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The pharmacognostic and antimicrobial study of methanolic extract of *Dialium guineense* leaf against organisms isolated from wound infection

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Abstract
There is a growing interest worldwide in the use of natural product of various plants as a natural antimicrobial agent especially now where the problems of emerging and reemerging resistant strains of microorganism are becoming the order of the day. The phytochemical screening and antimicrobial activities of methanolic extract of *Dialium guineense* leaf was tested to provide a basis for their adoption as an alternative control measure in combating this incidence. The Pharmacognostic/phytochemical screening were carried out using standard methods. Antimicrobial activity was tested against clinical isolates of *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus fumigatus* and *Candida albicans* using agar well diffusion method. Results of the Pharmacognostic studies revealed the presence of anticlinal walls which are thick and straight, numerous Uniseriate covering trichomes in both upper and lower epidermis. The phytochemical analysis showed presence of carbohydrate, tannins and alkaloids. The antimicrobial inhibition zone values of the extract ranged between 4 and 18 mm, with *E. coli* being most sensitive, followed by *A. fumigatus* and *P. mirabilis with least effect*. The finding suggests that *D. guineense* might be a good candidate in the search for a natural antimicrobial agent and further supports its popular and wide traditional applications in the treatment of various illnesses. Further study is ongoing to identify the specific secondary metabolites present in *D. guineense* which could be responsible for its antimicrobial activity. Study also needs to be done to determine the toxicological profile of the active principles in this plant.

Keywords: *Dialium guineense*, wound infection, phytochemical screening, antimicrobial activities, clinical isolates, medicinal plants

1. Introduction
The ubiquity of pathogenic organisms leaves us open to developing all sorts of ailments and the resulting use of antimicrobial agents for treating them is a worldwide practice [1]. As more and more of these agents are being used, pathogenic organisms are also changing, developing into different forms, becoming immune [2, 3], and of great concern globally [1, 4]. Several reports have also established that increased resistance to commonly used antimicrobial agents may be due to their indiscriminate use in the environment [2, 5-8]. Although it may be impossible to eliminate completely these organisms from the environment, but the menace they cause can be minimized through the adoption of alternative control measures such as the use of plants and their products. The role of plants in health care seems to be gaining popularity worldwide and has led to their classification as essential sources of medicinal agents while their products have also been used in traditional medicine [8]. Plants with medicinal values are globally available but majority of them are supposedly found in tropical countries. [8] Several of these natural products [9-12] have been reported to exert potential to produce a large number of organic chemicals of high structural diversity- the so called secondary metabolites that serve as defense agents against invading microorganisms [6, 8, 13, 14]. Some of such secondary metabolites with antimicrobial properties, includes tannins, terpenoids, alkaloids and flavonoids [8, 11, 15, 16]. This suggests that fruits, leaves, roots or whole extract from natural products may provide a new source of antimicrobial agents with potentially novel mechanisms of action as previously reported [6, 17]. The therapeutic properties of these natural products could be as a result of the antioxidant, antimicrobial, antipyretic and/or analgesic effects of the compounds present [18]. These compounds act by the rupturing of cell walls and membranes and irregular disruption of the intracellular matrix [9, 11].

Presently, the use of plant natural products in pharmaceutical care for the treatment of various diseases have been receiving increasing attention [19, 20]. The properties of a good number of these plants have not been completely assessed. One of such is *Dialium guineense* also known as Black Velvet Tamarind (BVT) of the family Leguminosae. As an indigenous tropical forest fruit tree, they grow in dense savannah forest, shadowy canyons and gallery forests measuring about 20 m high, 0.8 m in diameter, low-branching, rarely straight, bearing a compact densely leafy crown. [21]. They bear abundant of fruits that are circular and flattened, but sometimes near the apex, with a brittle shell enclosing their seed, embedded in a dry, brownish, sweetly acidic, edible pulp as previously described. [22] Oral interview with African traditional medicine dealers in Imo state Nigeria showed that *D. guineense* leaf is used traditionally in treatment of wound and infection Most of the potentials of this fruit tree are well documented and has gained lot of importance in both its nutritive and medicinal value. [21] According to Ogu and Amiebenomo, [23] Bero *et al*. [24] Odukoya *et al*. [25] *D. guineense* are being used as food supplement and remedies for stomach aches, bronchitis, diarrhoea, severe cough, malaria fever, antiulcer, haemorrhoids, jaundice as well as molluscicides. Similar studies by David *et al*. [26], Ezeja *et al*. [27] Ogu and Amiebenomo [23] also showed the analgesic, anti-vibrio, antidiarrhoeal potentials of methanolic leaf and stem bark extracts of *D. guineense*. There is the need for further research in order to exploit the full potential that may influence the extensive consumption, production, improvement, storage and domestication of *D. guineense* fruit plant. Therefore, this study was carried out to investigate the phytochemical constituents and evaluate antimicrobial activity of methanol crude extract of the *D. guineense* leaf by testing against resistant strains isolated from chronic wound infection. This study also aimed at establishment of a scientific proof to authenticate the traditional medicine claim on the use of *D. guineense* leaf in treatment of wounds and infection.

2. Materials and Methods

2.1 Plant collection and identification

Dialium guineense leaves were collected at Obuno village of Igbo-Ukwu Town, Anambra State located in the South-east region of Nigeria. The plant material was authenticated by H. Onyeachusim and voucher specimens deposited at the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Madonna University, Elele, Nigeria.

2.2 Pharmacognostic study-Macroscopy

Size, Shape, Surface, Venation, Petiole, Apex, Margin, Base, Texture, Odour and Colour of *Dialium guineense* leaf was noted as previously described [28, 29]

2.3 Pharmacognostic study -microscopy

The outer epidermal membranous layer (in fragments) were cleared in chloral hydrate, mounted with glycerin covered with a cover slip and observed under microscope for the presence and absence of epidermal cells, stomata, trichomes and cell inclusions as previously reported [30, 31]. A transverse section through the midrib and lamina of the freshly collected leaves were also made, cleared, mounted and viewed under the microscope [30, 31].

2.4 Post collection process

The leaf samples of *Dialium guineense* were processed by washing in tap water, dried at room temperature for three weeks and placed into a blender to be grounded into powder. The grounded leaves materials were transferred onto a closed tight container and stored at – 4 ºC until ready for use

2.5 Extraction of *Dialium guineense* leaf

200 g of *Dialium guineense* powder was macerated in 900 ml of 95 % cold methanol by cold extraction for 48 hours. The extract was filtered and concentrated to a small volume to remove all the solvent using a rotary evaporator at 40 °C as previously described [6]

2.6 Phytochemical analysis

The following test, lignin, starch**,** mucilage, calcium oxalate crystals, cellulose, carbohydrates (Molisch, Fehling's), cardiac glycosides (Keller-Killiani), cyanogenetic glycosides, saponins, tannins (general, phenazone, iron complex, formaldehyde, modified Iron Complex), alkaloids were performed using standard methods as previously described [11, 28, 30, 32-36].

2.6.1 Test for lignin

A few drops of phloroglucinol and concentrated hydrochloric acid were mixed with a portion of the extract on a clean slide and observed under the microscope for the presence or absence of a pink colouration.

2.6.2 Test for starch

N/50 iodine solution was mixed with extract samples of *Dialium guineense* mounted in a slide and observed for the presence of a blue-black colouration.

2.6.3 Test for mucilage

Extracts of *Dialium guineense* was mixed with ruthenium red solution and observed under the microscope for the presence or absence of pinkish colouration.

2.6.4 Test for calcium oxalate crystals

A few drops of concentrated H_2SO_4 was mixed with extract of *Dialium guineense* sample already cleared in chloral hydrate solution, mounted in a clean slide and viewed under the microscope for the presence of absence of oxalate crystals.

2.6.5 Test for cellulose

A few drops of iodine and concentrated H_2SO_4 were mixed with a powdered portion of the extract on a clean slide and observed under the microscope for the presence or absence of a blue-black colouration

2.7 Determination of moisture content of *Dialium guineense*.

2 g of powdered leaves of *Dialium guineense* was weighed into six different clean crucibles placed in an oven at a temperature of 105 ºC for 4 hours. Allowed to cool in a desiccator and weight of the powder determined. The procedure was repeated until there was no further loss in relation to the air-dried powdered leaf and the percentage moisture content recorded as,

$$\frac{W2 - W3}{W2 - W1} \times 100$$

Where

W1 = weight of container with lid;
W2 = weight of container with lid and sample before drying; and
W3 = weight of container with lid and sample after drying

2.8 Microbial isolates

The test organisms (*Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus fumigatus* and *Candida albicans*) were obtained from the University Port Harcourt Teaching Hospital, Port Harcourt, Nigeria. All isolates were sub-cultured onto selected culturing media to ensure purity and confirm their identification [6].

2.9 Evaluation of antimicrobial activity of the leaf extract of *Dialium guineense.*

The test organisms were aseptically cultured onto fresh Nutrient broth and incubated for 6-8 hours to ensure that the organisms were at their exponential phase of growth before carrying out the antimicrobial test on Mueller Hinton Agar (MHA) (for bacterial isolates) and Saubouraud Dextrose Agar (SDA) (for fungal isolates). The media were constituted according to manufacturer's specification, distributed accordingly to required volumes and sterilized by autoclaving at 121 °C for 15 min and maintained in molten form until ready for use [37-39]. The method of McFarland was modified for the preparation of the inoculum before seeding onto the appropriate media and allowed to set [7]. Wells measuring 6 mm in diameter were aseptically bored in the MHA and SDA media using sterile cork borer. 0.2 ml of the extract was dispensed into each well previously inoculated with the test organisms as previously reported [7]. 0.2 ml of Ofloxacin at 50 µg/ml was used as positive control while organism seeded-plates without extract served as negative control. The plates were incubated at 37 °C for 24 hours for bacterial isolates and at 25 °C for 7 days for fungal isolates. All samples were tested in duplicate and the diameters of zones of inhibition were recorded as the mean inhibition zone [40].

3. Results

3.1 Macroscopy

Table 1 shows the summarized description of *Dialium guineense* leaf under study. Results of the transverse section across the mid–rib, shows an upper and lower epidermis consisting of cells of same sizes. There are uniseriate covering trichomes on both surfaces. It has a bifacial surface i.e. there are two different surfaces with different identities, hence dorsiventral. The mesophyll consists of a palisade and the spongy mesophyll, embedding a crystal sheath. There are cluster crystals of calcium oxalate in the spongy mesophyll. The mid–rib bundle is surrounded by a zone of collenchyma on both surfaces, while the phloem vessels embed the xylem vessels.

Table 1: Macroscopic description of *Dialium guineense* leaf

Colour	Condition	Lamina						
		Apex	Margin	Venation	Base	Composition	Shape	Taste
Green	Fresh	Acuminate	Entire	Net	Symmetrical, Rounded	Simple	Obovate	Bitter

3.2 Microscopy

The microscopic features revealed that anticlinal walls are thick and straight and having numerous uniseriate covering trichomes in both upper and lower epidermis, most of them being unicellular or multicellular. Stomata are present in both lower and upper epidermis. The stoma is surrounded by two epidermal cells whose axis is parallel to the axis of the stomata pore.

3.3 Phytochemical analysis

Results of chemo microscopic investigations indicates the presence of lignin, starch, mucilage, calcium oxalate, cellulose, carbohydrate. The analysis further revealed the presence of carbohydrate, tannins and alkaloids. (Table 2).

Table 2: Results of phytochemical screening

Phytochemicals	Test	Inference
Carbohydrates	Molisch	++
	Fehling's	++
Glycosides (Bontrager's test for combined anthraquinone glycosides)	Hydrolysis with water	---
	Hydrolysis with dilute acid	---
	Hydrolysis with dilute acid and oxidation with H_2O_2	---
	Oxidative hydrolysis with ferric chloride as catalyst	---
Cardiac Glycosides	Keller-Killian's	---
	Kadde Test	---
Cyanogenetic Glycosides	Cyanogenetic Glycosides	---
Tannins	General	+++
	Phenazone	+++
	Iron Complex	+++
	Formaldehyde	+++
	Modified Iron Complex	+++
Saponins	Saponins glycoside	---
Alkaloids	Dragendorff's reagent	+++
	Wagner's reagent	+++
	Hager's reagent	+++
	Mayer's reagent	+++

+++ = Abundant, ++ = Moderate, --- = Absent

3.4 Antimicrobial activity of the leaf extract of *Dialium guineense*

Figure 1 and 2 shows the summarized antimicrobial effect of *Dialium guineense* leaf extract under study.

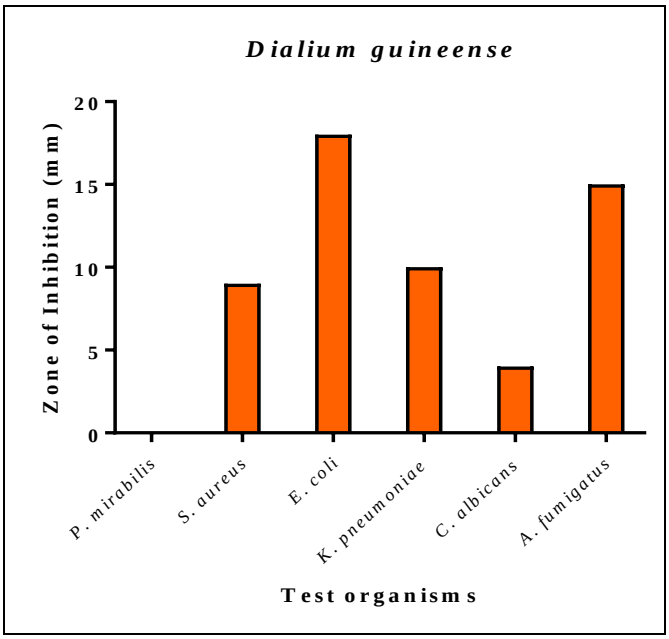

Fig 1: Inhibitory concentration produced by *Dialium guineense* against test organisms

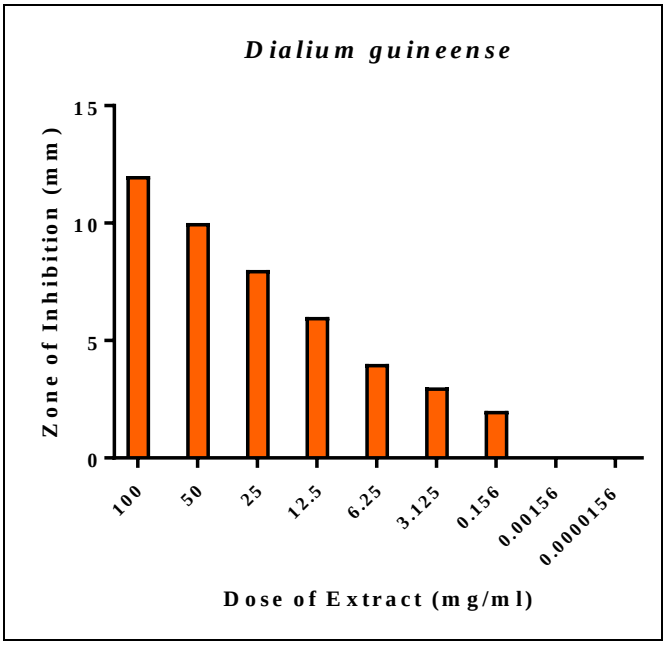

Fig 2: Varying inhibitory concentrations produced by *Dialium guineense* extract against *S. aureus*.

4. Discussion

In this study, several phytochemical constituents have been identified to include the presence of lignin, starch, mucilage, calcium oxalate, cellulose, carbohydrate, cutin, suberin, carbohydrate, tannins and alkaloids, but lacking glycosides. These secondary metabolites are known to possess various pharmacological effects [11, 14, 16, 30, 41] and may be responsible for the observed antimicrobial effect against the test isolates. Previous studies show that these metabolites are usually responsible for the pharmacological activities of medicinal plants and possible precursors for clinically useful drugs. [42] For example, tannins are polyphenolic compounds that bind to proline rich protein that interferes with protein synthesis and capable of anti-inflammatory and antimicrobial activities. [11] Previous report suggests that they play an active role in wound healing activity by way of suppressing inflammatory reactions invoked by the injured tissues. [43] In addition to this, tannins, have also been implicated in the haemostatic activity of plants where they arrest bleeding from damaged or injured vessels by precipitating proteins to form vascular plugs. [43] Obviously, there are concerns about the rapid rise in resistance of several pathogenic strains to available antimicrobial agents. This has also increased the tempo in medicinal research with the hope of discovering bioactive compounds for treating these resistant strains. The *in vitro* antimicrobial activities of methanolic leaf extract of *Dialium guineense* was studied in order to provide a pharmacological basis for their ethnomedicinal applications. According to Arullappan *et al*. [6] and Brantner *et al*. [44] the disc diffusion methods seems to be most frequently used method for studying the antibacterial activities of natural antimicrobial substances and plant extracts. These assays are based on the use of discs as reservoirs containing the solution of substances to be examined. [6] In this study, organisms previously isolated from wound infection were evaluated by measuring the diameter of the zone of inhibition against the extract. The results obtained in milimetre were compared with that Ofloxacin (50 µg/mL). The antimicrobial diameter zones of inhibition ranged between 4 and 18 mm (Fig. 1). As shown in the results, the extract had considerable effect on growth of *E. coli*, *A. fumigatus*, moderate effect on *S. aureus*, *K. pneumoniae*, *C. albicans*, and little or no effect on *P. mirabilis*. The medicinal value of plants has been suggested to lie in the ability of certain chemical substances that produce a definite physiological action against invading pathogens. [6] The minimum inhibitory concentration of *D. guineense* leaf extract was 6.25 mg/mL against *S. aureus*. The antimicrobial activity results presented in Fig. 2 showed that the test micro-organism was inhibited in a concentration dependent pattern at the various dilutions of the plant extracts. This seems to agree with previous report showing a correlation between concentration and molecular weight of the extract. The higher molecular weight, the slower the rate of diffusion as opposed an extract of low molecular weight diffusing faster and at a reduced time. [45, 46] Since the potency of medicinal plants is attributed to the action of the phytochemical constituents, the result of the antimicrobial activities suggests that the constituents of the leaves may play a useful role as alternative therapy in wound healing as previously reported. [43]

5. Conclusions

The findings from this study suggests that the methanolic leaf extract of *D. guineense* could be a good candidate in the search for a natural antimicrobial agent against infections and/or diseases caused by *E. coli* and *A. fumigatus*. In addition to its usefulness as a good source of food. This result also provides justification for the use of *D. guineense* leaves in wound management as currently being applied in traditional medicine practice. The plant may therefore be exploited further to understand better the mechanisms responsible for the antimicrobial activity revealed in this study. However, further study needs to be done to identify the specific secondary metabolites such as alkaloids and tannins present in *D. guineense* which could be responsible for its antimicrobial activity and to determine the toxicological profile of the active principles.

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