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PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITIES OF *NEWBOULDIA LAEVIS'* LEAVES ON ISOLATES FROM WOUND INFECTIONS.

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ABSTRACT

The leaves of Newbouldia laevis were subjected to ethanolic, hot, and cold aqueous extraction. Standard methods were used for both extraction and phytochemical screening. The antimicrobial activity of the plant extract was done using the modified agar well diffusion method. The extracts (ethanolic, hot, and cold aqueous) at a concentration of 100 mg/ml, and varying concentrations (100mg/ml, 50mg/ml, 25mg/ml) were used to challenge bacterial isolates in duplicates. The test organisms were Escherichia coli, Klebsiella pneumoniae, Proteus sp, Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Candida albicans.The ethanolic extract was screened phytochemically and revealed the presence of flavonoids, tannins, terpenes, steroidal and cardiac glycosides.The ethanolic extract showed highest antimicrobial activity of 14.5mm on the enteric gram negative organisms (Proteussp, K. pneumoniae and Escherichia coli), followed by the hot aqueous extract (12.5mm) on the enteric gram negative organisms and Staphylococcus aureus. The cold aqueous extract exerted the least inhibition of 11mm on the enteric bacteria. The zones of inhibition of the ethanolic extract for Staphylococcus aureus and Pseudomonas aeruginosa were 13.85mm and 13.86mm respectively; it was 12.5mm against C.albicans and 12mm against S.epidermidis. The hot aqueous extracts had the same diameter zones of inhibition of 12.5mm on Proteus sp, K. pneumoniae, E.coli and S.aureus, and it exerted the same inhibition zone of 11mm on both S. epidermidis and C. albicans, while it had 11.9mm on P.aeruginosa. The cold aqueous extract exerted the least zones of inhibition of 11mm against the enteric bacteria, 10.83mm and 10.8mm on S.aureus and P. aeruginosa respectively; and same inhibition of 10mm on both S. epidermidis and C. albcans. The extracts were subjected to MIC evaluation and the findings showed that E.coli was the most sensitive; followed by K. pneumoniae and Proteus sp, followed by S. aureus and P. aeruginosa, while S. epidermidis and C. albicans were the least affected by the antimicrobial effects of the leaves' extracts of N.laevis.There was no value for MBC. This work indicates that N. laevis' leaves possess phytochemicals with antimicrobial activity and could be used to treat wound infections when purified to the appropriate pharmacological level.

Key words: *Newbouldia laevis,* Phytochemical, Antimicrobial, Wound isolates.

INTRODUCTION

Newbouldialaevis (African Border Tree, Akoko Tree, *Newbouldia)* is referred to as *Newbouldialaevis*Seem, because it was Seem who was the first botanist (biologist) to give this plant a name – *Newbouldialaevis.Newbouldialaevis* is a medicinal plant. From non – structural

interview it was said that prior to 'ofor', *Newbouldialaevis'* tree was used for all the functions that 'ofor ' is being used today, like pouring libations, 'nsu' and offering of prayers by the Idol worshippers, etc, in Eastern Nigeria.Apart from its use for ritual and religious activities, it was popularly used for therapeutic purposes.Today, the leaves of these plants are being used by the herbalists for the treatment of ailments such as diarrhoea, dysentery, sexually transmitted diseases, jaundice and several other diseases caused by microorganisms (Akinperuetal. 2009). Preliminary phytochemical screening of the methanolic leaf extract in Nigeria by Usman and Osuji, (2007) revealed the presence of flavonoids, tannins, terpenes, steroidal and cardiac glycosides and the extract inhibited the growth of several microorganisms.There are many scientific reports crediting medicinal plants such as *Newbouldialaevis* in treating wound infections. Wounds are injuries sustained by individuals, which bring about breakage in the continuity of the skin, and allow organisms to gain access to tissues and cause infections. Ezebialuetal. (2010) reported that wound infections occur when virulent factors expressed by one or more microorganisms in a wound overcome the host natural immune system.The huge success recorded by quinine and quinidine isolated from *Cinchona* tree bark and recently artemisinin from *Artemisia annua*in the treatment of malaria (Di Flumerietal. 2000) had triggered interest in scientific search for medicinal plants. *N. laevis*is a potential source of novel drugs. This work is aimed at the phytochemical and antimicrobial potential of *N. laevis' leaves.*

MATERIALS AND METHODS

Collection and Preparation of the Plant Extracts

The leaves of *Newbouldialaevis*were collected at Okigwe area of Imo State, Nigeria. The identity of the plant was authenticated by Dr. Ezuma - the Head of Department of Plant and Animal Science, Abia State University, Uturu, Nigeria. The fresh leaves, after collection, were washed, air-dried for 5 days and ground to powder.

Extraction of Plant Leaves

The plant's material was subjected to ethanol, hot, and cold maceration technique for extraction.Theleafpowder (150g) was suspended in 900 ml of ethanol at room temperature. After 48 hours the crude extract was then decanted and filtered under vacuum through Whatman filter paper No. 1. The filterate was evaporated under vacuum, using rotary evaporator at 45^0C.The concentrated ethanol extract was dried further in a water bath for 48 hours. Fifty grammes of the leaf powder were infused in 300 ml of hot sterile distilled water and another 50g in 300 ml of cold sterile distilled water at room temperature. After 48 hours the crude extracts weredecanted and filtered under vacuum through Whatman filter paper No. 1. The filterates were then dried in a water bath for about 4 – 5 days. The quantities of extracts yieldedfrom ethanol, hot and cold aqueous extractions were 16.47g, 6.03g, and 5.946g respectively. The solid residue was stored in glass vials in a refrigerator (4^0C) from which portions were taken for each of the experiments.

Source of Organisms

Most of the microbial cultures used were from the Medical Microbiology Department of the University of Port Harcourt Teaching Hospital. Specimen collection, transport, and processing were carried out using conventional methods. All the isolates

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(microbial organisms) were identified by their cultural, microscopic and biochemical characteristics using standard methods (Tortoraetal.,2013; Willey etal ., 2011; Forbes etal.,2007;Finegold and Martin, 1982; Cheesbrough, 2002).

Antimicrobial Assays

Preparation of Microbial Inocula

About 4–5 colonies of 16 to 24 hours of age of the test organisms were selected from an agar plate and suspended in Mueller Hinton broth solution.The cultures were allowed to achieve good active growth, indicated by a turbid suspension. (Willey etal ., 2011; Forbes etal.,2007; Finegold and Martin, 1982; Cheesbrough, 2002). The use of standard inoculum size is as important as culture purity and was achieved by comparison of the turbidity of the organism suspension with a turbidity standard. Use was made with commercially prepared 0.5 Mcfarland which provided an optical density comparable to the density of a bacterial suspension of 1.5 x 10^8 colony forming units (CFU) / ml. A hundred fold dilution was made to achieve a x 10^6 colony forming units (CFU) / ml.

Screening for Antimicrobial Activity

A modified agar-well diffusion method was adopted in which the screening of the ethanol extract, the hot aqueous and cold aqueous extracts, and the standard antibacterial agents were carried out. About 25ml of sterile molten Mueller Hinton agar were cooled to about 40^0C and aseptically seeded with 100 µl of the desired organism at a turbidity of approximately 10^6 CFU / ml. The seeded agar was aseptically poured into sterile Petri dishes of 8.5cm (85mm) in diameter and allowed to set at room temperature. With the aid of a sterile cork borer, four uniform wells of 7mm in diameter were punched in each plate. The wells were each filled with 0.1 ml of a stock solution of the different extracts to give a final strength of 10 mg/ml of each of the extracts; a 0.1 ml of gentamicin solution to give a final concentration of 10µg/ml which was used as positive control. A pre-diffusion time of 1 hour was allowed before the plates were incubated aerobically at 37^0C for 18 – 24 hours. The diameter of the zones of inhibition was measured to the nearest millimeter with a transparent millimeter rule. Antimicrobial screening, using the varying concentrations (100 mg / ml, 50 mg / ml, 25 mg / ml) of the different extracts was conducted using the modified agar well diffusion method. The set up for the antimicrobial activity including the varying concentrations of the plant extracts (100mg/ml, 50mg/ml, & 25mg/ml) and control antibiotic agent was done in duplicate.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal(MICROBICIDAL) Concentration (MBC)

The modified broth method, as used by Sofowora, (2008), Akereleetal. (2011), was used to determine the MIC of the ethanolic, hot aqueous, and cold aqueous extracts. Serial two-fold dilutions of 2ml each were made in a concentration range of 50 to 0.391 mg/ml of the ethanolicextract and 25 to 1.5625 mg/ml of the aqueous fraction using double strength nutrient broth (Sabouraud's dextrose broth was prepared for fungal analysis) supplemented with 10 % glucose. The tubes were inoculated with 100 µl of thetest organisms (at a turbidity of approximately 10^6 CFU / ml) and were incubated aerobically at 37^0C for 18 – 24 hours. Microbial growth was determined by adding 20 µl of phenol red (0.2 %) indicator

and observing a change in colour from red to yellow when there was microbial growth. The lowest concentration that showed no change in colour was considered the MIC. For minimum microbicidal concentration or minimum bactericidal concentration – MBC determination, 20 µl of the liquid from each tube that showed no change in colour for the three extracts were plated on antimicrobial agent – free determining agar (eg. nutrient agar, Sabouraud's dextrose agar).The plates were incubated aerobically at 37^0C for 18 – 24hours. The lowest concentration that yielded no growth was recorded as the minimum microbicidal concentration or minimum bactericidal concentration (MBC).

Phytochemical Tests

These were analysed using standard methods (Akereleetal., 2011;Usman and Osuji, 2007;Tease and Evans, 1989; Harbone and Baxter, 1993;).

RESULTS

Distribution of Test Organisms

Fifty (50) microbial isolates were recovered out of the one hundred and fifty (150) samples from various suspected infected wounds. The most prevalent isolate was *P.aeruginosa* followed by *S.aureus* and *Proteus* sp. Then followed by *Klebsiellapneumoniae, E.coli, Candida albicans*and *S.epidermidis*. Gram positive bacterial isolates were *S. aureus* (18%), *S. epidermidis* (2%); and the Gram negative bacterial isolates, were *Proteus* sp(18%); *P. aeruginosa* (28%); *Klebsiellapneumoniae* (16%) and*E. coli* (14%). The frequency distribution of microbial fungal isolates was two isolates of *Candida albicans,* which gave 4%. The distribution of microorganisms among different wound types is shown in Table 1. Out of the fifty microbial isolates, 17 (34%) bacterial isolates were isolated from soft tissue infections. There were 7 (14%) and 4 (8%) bacterial isolates from burns and diabetic ulcers respectively. The leg and pressure ulcers accounted for 5 (10%) bacterial isolates, while the surgical wounds accounted for 6 (12%) bacterial isolates. Ten(ie, 20%) of the microbial isolates were from unknown or unrecorded sources (these included 2 *Candida albicans* and 1 *Staphylococcus epidermidis*). The frequency of isolation of microorganisms from the different wounds is shown in Table 2. Out of the 150 samples, positive growth was observed in 49 (32.7%), out of which, 48 (32%) samples showed monomicrobial growth, while only 1 (0.67%) sample showed polymicrobial growth of *S.aureus and S.epidermidis*. Ten,6.67% wounds showed no growth. Majority of the wounds (91 samples representing 60.67%), mostly from private hospitals and laboratories were contaminated.

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Table 1: Distribution of Microorganisms among Different Wound Types at the Upth

Wound types	a	b	c	d	e	f	g	Total
Surgical	1(16.7)	0(0)	3(50)	1(16.7)	1(16.7)	0(0)	0(0)	6
Burns	2(28.6)	2(28.6)	2(28.6)	1(14.3)	0(0)	0(0)	0(0)	7
Acute soft tissue	3(17.7)	5(29.4)	5(29.4)	2(11.7)	2(11.7)	0(0)	0(0)	17
Diabetic ulcer	1(20)	0(0)	1(20)	1(40)	1(20)	0(0)	0(0)	4
Leg & pressure Ulcers	1(20)	1(20)	1(20)	0(0)	2(40)	0(0)	0(0)	5
Non defined	1(10)	1(10)	2(20)	2(20)	1(10)	1(10)	2(20)	10
Total	9	9	14	8	7	1	2	50

Key: a= *Staphyloccocus aureus*
b= Proteus sp
c= Pseudomonas aeruginosa
d= Klebsiella pneumoniae
e= E.coli
f = S.epidermidis
g = Candida albicans

Figures in brackets are percentages of the microorganisms in different wound types and non-defined are wound isolates from stock culture that had no record of wound types.

Table 2: Frequency of Isolation of Microorganisms from Different Wound Types at the Upth

Wound Types	Positive Samples	Monomicrobial	Polymicrobial	Contaminants	No Growth	Total Samples
Surgical	6 (12.25)	6 (12.5)	none	none	1(10)	7 (4.7)
Burn	7 (14.3)	7 (14.6)	none	none	4(40)	11(7.3)
AST	17 (34.7)	17 (35.4)	none	40 (44)	2(20)	59(39.3)
D. ulcers	4 (8.2)	4 (8.3)	none	none	1(10)	5 (3.3)
L&P ulcers	5 (10.2)	5 (10.4)	none	51(56)	2(10)	58 (38.7)
Non-defined	10 (18.4)	9 (18.8)	1 (100)	none	none	10 (6.7)
Total	49	= (48 +	1)	91	10	150

Key: AST = Acute soft tissue; D. ulcers = Diabetic ulcers; L&P ulcers =Leg and pressure ulcers; Figures in brackets are percentages of the frequency of the isolation of the microorganisms from different wound types.

Table 3: The Minimum Inhibitory Concentration of the Ethanolic Leaves' Extract of Newbouldia Laevis.

Test organisms	Concentrations (mg /ml)							
	50	25	12.5	6.25	3.125	1.563	0.781	0.391
S.aureus (1)	-	-	-*	+	+	+	+	+
S.aureus (2)	-	-	-*	+	+	+	+	+
Proteus sp.	-	-	-	-*	+	+	+	+
P. aeruginosa (1)	-	-	-*	+	+	+	+	+
P. aeruginosa (2)	-	-	-*	+	+	+	+	+
K. pneumoniae	-	-	-	-*	+	+	+	+
E. coli	-	-	-	-	-*	+	+	+
S. epidermidis	-	-*	+	+	+	+	+	+
Candida albicans	-*	+	+	+	+	+	+	+

Key:
Values are means of replicates (n = 2)
– = No turbidity
+ = Turbidity
-* = MIC values, which are 12.5; 12.5; 6.25; 12.5; 12.5; 6.25; 3.125; 25; 50 mg /ml; against*S.aureus (1), S.aureus (2),Proteus* sp, *P. aeruginosa (1), P. aeruginosa (2), K.pneumoniae, E. coli, S. epidermidis*, *Candida albicans*, respectively.

Table 4: The Minimum Bactericidal (Microbicidal) Concentration of the Potent Ethanolic Leaves' Extract of *Newbouldia Laevis*

Test organisms	Concentrations (mg /ml)							
	50	25	12.5	6.25	3.125	1.563	0.781	0.391
S.aureus (1)	+	+	+	-	-	-	-	-
S.aureus (2)	+	+	+	-	-	-	-	-
Proteus sp.	+	+	+	+	-	-	-	-
P. aeruginosa (1)	+	+	+	-	-	-	-	-
P. aeruginosa (2)	+	+	+	-	-	-	-	-
K. pneumoniae		+	+	+	+	-	-	-
E. coli	+	+	+	+	+	-	-	-
S. epidermidis	+	+	-	-	-	-	-	-
Candida albicans	+	-	-	-	-	-	-	-

Key: Values are means of replicates (n = 2); - = Not applicable (ie. the tubes that had growth in MIC that needed not to be tested for MBC). + = Growth (ie. No bactericidal effect or microbicidal effect or killing effect).

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DISCUSSION

The least antimicrobial activity was observed on *Candida albicans*. Other workers have reported similar results (Usman and Osuji, 2007). As regards the potency of these extracts, the ethanolicextract was more potent than the aqueous extracts, and the hot more than the cold aqueous extracts; this potency (high activity) may correspond to their respective solubility in the solvents.The solvent plays an important role in the extraction of the active principle or ingredient. The higher microbial activity of ethanolic extract over the aqueous extracts might be as a result of more active chemical constituents being dissolved and being recovered in alcoholic solvent than the aqueous,ie; the activity of the extracts depends on the method used to obtain the extract(Unaeze et al. 1986).

Some of the active principles or ingredients of some plants'leaves like *Aspila africana* are not soluble in cold water and this may justify the preference for the local gin " kayi-kayi " or " ogogoro " as extractant in the preparation of crude drugs from medicinal plant material by traditional healers (Anibijuwonetal. 2010). Apart from herbal application, there is need for use of appropriate solvents in the dressing and treatment of wound infections. This proves and supports the medical practice of cleansing the wounds with spirit (70% alcohol), which possibly aids and promotes healing processes apart from acting as an antimicrobial agent. All the extracts of the leaves of *Newbouldia laevis'* at concentrations of 100mg/ml, 50mg/ml, 25mg/ml showed growth inhibition on all the tested organisms; with ethanolic extract exerting more diameter zone of inhibition than the hot and cold aqueous extracts. However, hot aqueous extract had wider diameter zone of inhibition than the cold extract. The degree of antimicrobial activities obtained vary from one researcher to another because they may use different strains. The different methods of purification yield different amounts of active ingredients. Our study had results similar to those of most researchers (Emeruwa, 1982;Akerele et al. 2011;Anibijuwon et al . 2010). Usman and Osuji, (2007) had wider diameters against some test organisms;Unaeze et al. (1986) stated that the potency of the extract depends on the method used to obtain the extract. Other research works have shown that the age of the plant when harvested and the season determine the amount of the active constituents, hence the active ingredients of plants can vary in quality and quantity from season to season (Sofowora, 1982). Many reports have confirmed that constitutional (conventional) antibiotics are more active than plant extracts (Emeruwa, 1982; Akerele et al. 2011; Anibijuwon et al . 2010). This is also true in the present case as the conventional antibiotic (gentamycin) exerted wider zone of inhibition than the plant's extracts. However, some like Usman and Osuji, (2007) had wider zones of inhibition (in some organisms) with plant extracts than some conventional antibiotics. Generally, most of the reports agree that both plant extracts and conventional antibiotics have higher antimicrobial effect on the bacteria than on the fungal organisms, similar to our results in this study. However, the most important factor in comparing the effect of conventional antibiotics and plant extracts is the incomparable concentrations of the two cases. This disparity in concentrations is also recognized in reports of plant extracts showing wider zones of inhibition than the conventional antibiotic (Usman and Osuji, 2007). This is also the problem encountered in the invivo effectiveness of these plant materials at the

same concentrations as the conventional antibiotics. Our study suggests that the active principles or ingredients obtained from the *N. laevis'* plant at this time of the year (October and November), showed only bacteriostatic effects and not bactericidal.

Ethanolic extracts of *N. laevis'* proved to be more active than the hot and cold aqueous extracts. Scientific knowledge is useful in harnessing the potency of medicinal plants (eg. *Newbouldialaevis)* for better cure and application.Therefore traditionalpractitioners can be assisted if they can come forward with their historical knowledge of these plants. So, when both parties (traditional practitioners and modern science) work together, knowledge, preparation and administration of correct doses and standardization can be improved and achieved. Finally, *Newbouldialaevis'* plant promise for antimicrobial agents for treating wound infections.

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