigeria and occassionally in the savanna region **(Okwu *et al*., 2005)**. The plant flourishes at the onset of the rain, from April through June **(Umoh, 1998).** It is a woody plant of about 7 m in height with simple leaves. The young leaves are chewed on account of their pungent spicy taste. The fruits are initially green then turn red on ripening between April and May and have a peppery spicy taste and are chewed for this property. The leaves are used to treat mild fever with other herbs such as the leaves of mango **(Gill, 1992).** The young stems of this plant are also used as chewing stick by patients with fever to help improve their appetite. Pharmacologically, the oil extracted from the fruits of the plant when mixed with fresh leaves of mango is used in treating fever **(Gill, 1992).**
The aim and objective of this study was to determine the antimicrobial activity and the phytochemical consitiuents of hexane and aqueous extracts of bark and roots of *D. tripetala*.

MATERIAL AND METHODS

Plant materials

The barks and roots of *D. tripetala* were collected in the month of August, 2010 from Umudioka in Dunukofia L.G.A. of Anambra State and was identified by Professor M. Idu of the Department of Plant Biology and Biotechnology, University of Benin, Benin City. The plant parts were dried at an average temperature of 45^{O}C for 3 days using a 50L GALLENKAMP hot box oven (model; N9615-50, 250^{o}C) manufactured by Rigal Bennett. The dried samples were macerated to fine particles. The weighed powdered samples (550g of bark and 165g of root) were extracted with 2100 ml hexane for bark and 700 ml for root with occasional stirring for 48 hrs for maximum dissolution after which it was sieved through a Whatman filter paper to obtain a solution. The solution was concentrated to dryness using 10L GALLENKAMP water bath(model; S8689-50) manufactured by Rigal Bennett. The residue were air-dried to remove any remaining hexane before they were subjected to another extraction using distilled water. The extraction was done for 18 h using 2800 ml of boiled distilled water. They were filtered and then concentrated over Gallenkamp water bath. The four extracts were stored in refrigerator until required for use.

Standardization and preparation of the microbial innocula

The stock culture were obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin. Five (5) bacterial strains; *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis* and two (2) fungi strains; *Candida albicans and Microsporium audouinii* were used.
All the test organisms (bacterial and fungal isolates) were sub-cultured on freshly prepared nutrient agar plates and potato dextrose agar plates and incubated for 24h and 48h respectively. Following the slightly modified method of Vandepitte *et al*. (2003), the inocula were standardized by transfering parts of the streaked colonies into 5ml of sterile nutrient broth in test tubes and incubated for 3h at 37^{O}C. The bacteria and fungi suspension growth were appropriately compared to that of a freshly prepared barium sulphate solution(0.5ml of 1% barium in Chloride to 99.5ml of 1% H_2SO_4 (0.36 Normal). The obtained turbidity was adjusted by adding more sterile nutrient broth to match the 0.5 Mcfarland

standard (10^6cfu/ml). After incubation, 1ml of the standardized cultures of the microbial isolates were inoculated onto the surface of freshly prepared nutrient agar plates (for the bacterial isolates) and sabouraud dextrose agar plates (for the fungal isolates) with the aid of sterile bent glass rod.

Test for antimicrobial activity

The diluted aqueous and hexane extracts of the root and bark of the plant were tested for their antimicrobial properties using the punch hole method **(Stoke, 1975).** The concentrations used were 400, 200, 100 and 50 mg/ml. The antibiotics; gentamicin (at a concentration of 5 mg/ml) and commercial fluconazole (at a concentration of 5 mg/ml), were used as positive controls for bacterial and fungal isolates respectively. The standard antiobiotic sensitivity discs (Gentamicin and Fluconazole) made by Asodisks Atlas Diagnostics, Enugu, Nigeria were purchased from a chemical laboratory store in Benin city and used, after which the plates were incubated overnight at $37^OC \pm 2^OC$ and $28^OC \pm 2^OC$ for bacterial and fungal cultures respectively. At the end of the incubation period, the diameter of the inhibition zone(s) were measured using meter rule and recorded.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of the crude extracts were determined by adapting the punch hole method as described by **Stokes (1975)** and decreased **c**oncentrations of the extracts i.e. 200, 100, 50, 25 and 12.5 mg/ml were utilized. The least concentration of the extract which inhibited the growth of the inocula was considered as the minimum inhibitory concentration.

Phytochemical screening of the extracts of the bark and root

Phytochemical screening of the aqueous and hexane extracts of the bark and root were conducted according to standard procedures as described by **Trease and Evans (1996).** The respective extracts were analysed for the presence of alkaloids, saponins, tannins, volatile oils, anthracene, flavonoids, reducing sugars, carbohydrates, cyanogenetic glycerides, phenolic compounds.

Data analysis

Results were expressed as means ± standard error of means [S.E.M] and level of significance between means were computed by student's t-test using SPSS 14.00 computer software package. The level of significance was determined at 0.05.

RESULTS

The test microbial isolates showed varying degrees of response towards various concentrations of *Dennettia tripetala* aqueous extract of the bark. The maximal or highest inhibitory zone (0.09 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of aqueous bark extract (Tab. 1). *P. aeruginosa* , *E. faecalis* and *E. coli* elicited the least zone of inhibition (0.01 mm±0.00) against 50 mg/ml and 100 mg/ml aqueous bark extract concentrations (Tab.1). The observed differences in the mean inhibitory zones was significant with the zone elicited by *P. aeruginosa* and *E. coli* being responsible**.**

Table 1 The effect of aqueous extract of the bark of *D. tripetala* on the test organisms at various concentrations

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	0.03^a±0.02	0.03^a±0.01	NMZI	NMZI
Pseudomonas aeruginosa	0.09^a±0.01	0.05^a±0.01	0.02^b±0.01	0.01^b±0.00
Escherichia coli	0.02^a±0.01	NMZI	0.01^a±0.00	NMZI
Staphylococcus aureus	0.05^a±0.01	0.04^a±0.00	0.04^a±0.01	NMZI
Enterococcus feacalis	0.05^a±0.01	0.03^b±0.00	0.02^b±0.03	0.01^b±0.00
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (0.05 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of hexane bark extract (Tab. 2). *B. subtilis* and *S. aureus* elicited the least zone of inhibition (0.02 mm±0.01) against 200 mg/ml and 400 mg/ml hexane bark extract concentrations (Tab.2). The observed differences in the mean inhibitory zones was not significant with the zone elicited by *P. aeruginosa* and *B. subtilis* being responsible.

Table 2 The effect of hexane extract of the bark of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	0.04^a±0.01	0.02^a±0.01	NMZI	NMZI
Pseudomonas aeruginosa	0.05^a±0.01	0.03^a±0.01	NMZI	NMZI
Escherichia coli	0.05^a±0.01	NMZI	NMZI	NMZI
Staphylococcus aureus	0.02^a±0.01	NMZI	NMZI	NMZI
Enterococcus feacalis	0.03^a±0.01	NMZI	NMZI	NMZI
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (30.00 mm±3.00) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of aqueous root extract (Tab. 3). *S. aureus* and *E. coli* elicited the least zone of inhibition (0.02 mm±0.01) against 50 mg/ml and 100 mg/ml aqueous root extract concentrations (Tab.3). The observed differences in the mean inhibitory zones was significant with the zone elicited by *E. coli*, *S. aureus, E. faecalis* and *B. subtilis* being responsible**.**

Table 3 The effect of aqueous extract of the root of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	25.00^a±1.00	23.00^a±1.00	0.09^b±0.01	0.04^b±0.01
Pseudomonas aeruginosa	30.00^a±3.00	27.00^a±2.00	25.00^a±0.50	20.00^a±0.75
Escherichia coli	13.00^a±1.55	10.00^a±0.35	0.02^b±0.01	NMZI
Staphylococcus aureus	22.00^a±0.10	18.00^a±0.20	16.00^a±1.56	0.02^b±0.01
Enterococcus feacalis	20.00^a±1.00	17.00^a±0.20	16.00^a±0.78	15.00^a±050
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (0.08 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of hexane root extract (Tab. 4). *B.subtilis*and *E. faecalis* elicited the least zone of inhibition (0.02 mm±0.01) against 100 mg/ml and 200 mg/ml hexane root extract concentrations (Tab.4). The observed differences in the mean inhibitory zones was significant with the zone elicited by *B. subtilis* being responsible**.**

The highest inhibitory zone (0.08mm) was shown by *P. aeruginosa* at 400mg/ml whilst the least inhibition zone (0.02mm) was displayed by*B.subtilis* and *E. faecalis* at100mg/ml and 200mg/ml respectively. The differences in the mean inhibitory zones shown by *B. subtilis* was not significant (P>0.05) (Tab. 4).

Table 4 The effect of hexane extract of the root of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	0.05^a±0.01	0.02^a±0.01	0.02^a±0.01	NMZI
Pseudomonas aeruginosa	0.08^a±0.01	0.05^b±0.01	NMZI	NMZI
Escherichia coli	0.05^a±0.01	0.04^a±0.01	NMZI	NMZI
Staphylococcus aureus	0.03^a±0.01	NMZI	NMZI	NMZI
Enterococcus feacalis	0.04^a±0.01	0.02^a±0.01	NMZI	NMZI
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

Gentamicin exhibited inhibitory activity against all the exposed bacterial cultures except *B. subtilis.* The highest inhibition zone (50 mm) against gentamicin disc was shown by *S. aureus*. Fluconazole did not show any significance inhibition on the fungal test organisms (Tab. 5).

Table 5 Effects of the antibiotics on the test organisms

Test organisms	Zone of inhibition (mm)	
	Gentamicin (5mg/ml)	Fluconazole (5mg/ml)
Bacillus subtilis	NMZI	NA
Pseudomonas aeruginosa	13	NA
Escherichia coli	29	NA
Staphylococcus aureus	50	NA
Enterococcus feacalis	20	NA
Candida albicans	NA	NMZI
Microsporium audouinii	NA	NMZI

NMZI- No Measurable Zone of Inhibition; NA- Not Applicable

The hexane extract of root and bark gave a negative result for all the secondary metabolites whereas the aqueous extracts of root and bark gave positive result for some phytochemicals (Saponin, flavonoids, reducing sugar, carbohydrates, volatile oils, phenolic compounds) (Tab. 6).

Table 6 Summary of the result from the phytochemical analysis of aqueous and hexane extracts of root and bark of *Dennettia tripetala*

Phytochemical constituents	Aqueous		Hexane	
	Bark	Root	Bark	Root
Alkaloids	-	-	-	-
Saponins	+	+	-	-
Tannins	-	-	-	-
Volatile oils	+	+	-	-
Anthracene	-	-	-	-
Flavonoids	+	+	-	-
Reducing sugars	+	+	-	-
Carbohydrates	+	+	-	-
Cyanogenetic glycerides	-	-	-	-
Phenolic compounds	+	+	-	-

\+ - Present.
\- Absent.

The bacterial isolates all displayed an MIC value against hexane and aqueous root and bark extracts. The minimum inhibitory concentration of the aqueous extract of root to inhibit the bacteria test organisms ranged from 50 - 400 mg/ml. The lowest MIC of 50 mg/ml was recorded against *S. aureus*, *B. subtilis*, *E. faecalis* and *P. aeruginosa* while the highest MIC of 400 mg/ml was recorded against *E. coli*, *S. aureus* and *E. feacalis.*

Table 7 The minimum inhibitory concentration of aqueous extract of the root of *D. tripetala* on test organisms.

Test organisms	Concentration of extracts (mg/ml)			
	Aqeous		Hexane	
	Root	Bark	Root	Bark
Staphylococcus aureus	50	100	400	400
Bacillus subtilis	50	100	100	200
Escherichia coli	100	100	200	400
Enterococcus faecalis	50	50	200	400
Pseudomonas aeruginosa	50	50	200	200
Candida albicans	>400	>400	>400	>400
Microsporum audouinii	>400	>400	>400	>400

DISCUSSION

The aqueous preparation of *D. tripetala* roots exhibited a greater antibacterial activity in comparision with other extracts prepared from the same plant. The antimicrobial activity of *D. tripetala* root can be attributed to the metabolites which it contains. It was observed that sensitivity increased with increasing concentration of the aqueous root extract. The hexane extracts showed little or no activity against the test microorganisms. This might be attributable to the fact that there was no phytochemicals extracted for it to show activity against the test microorganisms. The potency of the extract was comparable to those of antibiotics which are pure substances. The extracts did not show any activity against fungi (i.e. no antifungal activity) probaably due to the high resistant nature of the fungal strain used. The antifungal agent, fluconazole did not show activity against the fungal isolates probably because the strains of the fungi used are resistant to the activity of the antibiotic.

Based on the statistical analysis, there was significant difference between the inhibitory effect of the aqeous root and bark extracts against *P. aeruginosa*, *B. subtilis*, *E. coli*, *S. aureus*, *E. faecalis*. However, there was no significant difference between the inhibitory effect of the hexane bark and root extract. Considering the minimum inhibitory concentration (MIC) values of the aqueous root extract of the plant, the most potent activities were against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. The ability of the extract to inhibit some of the test bacterial isolates further confirm its traditional medical use by the traditional medical practitioners for the treatment of stomach disturbance and skin infections. *Staphyloccocus aureus* is known to play a significant role in skin diseases including superficial and deep follicular lesion, so the strong activity of the aqueous root extract of *D. tripetala* indicated that it could be effective against skin diseases. The antimicrobial activity observed for the aqueous extract of the root may be due to the fact that roots store more of the chemical compounds produced by plants than the bark. The root has cells that function in storing or accumulating certain active secondary metabolites and also due to the solvent used in extraction (i.e. distilled water) which is a polar solvent. However, the mechanisms through which the extract from the present study exert its antimicrobial activities requires further elucidation. The hexane extracts of the root and bark showed no activity against the test organisms.

A great number of chemicals found in plants have been said to be responsible for the medicinal properties of the plants. Saponins have been known to provide the starting material for the synthesis of corticosteroids and oral contraceptives **(Trease and Evans, 1996),** which are drugs that affect the female hormones. This observation supported the usage of *D. tripetala* in preparation of dishes for pregnant and postpartum women and also in prevention of nausea in pregnant women according to **Nwinuka and Nwiloh (2009)**. Volatile oil (i.e. B-phenylnitroethane) present in the plant parts, gives it its typical fragrance as well as for its pungency. These volatile oils together with phenolic compounds have antibacterial property which supports the use of the plant as antibacterial. Flavonoids are strong antioxidants, also found to be effective antimicrobial substances *in vitro* againsta wide range of microorganisms by inhibiting the membrane bound enzymes **(Cowan, 1999).** This support the use of the plant as antibacterial drugs. **Ejechi and Akpomedaye, (2005)** reported that essential oil and phenolic acid of pepper fruit can play a significant role in food preservation and protection against pathogens when they tested the extracts on fresh beef. The findings in this study agree with earlier studies that, not all phytochemicals are present in all plant parts and that those present differ according to the type of extracting solvent used **(Ayinde *et al*., 2007 ; Tijjani *et al*., 2009).** Based on this, **Okwu *et al*., (2005**) using ethanolic extract of the fruit of *D. tripetala* isolated phenanthrene alkaloid; uvariopsine, which was the first report of isolation of uvariopsine from the family Annonaceae. However, in the present study, the aqueous and hexane extracts did not show the presence of alkaloids (Table 6).

CONCLUSION

The presence of the phytochemicals in the aqueous extract of root has shown that *D. tripetala* root can be very effective against some bacteria making it a good antibacterial agent. The aqueous extract of the root contained active phytochemicals as measured by the degree of inhibition and number of microorganisms inhibited. Therefore it may be considered as potential antimicrobial agents for use in food products and post harvest disease control. However, further studies are recommended on the chemical characterization as well as bio-activity guided studies of the aqeous extract of the root.

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