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Phytochemical and Anti-microbial Study of Seventy Percent Methanol Leaf Extract of *Terminalia microptera* against Selected Pathogenic Bacteria

Daniel Augustine Innalegwu[1*], Ewansiha Joel Uyi[2], Amuda Oladuni Amuda[2] and Nathaniel Vivian[2]

[1]Centre for Genetic Engineering and Biotechnology (Drug and Vaccine Discovery Unit), Federal University of Technology, Minna, Niger State, Nigeria.
[2]Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author DAI designed the study and wrote the protocol. Authors EJU and DAI managed the organisms, collected all data, performed the statistical analysis, wrote the first draft of the manuscript and also wrote part of the manuscript. Authors DAI, EJU, AOA and NV did the literature search. All authors read and approved the final manuscript.

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ABSTRACT

The phytochemical and antimicrobial activity of *Terminalia microptera* leaf methanol (70%) crude extract was determined against some selected pathogenic microorganisms using the qualitative phytochemical, tube dilution and agar well diffusion methods respectively. The results of the phytochemical analysis shows that all the phytochemicals analysed for were present; these include alkaloid, flavonoids, saponins, cardiac glycosides, anthraquinons, sterols, phlobatanins and terpenes. All organisms screened were found to be sensitive to the extract at all the concentrations. *Klebsiella pneumoniae* was more susceptible to the extract with mean zones of inhibitions of 24.33±0.88, 29.33±0.33 and 33.33±0.88 for 20, 30 and 40 mg/ml respectively followed closely by *Streptococcus pyogenese* and *Salmonella typhi* with mean zones of inhibitions of 17.67±0.88,

**Corresponding author: E-mail: augustinedaniel36@gmail.com;*

25.00±0.58 & 28.67±0.88 and 17.00±0.58, 18.33±0.67 & 21.33±0.67 respectively while *Micrococcus luteus, Pseudomonas aeruginosa and Streptococcus mutans* sensitivity were seen to be on the lower side with mean zones of inhibitions of 15.67±0.33, 18.67±0.88 and 17.33±0.67 respectively at 40 mg/ml. In the same vein, *Klebsiella pneumoniae* had the lowest minimum inhibitory concentration of 1.25 mg/ml and minimum bactericidal concentration of 10 mg/ml i.e, it is more susceptible to the plant extract while *Pseudomonas aeruginosa, Streptococcus mutans, Salmonella typhi* and *Salmonella paratyphi* C were more resistant to the extract with MIC of 10 mg/ml. The result of this study shows that extracts from *Terminalia microptera* had antimicrobial activity against the test organisms and therefore can be used to develop drugs that can be used to treat infections caused by these organisms.

Keywords: Terminalia microptera; phlobatanins; Klebsiella pneumonia; minimum inhibitory concentration; Combretaceae.

1. INTRODUCTION

Natural products and plants-derived substances have recently become of great importance due to their universal applications. There have been a dramatic surge in microbial infections in the past 20 years due to compromise in human immune systems by factors such as AIDs, aging, organ transplant and cancer therapy. Also, increase in the rate of microbial infections have been considered as one of the major problem confronting mankind [1].

The global problems of multiple antibiotics resistance as well as emergence of new and resurfacing of previously eradicated diseases also contribute to this problem. Most of the current antimicrobial drugs are simply biostatic while others are xenobiotic or foreign materials which are harmful to the kidney, the hematopoietic and central nervous system (CNS) [2]. Furthermore, antimicrobial resistance among enteric pathogens is of great concern as it poses a great threat to human health and even the economy of an individual as well as a nation [3]. New microbial strains are also emerging which strongly opposed the current arsenal of drugs [4]. This is because the organisms have become resistant to this antimicrobial agents as a result of the regular use, misuse and reuse of this agents leading to therapeutic failure [5]. This among many factors have made it mandatory to fight against emerging and re-emerging infectious diseases with a view of discovering and inventing new agents of greater therapeutic potentials to mitigate the frequent outbreak of this diseases which has posed a new threat to global health security [6]. With the rise in problem of side effects and limited efficacy of antibiotic drugs [1], there is an urgent need for the development of alternative antimicrobial substances and researchers and of course the entire global world are now turning to natural product from plants and other micro-organisms as their main source of bioactive compounds with antimicrobial properties to complement the existing synthetic antimicrobial drugs that are gradually losing their potentials against pathogenic micro-organisms in the microbial world [1]. Anti-microbial agents are substances that kill microorganisms or inhibit their growth. They are widely employed to treat bacteria diseases. Antimicrobial agents that reversibly inhibit growth of bacteria are called bacteriostatic whereas those with irreversible lethal action on bacteria are known as bactericidal [7]. Ideally, antimicrobial agents disrupt microbial processes or structures that differ from those of the host. They may damage pathogens by hampering cell wall synthesis, inhibiting microbial protein and nucleic acid synthesis, disrupting microbial membrane structure and function, or blocking metabolic pathways through inhibition of key enzymes [8].

Haslam et al. [9] reported that plant extracts and their products are used in many parts of the world as the active principles in herbal remedies. They are used locally in the treatment of malady, many centuries before the evolution of scientific studies were discovered [7].

The current evolution of bacterial resistance to currently available antibiotics has necessitate the search for novel and more potent antimicrobial compounds which will pose less harm to the human system [10], to which these micro-organisms are yet to develop resistance [3].

Medicinal plants are the richest source of bioactive substance which are of great importance in traditional and modern medicine [1].

Terminalia macroptera is a tree belonging to the family *Combretaceae*. The plant is up to 20 m in hieght and 30 m in girth with an open spreading

crown which may be recognised readily by the prominent tuffs of nearly stalkless pale green leaves and by its large fruits. It is commonly found in Ghana, Senegal, Sudan, Uganda and Nigeria and locally known by the Hausas in Nigeria as Kwandari. In Mali *T. macroptera* is used against a variety of ailments, and more than 30 different indications have been mentioned by the traditional healers in ethno pharmacological studies. The stem bark and leaves are most commonly used against sores and wounds, pain, cough, tuberculosis and hepatitis [11]. The roots are used against hepatitis, gonorrhea and various infectious diseases, including *H. pylori*-associated diseases [11]. Flavonoids, triterpenoids, ellagitannins and related phenolics [11], have been identified from different parts of *T. macroptera*. Ellagitannins are known antimicrobial compounds which may be related to the use of the leaves against wounds and other microbial infections [12].

Water decoctions of *T. macroptera*, administered orally, are the most common preparations used by the traditional healers in Mali [13]. Plant polysaccharides isolated from crude water extracts have shown effects related to the immune system by different *in vitro* and *in vivo* test systems [14]. The chemical characteristics and biological activities of polysaccharides, especially those from plants used in the treatment of wounds, ulcer and cancer have been reported [15].

This high interest of plants derived drugs is due to the fact that herbal drugs or herbal medicines are judged to be safer and less costly than the synthetic drugs which possess serious side effects. It is therefore of great importance to screen plants for potential and promising biological activity [16]. The claims by both the local marketers, consumers and literature have necessitated this study, to assess the in vitro antimicrobial potency of *T. macroptera* against selected microbial pathogens.

2. MATERIALS AND METHODS

2.1 Plants Materials

The leaves of *T. microptera* were collected from Akoko-Edo in Ondo State of Nigeria and was authenticated in the Biological Sciences Department of Federal University of Technology, Minna, Niger State by Dr. Dawud Yusuf.

2.2 Bacterial Strains

The bacteria use for the biological test include the gram positive *Streptococcus mutans, Streptococcus pyogenes* and gram negative *Pseudomonas aeruginosa, Klebsiella pneumonia, Micrococcus luteus, Salmonella typhi,* and *Salmonella paratyphi* A, B and C*.*

2.3 Methods

2.3.1 Preparation and extraction of plant material

The leaves of *T. microptera* were collected washed and air-dried at room temperature at the Centre for Genetic Engineering and Biotechnology, Federal University of technology, Minna, Niger State. The dried leaves were then blended using kitchen type blender to obtain a fine powder using the method described by [17]. One hundred grams (100 g) of the plant powder was extracted with 70% methanol using reflux method at a temperature of 45℃ for 2 hours and the extract was filtered using muslin cloth followed by further filtration using whattman No 1 filter paper with pore size of 0.7 µm to obtain a fine filtrate. The filtrate was then concentrated using RE-6000 rotary evaporator at 50℃. The concentrate was then freeze dried using the LGJ-10 lyophilizer at a temperature of -30℃ to a fine powder which was stored in an air-tight amber bottle and kept in the refrigerator for further analysis.

2.3.2 Phytochemical analysis

The phytochemical analysis of the extract of *T. macroptera* was carried out based on coloration and precipitation test as described by [18] and [19].

2.3.3 Test for alkaloids

Zero point five gram (0.5 g) of extract was diluted into 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

2.3.4 Test for phenols

One millilitre (1 ml) of crude extract and Iron (III) chloride were mixed for 2 minutes. Formation of a deep bluish green colouration of the mixture indicate the presence of phenols.

2.3.5 Test for tannins

Zero point five gram (0.5 g) of the extract was boiled with 10 ml of distilled water in a test tube and then filtered. A few drops of 10% of ferric chloride was added and observed for brownish green or a blue-black coloration.

2.3.6 Test for terpenoids (Salkowski test)

To 0.5 g of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

2.3.7 Test for cardiac glycosides

One gram (1 g) of the extracts was treated with 2 ml of glacial acetic acid, a drop of 10% $FeCl_3$ and 1 ml of concentrated H_2SO_4. The appearance of brown coloration indicates the glycosides.

2.3.8 Test for flavonoids

Five millilitre (5 ml) of dilute ammonia was added to the aqueous portion of the extract followed by concentrated sulphuric acid (1 ml). A yellow coloration that disappears on standing indicates the presence of flavonoids.

2.3.9 Test for saponins

To 0.5 g of extract was dissolved in 5 ml of distilled water in test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

2.3.10 Test for anthraquinones

One millilitre of the plant crude extract was mixed with 1 ml of chloroform, then 10% NH_3 solution was added to the mixture. A brick red precipitate indicate the presence of anthraquinones.

2.3.11 Test for phlobatannins

Zero point two grams (0.2 g) of the crude extract was mixed with 5 ml of 1% HCl in a test-tube and heated for 2 minutes. A red precipitate indicates the presence of phlobatannins.

2.3.12 Test for steroids

Five drops of concentrated H_2SO_4 was added to 0.2 g of the extract. A reddish brown colour indicates the presence of steroids.

2.3.13 Statistical analysis

The data are presented as mean ± S.E.M. All the data were analysed by one-way ANOVA and differences between the means were assessed with Duncan Multiple comparison test. Differences were considered significant at $p< 0.05$. All analyses were carried out using Statistical Package for Social Science (SPSS) version 20 (USA).

2.4 Antimicrobial Susceptibility Screening

2.4.1 Standardization of inoculum

One loop full of eighteen hour broth culture of the test organisms was suspended in sterile nutrient broth. It was standardized according to Clinical Laboratory Standards Institute [20] by gradually adding normal saline to compare its turbidity to McFarland standard number 0.5 which is equivalent to density of $1.0x10^6$cfu/ml.

2.4.2 Susceptibility testing of plant extracts

Thirty-nine gram (39 gm) of the Mueller Hinton agar was dissolved into 1 liter of distilled water in a conical flask. The medium was sterilized in the autoclave at 121℃ for 15 minutes and the media was allowed to cool (45℃). 20 ml the Muller Hinton agar was dispensed into sterile petri dishes of 90 mm and allowed to set. A sterile cork borer of diameter 6 mm was used to bore equidistant wells onto the agar plates. One drop of the molten agar was used to seal the bottom of the bored wells so as to prevent the extract from sipping beneath the agar. The medium was then inoculated with the test organisms using the spread plate method and 200 µl of the extract (40 mg/ml) of methanol and aqueous extract were transferred separately into the wells and 200 µl at 1 mg/ml of the standard drug (Chloramphenicol (30 µg)) were used as the positive control while dimethylsulfuroxide (DMSO) served as the negative control. One hour pre-diffusion time was allowed after which the plates were incubated at 37℃ for 18 hours.

The zones of inhibition were then measured to the nearest millimetre using a calibrated veneer calliper. The above method was carried out in triplicates while the mean and standard error of the mean were taken.

2.4.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Tube dilution method was used to determine the MIC and MBC of the active extracts. A two fold and seven series (40, 20, 10, 5, 2.5, 1.25 and 0.625 mg/ml) dilutions of each extract were prepared in Muller Hinton broth. Zero point one millilitre (0.1 ml) of each of the standardized test organisms (0.5 McFarland turbidity standard) was added to each dilution. One control tube was also prepared which contain only sterile medium without the test organisms or the extract after which all tubes were incubated in a water bath with shaker at 37℃ for 24 hours. After incubation, the tubes were observed for turbidity and the tube without visible turbidity, as compared to the control tube was recoded as the minimum inhibitory concentration while the minimum bactericidal concentration was determined by subculturing the content of the tubes without visible turbidity unto freshly prepared Mueller Hinton medium beginning with the minimum inhibitory concentration and above. The culture was incubated at 37℃ for 24 hours. The lowest concentration of the sub cultured medium without visible growth was recoded as the minimum bactericidal concentration (MBC) [21].

2.5 Statistical Analysis

The data generated for zones of inhibition were subjected to Analysis of Variance (ANOVA) using IBM SPSS version 20 to check if there were significant differences between the different concentration of the extract and the standard drug used.

3. RESULTS AND DISCUSSION

The result of the phytochemical analysis revealed the presence of 9 phytochemicals which include alkaloid, flavonoids, saponins, cardiac glycosides, anthraquinons, sterols, phlobatanins and terpenes Table 1. The presence of these constituents no doubt may be responsible for the plant to be medicinal in nature oweing to the fact that most of the phytochemical constituents present are reported to be used for the treatment of one ailment or the other as reported by [22]. Saponins which is present in the plant extract are also reported to possess immune boosting capacity and anti-inflammatory properties [22]. [12] also reported on the antimicrobial activity of alkaloids which is also very present in the plant leaf extract of *T. microptera*. [23] reported on the mechanism of action of tannins in the precipitation of protein to for water soluble compounds and as a result, bacterial are inactivated by the direct damage done to their cell membrane. In Table 2, the antibacterial activity of the *T. microptera* clearly revealed how active the plant extract is to the test organisms, this is well understood when compared with the activity of the standard drug. Despite the fact that the plant extract is still in the crude form, it shows a considerable level of activity. The specification for susceptibility for the standard drug is zones of inhibition from 18 mm above, which is recorded for the extract against the test organisms from 30 mg/ml and above except for *Salmonella paratyphi* C at 20 mg/ml. The plant extract was observed also to be concentration dependent such that, increase in the concentration gives a direct increase in the inhibition zones which cut across all the test organisms. *Klebsiella pneumoniae* was the most susceptible organism

Table 1. Qualitative phytochemical components of 70% methanol extract of *T. microptera*

Phytochemicals	Inference
Alkaloid	Present
Flavonoid	Present
Tannins	Present
Saponins	Present
Phenols	Present
Cardiac glycosides	Present
Anthraquinones	Present
Steroids	Present
Phlobatannins	Present
Terpenes	Present

Key: + = present

Table 2. Mean zones of inhibition of 70% methanol extract of *T. microptera*

Organisms	20 mg/ml	30 mg/ml	40 mg/ml	Chloramphenicol (30 µg)
M. luteus	8.67±0.33^{a}	12.33±1.20^{b}	15.67±0.33^{c}	16.33±0.57^{c}
P. aeruginosa	8.00±0.57^{a}	13.00±1.00^{b}	18.67±0.88^{c}	21.00±1.00^{c}
S. mutans	9.00±0.58^{a}	13.33±0.67^{b}	17.33±0.67^{c}	16.00±0.00^{c}
K. pneumonia	24.33±0.88^{b}	29.33±0.33^{c}	33.33±0.88^{d}	16.67±0.57^{a}
S. pyogenes	17.67±0.88^{a}	25.00±0.58^{b}	28.67±0.88^{c}	17.67±0.57^{a}
S. typhi	17.00±0.58^{b}	18.33±0.67^{b}	21.33±0.67^{c}	14.33±1.00^{a}
S. paratyphi A	16.67±0.67^{ab}	18.33±0.33^{b}	22.33±0.67^{c}	16.33±1.15^{a}
S. paratyphi B	14.67±0.33^{a}	15.67±0.67^{a}	19.00±0.58^{b}	21.00±1.00^{c}
S. paratyphi C	18.67±0.88^{b}	17.33±0.33^{b}	21.00±0.58^{c}	14.67±1.53^{a}

Values on the same column with different superscript are significantly different ($p<0.05$)
Specification for Chloramphenicol: ≤12 mm (resistant), 13-17 mm (intermediate), ≥18 mm (susceptible) (CLSI, 2012)

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of 70% methanol extract of *T. microptera* (mg/ml)

Bacterials	40	20	10	5	2.5	1.25	0.625	MIC	MBC
M. luteus	-	-	-	-	+	+	+	5	20
P. aeruginosa	-	-	-	+	+	+	+	10	20
S. mutans	-	-	-	+	+	+	+	10	20
K. pneumonia	-	-	-	-	-	-	+	1.25	10
S. pyogenes	-	-	-	-	+	+	+	5	20
S. typhi	-	-	-	+	+	+	+	10	40
S. paratyphi A	-	-	-	-	-	+	+	2.5	20
S. paratyphi B	-	-	-	-	-	+	+	2.5	10
S. paratyphi C	-	-	-	+	+	+	+	10	40

Key: + = turbid, - = no turbidity

having the highest mean zone of inhibition of 24.33±0.88 at 20 mg/ml. this was also evident in the MIC at 1.25 mg/ml and MBC of 10 mg/ml. The plant crude extract was active against *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Salmonella typhi* and *Salmonella paratyphi* C with a wide zones of inhibition when compared to the activity of the control drug considering the specification by [20]. This also is in agreement with the report of [24] on the activity of species of this plant against these organisms. The minimum inhibitory concentrations of the crude extract against the test organisms generally were low suggesting that the plant extract will be highly effective against the test organisms if developed as drugs for treatment of infections due to the test organisms and also it is a pointer to the fact that the fractions will be more active when purified by more sensitive methods like partitioning and column chromatography.

4. CONCLUSION

Based on the results of this study, *T. microptera* possesses phytochemical constituents, it can therefore be concluded that methanol leaf extract of the plant exact its antibacterial activities against the selected microorganisms and can therefore be used to develop drugs that can be employed for the treatment of infections caused by these organisms. Also, that the activity of the plant extract is dose dependent as an increase in activity was observed when the concentration was increased.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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