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ASSESSING THE ANTIMICROBIAL PROPERTIES OF METHANOL EXTRACTS OF SELECTED NIGERIAN MEDICINAL PLANTS USING TETRAZOLIUM MICROPLATE ASSAY

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ABSTRACT

Medicinal plants have made significant contributions to the treatment of infectious diseases. An upsurge in antimicrobial resistance has caused an upsurge in the demand for the identification and development of novel antimicrobial agents. This study aimed to investigate the antimicrobial activity of methanol leaf extracts of viz; *Deterium microcarpum* Guill. & Perr, *Lophira alata* Banks ex Gaertn, *Hibiscus tiliaceus* L. and *Ricinus communis* L. The Minimal Inhibitory Concentration (MIC) values for the test microorganisms, *Staphylococcus aureus* (ATCC 6571), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Bacillus subtilis* (ATCC 3366), and isolates of *Trichophyton rubrum* and *Candida albicans* were determined using the modified rapid p-iodonitrotetrazolium chloride (INT) colorimetric assay. The antimicrobial activity of the extracts varied against all the test microorganisms, with MIC values ranging between 125.00-7.81µg/mL. *Detarium microcarpum* extract exhibited complete growth inhibition at the lowest extract concentration with a MIC value of 7.81 µg/mL on *C. albican* while *Hibiscus tiliaceus* and *Ricinus communis* had the highest growth inhibition on *T. rubrum* at the MIC value of 15.63 µg/mL. After subculturing, all the extracts displayed Minimum Fungicidal Concentration (MFC) ranged from 31.25-62.50 µg/mL on *T. rubrum. Hibiscus tiliaceus, Detarium microcarpum* and *Lophira alata* exhibited strong Minimum Bactericidal Concentration (MBC) on *E. coli* with an MBC value of 62.50 µg/mL. This study justifies the usage of these plants in ethnomedicine to treat a variety of infectious disorders, based on the broad-spectrum antimicrobial activity these plants have demonstrated.

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INTRODUCTION

Global health stands at serious danger from antimicrobial resistance. The world is today facing a challenge due to the rise and quick spread of microbes that are resistant to almost all antimicrobial drugs [1]. Of the top ten leading causes of death and morbidity in the world is microbial infections. [2], Approximately half of all deaths in underdeveloped nations are caused by infectious diseases because of poor hygiene, crowded living conditions, and inadequate environmental sanitation, all of which have been linked to the spread of

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bacterial infections [2]. A number of medicinal plants had been tested for their antibacterial properties. Numerous researchers have reported positive results for antimicrobial testing of plant extracts at very high concentrations or at very small dilutions. Consequently, it was recommended that the amount of plant extracts in antimicrobial susceptible studies should be as low as 1 mg/mL. It is speculated that the extracts exhibit powerful antibacterial properties when their MIC values are less than 100µg/mL. [3-4]. Various laboratory methods could be used to measure *In vitro* susceptibility of microorganisms to antimicrobial agents. Broth dilution method is one of the antimicrobial susceptibility tests which allow the dilution of test samples to the bare minimum, which is one of the advantages of the method. This study enhanced the capability of broth dilution method with the introduction of P-INT (p-Iodonitrotetrazolium chloride) dye solution which could detect viability of microorganisms. P-INT (p-Iodonitrotetrazolium chloride) is a rapid and modern method in the determination of MIC with the use of microplate assay. P-iodonitrotetrazolium (P-INT) is a dye employed in biological assays as microbial growth indicator. The use of Tetrazolium dye in the evaluation of antimicrobial properties have been reported by many authors [5-10]. This method gives rapid results and safe the stress of measuring zones of inhibition in antimicrobial experiments. Its fundamental principle lies in assessing the metabolic activity or viability of cells or microorganisms through their ability to enzymatically reduce the yellow tetrazolium salt, p-iodonitrotetrazolium chloride (INT) solution, to formazan, a red/purple-red insoluble compound. [11-12].

Antimicrobial resistance has a greater effect in developing countries like Nigeria, where the expense of treating microorganisms' resistance diseases and the resulting fatalities are not taken into factor [13]. Medicinal plants have been used in folk medicines in the treatment of many ailments, and these plants are reported to be very rich in phytochemicals with diverse biological activities [14-19]. More so, the abundant bioactive constituents possess by medicinal plants acted as a substitute supply of antibacterial substances [20]. Medicinal plants have been utilised to cure infectious ailments in Nigeria and other African nations, and the majority of these plants have undergone scientific study [21]. *Detarium microcarpum* Guill. & Perr. Is a plant species in Fabaceae family. This shrub or tree, which may grow up to 10 meters in height, is known for its scaly bark, reddish-brown scaly back, and fractured twigs with a greenish-yellow sprout on the edge [22]. *Deterium microcarpum* is used traditionally for the treatment of diarrhoea, meningitis, haemorrhoids and tuberculosis [23]. In the northern region of Nigeria, snake bites are treated using *Deterium microcarpum* leaves [24]. *Lophira alata* Banks ex Gaertn. is a plant species that belongs to the Ochnaceae. Based on reports, the stem bark decoction is used in the treatment of ovarian cysts, typhoid fever, diarrhoea, and vaginal discharge [25]. The leaves of *Lophira alata* are boiled in water in North Western Nigeria and neighbouring Southern Niger, and the resulting extract is used for drinking and washing in order to assist pregnant women give birth with lesser difficulty [26]. *Hibiscus tiliaceus* L. (Malvaceae) is a common plant found in tropical Climates [27]. People utilised the plant as traditional medicine to cure typhoid, fever, coughs, dry throat, ear infections, chest congestion, diarrhoea, and dysentery [28]. According to reports, the *H. tiliaceus* extract possesses a number of intriguing pharmacological properties, including anthelmintic, antibacterial, anti-inflammatory, and antioxidant properties [29-30]. *Ricinus communis* L, is a flowering producing plant in the family of Euphorbiaceae [31]. It was discovered that cattle given *R. communis* leaves secreted more milk [32]. An infusion of the leaves has antifungal and antibacterial qualities. Oil gotten from the leaf part is used as therapy for newborn flatulence [33]. Therefore, this research was performed to determine the antimicrobial activities of the selected medicinal plants.

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MATERIALS AND METHODS

Plant collection and authentication

Fresh leaves of plants viz; *Deterium microcarpum, Lophira alata, Hibiscus tiliaceus* and *Ricinus communis* were collected and identified by Mr. Adebayo Sunday at the University of Ilorin Herbarium with the voucher numbers; UILH 001/771/2023, UILH 001/1129/2023, UILH 001/681/2023, UILH 001/665/2023 and voucher specimens deposited. The leaves were dried under the shade for four (4) weeks and pulverized into coarse powder.

Extraction of plant material

At room temperature, 20 grams of powdered material each were extracted using graded methanol over a 72-hour maceration process with being continuously shaken. The resulting extracts were filtered and dried using a rotary evaporator (Rotavapor® R-200 Buchi, Switzerland) at a lower pressure. Percentage (%) yield was calculated and extracts were stored in sterile sample bottles and kept in the refrigerator at 4^0 C until use.

Microorganisms used and culture preparation

The organisms were acquired from the University of Ibadan's Faculty of Pharmacy's Department of Pharmaceutical Microbiology. The organisms included four (4) bacteria; *Staphylococcus aureus* (ATCC6571), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* ((ATCC 27853), *Bacillus subtilis* (ATCC3366), two fungi; *Trichophyton rubrum* and *Candida albican* were received as clinical isolates from Ibadan's University College Hospital (UCH). Manufacturer's instructions for making MHA (Mueller Hinton agar) and SDA (Sucrose dextrose agar) media for bacterial and fungal cultures, respectively, were followed. For each organism, a single colony was introduced into 5 mL of SDA and MHA medium, respectively. From the original culture, organisms were subcultured and incubated overnight at 37°C for 24 hours for bacteria and 25°C for fungi.

Antimicrobial Assays

The test was carried out by broth micro-dilution method described by Jimoh and co-workers [34]. The antimicrobial assay was conducted using 96-well plates and the broth micro-dilution technique according to the standard. The samples were dissolved in DMSO and balanced up with dissolution solvent to prepare stock solutions of samples at 500 µg/mL. A final 1% DMSO content in the well was guaranteed. Initial tests using 1% (v/v) DMSO had no effect on the test organisms' growth or the colour shift of tetrazolium brought up this growth. In 96-well plates, double-fold serial dilution was carried out to obtain a range of five working concentrations; 250 – 15.63 µg/mL. The reference drugs Ciprofloxacin and ketoconazole (10 µg/mL) were diluted to yield working concentration of 10 - 0.3125 µg/mL. Ciprofloxacin was used as the reference drug control against bacterial strains, while ketoconazole served as reference drug control against fungal isolates. Each microplate well was inoculated with 40 µL of the microorganism's suspensions of bacteria and fungi cells (108 cfu/mL) and incubated at 37°C for bacterial growth for 24 hours and fungal growth for 48 hours at 25° C, respectively. Sterile control contained (culture broth and plant extract), negative control (culture broth and 1% DMSO without antimicrobial agent) and the negative control (broth and organism). The experiment was carried out in triplicate.

Evaluation of microbial growth inhibition by p - iodonitrotetrazollium violet (p -INT)

Extracts/reference drugs minimum inhibitory concentration (MIC) were determined on test microorganisms by the method of tetrazolium microplate assay as described by [35]. Forty microlitre (40 μL) of 0.2 mg/mL p -INT was added to each well and incubated for thirty (30) minutes, after the first incubation. By monitoring the changing of the yellow p-iodonitrotetrazolium chloride (INT) in the 96-well microplate into red or purple-red formazan upon reduction, the microbial growth was confirmed.

Minimum inhibitory concentration (MIC) determination

The colour shift of p-iodonitrotetrazolium chloride (PINT) in the 96-well microplate to red/purple-red formazan when there was clear growth, and remain yellow liquid when there was no clear growth was employed to verify microbial growth. MIC is referred to as the least concentrations of extracts/drugs when no color change (clear yellow) was observed and brought about complete inhibition of test organisms' growth [36]. The least concentrations of the extracts or drugs in which there was no obvious shift in the PINT dye solution from clear yellow to red/purple-red, indicating no growth of bacteria or fungi, were identified in the broth dilution visually as the Minimum Inhibitory Concentration values.

Minimum Bactericidal and Minimum Fungicidal Concentration Determination

The MBC and MFC were evaluated by subculturing aliquots of the preparations, which did not indicate any growth after incubation in MIC assays on Mueller Hinton agar and SDA prepared according to standard protocol. The subcultured mixtures were incubated at 37°C for 24 h for bacteria and 25° C for 48 hrs for fungi, respectively. MBC and MFC were regarded as the least concentration of extract which did not produce any microbial growth after the respective incubation period [36].

Statistical analysis

Experimental results were mean of three (3) values using Microsoft® Excel® 2018 (16.0.04266.1001) 32-bit).

RESULTS

The lowest concentrations of extracts/drugs at which there was clear yellow solution of PINT in the well and no clear microbial growth observed was considered as Minimum Inhibition Concentration (MICs). Table 1. All the extracts demonstrated varying degree of antimicrobial activity compared with the standard drugs. In contrast to other extracts, *Lophira alata* extract demonstrated significant antibacterial activity against the majority of microorganisms. Each microorganism had various levels of inhibitory ability, with MIC values ranging from 31.25 to 62.50 µg/mL. *Hibiscus tiliaceus*, *Ricinus communis* and *Detarium microcarpum* extracts displayed moderate growth inhibition with MIC values ranged between 7.81 - 125.00 µg/mL. Exceptionally, *Detarium microcarpum* extract exhibited complete growth inhibition at the lowest extract concentration with MIC value of 7.81 µg/mL on *C. albican*, *Hibiscus tiliaceus* and *Ricinus communis* had highest growth inhibition on *T. rubrum* at the MIC value of 15.63 µg/mL. After subcultured, all the extracts displayed Minimum Fungicidal Concentration (MFC) ranged from 31.25 – 62.50 µg/mL on *T. rubrum. Hibiscus tiliaceus, Detarium microcarpum* and *Lophira alat*a exhibited strong Minimum Bactericidal Concentration (MBC) on *E. coli* with MBC value of 62.50 µg/mL compared with standard drugs. Table 2 and 3.

Table 1: Minimum inhibitory concentration (MIC) of Selected Plant Extracts on Test Microorganisms

Sample	**MIC (µg/mL)**					
	S. aureus	***E. coli***	***P. aeruginosa***	***B. subtilis***	***T. rubrum***	***C. albican***
Hibiscus tiliaceus	31.25	31.25	62.50	62.50	15.63	125.00
Ricinus communis	125.00	62.50	62.50	125.00	15.63	125.00
Detarium microcarpum	62.50	31.250	125.00	31.25	62.50	7.81
Lophira alata	62.50	31.25	62.50	31.25	31.25	31.25
Ciprofloxacin (10 µg/mL)	0.625	0.625	0.625	0.625	-	-
Ketoconazole (10 µg/mL)	-	-	-	-	0.625	0.625

Bacterial strains: *S. aureus = Staphylococcus aureus; E. coli = Escherichia coli; P. aeruginosa = Pseudomonas aeruginosa; B. subtilis = Bacillus subtilis.* **Fungal isolates:** *T. rubrum = Trichophyton rubrum; C. albican = Candida albican.*

Table 2: Minimum Bactericidal Concentration (MBC) of Selected Plant Extracts on Test Microorganisms

Sample	**MBC (µg/mL)**			
	S. aureus	***E. coli***	***P. aeruginosa***	***B. subtilis***
Hibiscus tiliaceus	125.00	62.50	125.00	125.00
Ricinus communis	250.00	125.00	125.00	250
Detarium microcarpum	125.00	62.50	250.00	125.00
Lophira alata	125.00	62.50	125.00	125.00
Ciprofloxacin (10 µg/mL)	1.56	1.56	1.56	1.56

Bacterial strains: *S. aureus = Staphylococcus aureus; E. coli = Escherichia coli; P. aeruginosa = Pseudomonas aeruginosa; B. subtilis = Bacillus subtilis.*

Table 3: Minimum Fungicidal Concentration (MFC) of Selected Plant Extracts on Test Microorganisms

Sample	**MFC (µg/mL)**	
	T. rubrum	***C. albican***
Hibiscus tiliaceus	31.25	250.00
Ricinus communis	62.5	250.00
Detarium microcarpum	31.25	250.00
Lophira alata	62.50	125.00
Ketoconazole (10 µg/mL)	1.56	1.56

Fungal isolates: *T. rubrum = Trichophyton rubrum; C. albican = Candida albican*

DISCUSSION

The discovery of antimicrobial agents has been greatly aided by natural products. Some of the remedies are made entirely of natural ingredients, while others are used as a starting point for new drug development. Nevertheless, antimicrobial resistance, cost and other undesirable effects of available conventional drugs are alarming globally every year. This study presents the antimicrobial activities of methanol leaves extracts of *Hibiscus tiliaceus*, *Ricinus communis, Detarium microcarpum,* and *Lophira alata* on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Trichophyton rubrum* and *Candida albican*. Colorimetric test is a more cost-effective method of determining MIC and produces more repeatable results [36]. The broth dilution method's sensitivity and accuracy in determining MIC with formazan derivatives generated by living bacteria or fungi have been improved by the use of tetrazolium salt as a colorimetric indicator [37]. There are numerous methods for the screening of biological extracts for potential antimicrobial activity. According to [38], 96-well plate micro broth dilution susceptibility method has emerged as the preferred approach for drug susceptibility testing due to its low sample needs, affordability, and high throughput rate, which is more elaborate on employing the use of P-INT dye solution. This study employed the combined approach of broth dilution method and the use tetrazolium microplate colorimetric assay in evaluation of antimicrobial activities. In the reports of [39, 40] which also corroborate the work of [41-42], MIC values below 100 µg/mL indicate high antimicrobial ability, 100 ≤ MIC ≤ 625 µg/mL indicates moderate antimicrobial ability, and > 625 µg/mL indicates low antimicrobial ability. Similarly, compounds/drugs have significant antimicrobial activity when MIC< 10 µg/mL, moderate antimicrobial activity when 10 <MIC< 100 µg/mL, and low antimicrobial activity when MIC > 100 µg/mL.

All the extracts demonstrated varying degree of antimicrobial activity compared with the standard drugs. Extract of *Lophira alat*a showed strong antimicrobial activity compared with other extracts. The extract had MIC values ranging from 31.25 - 62.50 µg/mL on test microorganisms. Previous study had MIC of lophirones B and C isolated from stem bark of *Lophira alat*a against *E. coli*, *P. aeruginosa* and *S. aureus* to be 200, 100; 200 and 150-µg/mL, respectively [43]. *Detarium microcarpum* had moderate activity on test microorganism but exceptionally had strongest inhibition on *C. albican* with MIC value of 7.8 µg/mL. In the study of [44], MIC of aqueous stem bark extract of *Detarium microcarpum* was reported to be 50 mg/mL for *S. aureus* while ethanol stem bark extract indicted MIC for *S. aureus* at 25 mg/mL. Also, in the study of [45], methanol aqueous seeds coat (32.324%, w/w) extract of *Detarium microcarpum* evaluated against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomona aeruginosa, Klebsiella pneumonia, Salmonella paratyphi* and *Candida albicans* of clinical origin reported to have MIC values range of 1.05-2.89 mg/mL. Obtained methanol extract

from stem bark of *Detarium microcarpum* have antibacterial properties on *Escherichia coli, Staphylococcus aureus* and

Salmonella typhi with good activity on *S. typhi* at concentrations of 12.5 mg/mL [46]. *Ricinus communis* leaves methanol extract in this study also demonstrated good activity compared with previous studies. The extract displayed MIC ranged between 15.63-125 µg/mL with highest activity on *T. rubrum*. Ethyl acetate fraction of the methanol leaves extract of *Ricinus communis* showed MIC value of 1.56 mg/mL on *S. aureus* and 6.67 mg/mL on *Candida albicans* [47] While in another study, the minimum inhibitory concentration (MIC) of *Ricinus communi*s methanol, ethanol and aqueous leaf extracts on the pathogens *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumonia* ranged from 3.13 to 25.0 mg/mL [48]. Results showed MIC values of *Ricinus communis* seed aqueous extract in the work of [49] ranged between 8-32mg/mL while the MIC of the alcoholic extract of seed ranged between 8 - 16 mg/mL for all the tests microorganisms; *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus fecalis*). The study of [50] reported methanol stem bark extract of *Ricinus communis* with a MIC value of 8.3 µg/mL. This corroborates the result of this study on the leaf extract, justifying the use of different plant part of *Ricinus communis* as antimicrobial agent. *Hibiscus tiliaceus* also demonstrated good activity on test microorganisms with MIC ranged between 15.63 – 125 µg/mL. Both *Ricinus communis* and *Hibiscus tiliaceus* demonstrated the same inhibition capacities on *T. rubrum* with MIC value of 15.63 µg/mL. Previous reports on antimicrobial tests of *Hibiscus tiliaceus* showcased water fractions of flower, leaf and seed of *Hibiscus tiliaceus* with MIC values between 1.25 - 20 mg/mL on *Pseudomonas aeruginosa* and *Bacillus subtilis* [51]. *Hibiscus tiliaceus* methanol leaves extract reported to have strong antimicrobial activity against *S. aureus* and *C. albicans* with MIC values of 128 to 256 µg/mL according to Trung *et al*., 2023. The report of [52] on *Hibiscus tiliaceus* methanol leaves extract on *S. aureus* and *C. albicans* in microgram per milliliter (µg/mL) conformed with results in this study wherein *S. aureus* and *C. albicans* had 31.25 and 125.00 µg/mL MIC values, respectively. The disparity could be in different methods used in evaluating the antimicrobial activity. In addition, [53] declared ethanol leaves extract of *Hibiscus tiliaceus* from Bangladesh positively inhibited *E. coli* and

Salmonella paratyphi with MIC values of 500 µg/mL and 250 µg/mL, respectively. With MIC values of less than 100 µg/mL on the majority of tested organisms, the antimicrobial activities of all investigated plant extracts were found considerable. After subculturing, all the extracts displayed Minimum Fungicidal Concentration (MFC) ranged from 31.25 – 62.50 µg/mL on *T. rubrum.* This indicated all the extracts possess antifungicidal agents. *Hibiscus tiliaceus, Detarium microcarpum* and *Lophira alat*a exhibited strong Minimum Bactericidal Concentration (MBC) on *E. coli* with MBC value of 62.50 µg/mL compared with standard drugs.

The claims of previous authors on antimicrobial activities of the studied plants and on several other plants are not disputed. This further validate the antimicrobial activities of the studied plants. However, this study justified there could still be significant antimicrobial activities of plant extracts and drug even at lower concentration in microgram (µg) against in milligram (mg) measure employed in most antimicrobial researches that have been reported coupled with employment of broth dilution approached which allows dilution to bare minimum and could be easier in combination with a colorimetric assay. This indicates efficacy of antimicrobial agents in little concentration and encourages saving of samples. Results of this study conform with attributes of a good antimicrobial agents as reported by [54-57].

CONCLUSION

Methanol extracts of *Deterium microcarpum, Lophira alata, Hibiscus tiliaceus* and *Ricinus communis* possess potential antimicrobial effects against the test microorganisms which justifies their use in the traditional management of infectious diseases. The strong to moderate antimicrobial activities displayed by these plants were also in accordance with the attributes of MIC values which good antimicrobial agents should possess as reported in literature.

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AUTHORS' CONTRIBUTION

OOO and LOS designed the work. LOS and NAO carried out the study. LOS, RBI, ATA and COO drafted the manuscript, OOO supervised the study and revised the manuscript. All authors read, made comments, and agreed on the final manuscript.

CONFLICT OF INTEREST

The authors confirm no conflicts of interest to report.

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ABBREVIATION

CFU - colony forming unit: it is a unit that estimates the number of viable microbial cells (bacteria, fungi, viruses)
DMSO - Dimethyl sulfoxide

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