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Design and microbial screening of herbal ointment of *Phyllanthus amarus*

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Abstract
Objective: Herbal ointment containing methanol extract of the aerial part of *Phyllanthus amarus* was formulated and parameters including antimicrobial properties against some human pathogens such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Aspergilus niger* were evaluated.
Method: The extract was obtained by maceration of 1 part of pulverized dried leaves in 4 parts of methanol. Simple ointment BP was used as ointment base for dispersion of the plant extract. Parameters investigated include; spreadability, pH and zones of inhibition of extract and formulated ointment while chloramphenicol and fluconazole ointments were used as controls. The sensitivity of the organisms in ointment containing varying concentrations of the extract was also determined.
Result: The *pH* of the ointment was 6.17 ± 0.9 and the spreadability was 120 ± 0.5. *B. subtilis, P. aeruginosa and Staph aureus* were susceptible to ointment containing 0.1% w/v and 0.15% w/v extract concentration, while *E. coli* and *A. niger* were resistant. The organisms were all susceptible to the ointment containing 0.2% w/w extract concentration.
Conclusion: *Phyllanthus amarus* ointment has good antimicrobial properties and can be used for the treatment of wounds and skin infection caused by susceptible organisms.
Keywords: Herbal formulation, methanol extract, *Phyllanthus amarus*, ointment, antimicrobial activity.

1. Introduction
More than 50,000 different species of medicinal plants have been recognized in Africa and many of them are useful in traditional medicine for prophylaxis and cure of diseases[1]. An estimated 50% of modern drugs are of natural-product origin[2] and these products play important roles in drug development[3]. Notwithstanding great advances in modern medicine, plants still make an important contribution to health care. In recent times, the practice of herbalism has gained prominence[4]. Majority of the population still use medicinal plants for their primary health care due to either poverty or lack of access to modern medicine. Moreover, it is an important part of African culture. The World Health Organization (WHO) clearly recognizes these facts and the importance of medicinal plants, hence proposed their authentication the world over[5]. A wide range of diseases have been treated with medicinal plants, these include: diarrhea, dysentery, diabetes asthma, abdominal pains, sprains, bacterial and fungal infections[6]. The India system of Ayurvedic medicine and Chinese traditional medicine (CTM) are based largely on native plants easily found in the vicinity of most rural homes and were found to be effective and within the reach of the poor. Generally, up to 80% of available medicine comes from medicinal plant in rural communities of developing countries[7], these also provide raw material for industrial manufacture of pure chemical compounds[8]. These and many other reasons stimulate the interest in research and development work on medicinal plants. One of such well established herbal plant is *Phyllanthus amarus*.

Phyllanthus amarus Schum & Thonn (Euphorbiaceae) is a small herb growing up to 50 cm in height with small yellow flowers (5 white sepals and apical acute anther), leaves and fruits. The fruit has green capsules with smooth, fruiting pedicels. The Urhobo speaking people of Nigeria call it obukoiyeke locally[9], while it is known as Iyin-olobe among the Yorubas[10]. Phytochemical studies have shown the presence of many valuable compounds such as lignins, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids[11]. *Phyllanthus amarus* has diuretic, astringent and cooling properties. It also has antimicrobial, anti-diabetes and anticancer properties[12,13]. Its anti-inflammatory, anti-hepatotoxic, antispasmodic and analgesic properties are well documented[14]. Some researchers have also established its antiviral activity against hepatis B virus[15,16]. It also possesses anti-nociceptive activity[17]. Adeneye *et al*, had also established its anti-lipidamic potential[10]. Whole plant as well as parts such as the roots, stems and leaves has been used.

Based on these numerous claims, simple ointment of *Phyllanthus amarus* was formulated and evaluated *in vitro* to see if its antimicrobial properties are retained in the ointment formulation. Simple ointment consists of hydrocarbons (hard and soft paraffin) and absorption (wool fat and cetostearyl alcohol) bases which combine occlusive properties for longer action and emollient effect[18]. The specific

objectives of this work were to: (i) Formulate simple ointment with methanol extract of *Phyllanthus amarus*; (ii) Investigate the *in vitro* activity of the pure extract and the ointment of *Phyllanthus amarus* on *B. subtilis, S. aureus, P. aeruginosa, Escherichia coli* and *A. niger*.

2. Materials and methods

2.1 Materials

Fresh aerial parts of *Phyllanthus amarus* were collected from Faculty of Pharmacy botanical garden, Delta state University, Abraka and identified by the plant Curator Mr. Sunday Nimehe in the Department of Pharmacognosy, Faculty of Pharmacy, Delta state University, Abraka, Nigeria, where a voucher specimen was deposited for reference.

The herbs were washed, chopped into bits and air dried under shade. Wool fat, hard paraffin, cetostearyl alcohol, Tween 80, white soft paraffin (BP grade), chloramphenicol (Yangzhou Pharmaceutical Co. Ltd.) and fluconazole. Methanol (BDH, Poole England). Other reagents used were of analytical grade and were used without further purification.

2.2 Methods

2.2.1 Extraction

The dried leaves were pulverized into a coarse powder with the aid of laboratory milling machine. A 250 g quantity of the powder was extracted with 1000 mL of methanol using Soxlet apparatus. Excess solvent was removed with the aid of a Rotary Evaporator (CH – 9230 Flawil, Switzerland) and further concentrated in a vacuum oven (Labtech, India).

2.2.2 Preparation of ointments

Simple ointment BP was formulated according to the formula in **Table 1**[19]. *Phyllanthus amarus* ointment was prepared by melting 200 g quantity of simple ointment BP in a stainless steel jar over hot water bath maintained at 80°C. The molten ointment was removed from the hot water bath and 10 g of *Phyllanthus amarus* extract was added and triturated until cold. The formulation was transferred into an ointment jar and stored pending further investigations.

Table 1: Formula for the preparation of Simple ointment BP[19]

Ingredients	Formula (g)	Amount used (g)
Wool fat	50.0	12.5
Hard paraffin	50.0	12.5
Cetostearyl alcohol	50.0	12.5
White soft paraffin	850.0	212.5
Total	**1000.0**	**250.0**

2.2.3 Ointment spreadability test

One gram of the ointment was placed on one side of a slide and covered with a second slide. An object of known weight was placed on top of the second slide for 5 min. The second slide was allowed to slide over the first slide under gravity in a vertical position and the time taken noted. This procedure was done in triplicate and average value recorded.

2.2.4 Evaluation of pH of ointment

The pH of various formulations was determined using a Digital pH meter (Hanna Instruments, Wood socket RI USA). One gram of the weighed formulation was dispersed in 100 mL of diluted tween 80 and the pH wasdetermined directly[20, 21].

2.2.5 Assessment of antimicrobial activities of *Phyllanthus amarus* extract and ointment

(a) Collection of microorganisms

The organisms used for the antimicrobial activity were collected from Microbiology Department ofDelta State University Teaching Hospital Oghara, Delta State, Nigeria.

The antibacterial screening was done with crude methanol extract of *Phyllanthus amarus*, ointment formulated with extract (dissolve with tween 80) and chloramphenicol against four bacteria isolates (*Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* using Agar well diffusion according to the method described by Ogunjobi and Abiala[22].

(b) Antifungal screening

Active culture of the fungi made into slant was shaken vigorously to make fungal spores suspension. *A. niger* suspension was made in buffer peptone water with 24 h of incubation at 25 ± 0.5 ^{o}C. The suspension was mixed with sterile potato dextrose broth (PDB) poured into petri dishes and allowed to solidify. A sterile cork borer was then used to bore wells into the agar. Equal amount of the *Phyllanthus amarus* extract and fluconazole was dispensed into the agar, which was later observed for zone of inhibition[23].

(c) Control experiment

Control experiment was set up by using sterile distilled water in place of methanol extract. Chloramphenicol 0.01 to 0.2 mg/mL) and fluconazole (0.01 to 0.2 mg/mL) were used as positive control.

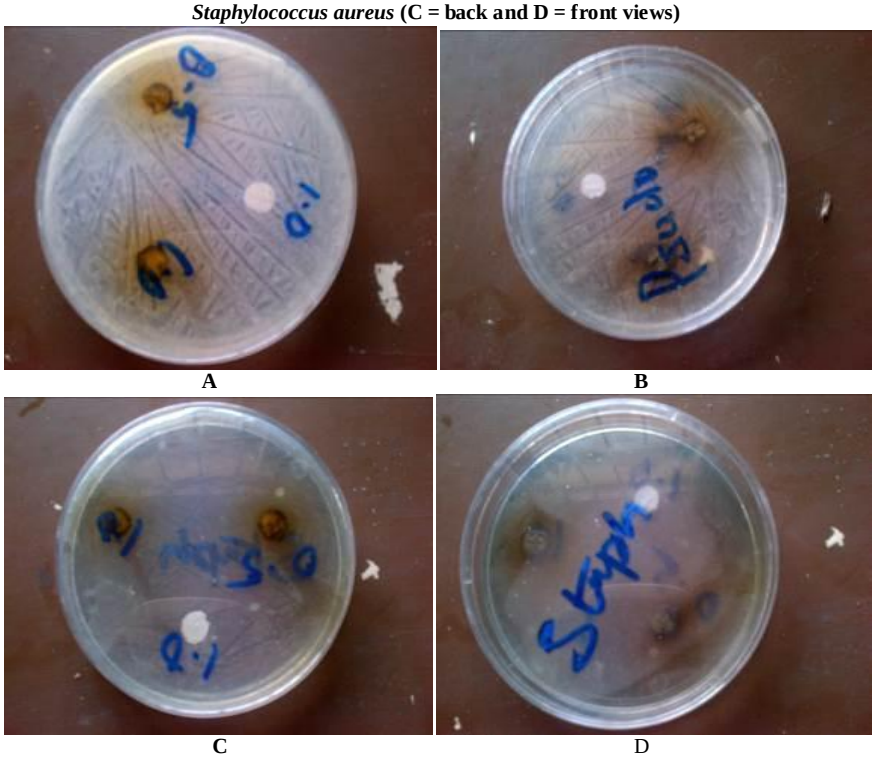
3. Results and Discussion

The pH of the methanol extract of *Phyllanthus amarus* ointment was 6.23 ± 0.1. It is slightly acidic probably due to the presence of gallic acid, amariinic acid and repandusinic acid, which are among the principal constituents of *Phyllanthus amarus* extract[24, 25]. The pH of the formulation lies within the normal range of the human skin (4.5 to 6.8)[21,26] and as such it will not likely produce any skin irritation such as erythema and edema[21].

The spreadability is 120 ± 0.1 sec, which indicates that the formulated ointment does not spread easily on the skin surfaces. This is expected since simple ointment is made up of mainly hydrocarbon bases (Soft paraffin and hard paraffin). Hydrocarbon bases are sticky, thus providing an occlusive effect for longer pharmacological action. This will limit the amount of phytochemicals absorbed into systemic circulation, thus reducing systemic side effects of these phytochemicals. It also contains components of absorption bases (wool fat and cetostearyl alcohol). Absorption bases not only provide emollient effect but also aid spreading and absorption of fat soluble active ingredients[18]

The methanol extract of *Phyllanthus amarus* and the formulated ointment demonstrated varying degree of antimicrobial activity against the test organisms. The methanol extract at concentration ≥0.1 % w/v show high activity against *E. coli, B. subtilis, P. aeruginosa* and *S. aureus*. But *A. niger* was resistant at <0.2% w/v (**Fig. 1 and Table 2**). However, the formulated ointment showed high inhibitory activity against *B. subtilis, P. aeruginosa* and *S. aureus* at ≥ 0.1 %w/v. At concentration 0.2 %w/v the test organisms were all susceptible to *Phyllanthus amarus* ointment.

Fig 1: **Disk positive results of *Pseudomonas aeruginosa* (A = back and B = front views) and *Staphylococcus aureus* (C = back and D = front views)**



A **B**

C D

Table 2: Zones of inhibition (mm) of extract and ointment of *Phyllanthus amarus* against clinical isolates

Concentration (%w/v)	*E. coli*		*B. subtilis*		*P. aeruginosa*		*S. aureus*		*A. niger*	
	PE	PO	PE	PO	PE	PO	PE	PO	PE	PO
0.01	7	6	8	6	16	9	13	10	8	0
0.05	10	8	10	6	14	12	14	13	12	0
0.10	17	15	16	18	19	18	20	19	15	15
0.15	16	13	15	19	11	18	22	19	8	15
0.20	16	16	18	19	20	18	23	20	16	16

PE: means *Phyllanthus amarus* extract; PO: means *Phyllanthus amarus* ointment; NOTE: ≥ 16 indicate susceptibility; ≤ 15 indicate resistance

Table 3: Antimicrobial activity (zone of inhibition in mm) of chloramphenicol, fluconazole and methanol against clinical isolates

Chloramphenicol (%w/v)	***E. Coli***	***B. subtilis***	***P. aeruginosa***	***S. aureus***	***A. niger***
0.01	15	14	13	13	-
0.05	15	15	14	14	-
0.10	16	16	17	16	-
0.15	16	17	17	18	-
0.20	17	19	22	23	-
Fluconazole (%w/v)					
0.01	-	-	-	-	15
0.05	-	-	-	-	17
0.10	-	-	-	-	18
0.15	-	-	-	-	16
0.20	-	-	-	-	17
Methanol (%v/v)					
0.01	7	4	5	7	0
0.05	7	5	5	6	0
0.10	8	7	6	8	0
0.15	9	5	4	8	0
0.20	12	11	10	13	0

- Means not applicable; NOTE: ≥ 16 indicates susceptibility; ≤ 15 indicates resistance.

It can be seen from these results, among the tested organisms, that bacteria were found to be more sensitive to the extract and the formulated ointment than fungi. These finding are in agreement with those of other researchers[13,27]. At concentrations of 0.01% w/v and 0.05 %w/v, all the test organisms showed resistance to the formulated ointment. This is a clear indication that *Phyllanthus amarus* ointment is dose dependent and that the extract is more potent than the formulated ointment. As the concentration of the ointment increased, there were larger zones of inhibition and this shows the effectiveness of the plant ointment against the test organisms (**Table 2**). Chloramphenicol, a standard antibiotic had similar antibacterial activity with that of the extract and ointment of the extract (**Table 3**). The results were similar to that obtained for fluconazole against *A. niger*. However, other researchers obtained higher activity when gentamycin and nystatin were used as positive control[14]. The blank test with tween 80 showed no antimicrobial properties whereas for the methanol, antimicrobial activity was observed but the

zones of inhibition were small when compared with the plant ointment (**Table 4**). Even other researchers observed significant activity when hot water was used for the extraction[13]. This helped to support our finding that the activity observed with extract was not due to the antimicrobial activity of methanol.

Table 4: **Antimicrobial activity (zone of inhibition in mm) of methanol against clinical isolates**

Methanol (%v/v)	*E. Coli*	*B. subtilis*	*P. aeruginosa*	*S. aureus*	*A. niger*
0.01	7	4	5	7	0
0.05	7	5	5	6	0
0.10	8	7	6	8	0
0.15	9	5	4	8	0
0.20	12	11	10	13	0

4. Conclusion

The crude methanol extract of *Phyllanthus amarus* and *Phyllanthus amarus* extract formulated as an ointment have antimicrobial activity against *B. subtilis, S. aureus, E. coli, P. aeruginosa* and *A. niger*. Our findings have justified the folkloric medicinal uses and speculations about the therapeutic values of this plant for combating topical infectious diseases. The activity against these organisms showed remarkable concentration dependence. The *Phyllanthus amarus* ointment can be used as a potential agent for the treatment of wounds and skin infections caused by these susceptible organisms.

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