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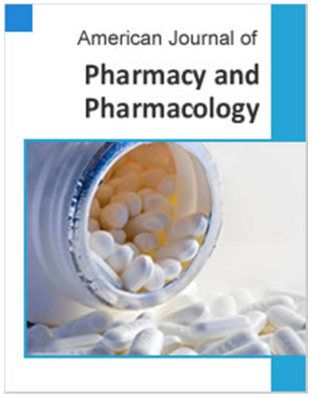

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Quantitative Phytochemical and Antibacterial Efficacy of Fractions of *Terminalia microptera* Leaf

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Abstract
Quantitative phytochemical analysis, solvent partitioning and agar well diffusion methods were employed to determine the phytoconstituents, fractions and antibacterial activity of the crude extract of *T. microptera* leaf respectively. Results obtained reveals the presence of phenols, tannins, alkaloids and saponins at a concentration of 481.75±0.17, 70.52±1.62, 541.00±0.61 and 2.13±0.32 mg/g respectively while flavonoid was not detected. Three fractions with corresponding yield of 1.67% (chloroform fraction), 24.39% (ethyl acetate fraction) and 52.17% (aqueous fraction) were obtained from the crude extract. Chloroform fraction was active on three Gram negative organisms (*S. typhi, S. paratyphi* A, B and C) with zones of inhibition ranging from 10.00±1.15 to 12.33±0.33mm while ethyl acetate and aqueous fractions were active on all the tested organisms with zones of inhibition ranging from 11.33±0.67 to 16.33±0.67mm and 14.33±0.33 to 16.33±1.00mm respectively. Considering the antimicrobial activity of the crude fractions as reported by previous studies, it can therefore be concluded that the fractions might be less active at their partially purified state and may exert considerable activity in their combined state or may be bacteriostatic in activity when applied for the control and treatment of infections caused by the test organisms.

1. Introduction

The emergence of multidrug resistant pathogens has been reported to be one of the leading causes of death worldwide [1] with infectious diseases responsible for 68% of all deaths globally in 2012 [2]. Many infectious microorganisms are becoming resistant to synthetic drugs and it has become the major concern for health institutions, pharmaceutical companies and governments all over the world; thus there is need for an alternative therapy [3]. Medicinal plants contain numerous active constituents of great therapeutic value and have been used as an exemplary source for centuries as an alternative remedy for treating human diseases [4].

Approximately 80% of the third world population depends on traditional medicines for maintaining general health and combating many diseases [5].

Terminalia macroptera is a tree belonging to the family Combretaceae. The plant is up

to 20m in height and 30m 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 [6]. The roots are used against hepatitis, gonorrhea and various infectious diseases, including *H. pylori*-associated diseases [6]. Flavonoids, triterpenoids, ellagitannins and related phenolics [6], 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 [7].

Water decoctions of *T. macroptera*, administered orally, are the most common preparations used by the traditional healers in Mali [8]. Plant polysaccharides isolated from crude water extracts have shown effects related to the immune system by different *in vitro* and *in vivo* test systems [9]. The chemical characteristics and biological activities of polysaccharides, especially those from plants used in the treatment of wounds, ulcer and cancer have been reported [10].

Due to high demand and dependence in herbal medicine to combat the death toll arising as a result of resistance of orthodox medicine by pathogens there is need to explore the wealth of the nature's bio-flora to screen plants that contains active components that can be of medicinal value to humanity. This research work is aimed at determining the quantitative phytochemicals of the crude extract and the antimicrobial activity of fractions obtained from *T. microptera* leaf extract using the partial purification technique.

2. Materials and Methods

2.1. Plants Materials

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

2.2. Bacterial Strains

The bacteria used for the biological test were obtained from Federal Institute for Industrial Research Oshodi (FIIRO), Lagos, Nigeria. They include Gram positive *Streptococcus mutans* ATCC 25175*, Streptococcus pneumoniae* ATCC 33400, *Streptococcus pyogenes* JCM 5674 and Gram negative *Pseudomonas aeruginosa* ATCC 10145*, Klebsiella pneumoniae* BYK-9*, Micrococcus luteus* ATC 10240*, Salmonella typhi* T4*,* and *Salmonella paratyphi* A SPA2, *Salmonella paratyphi* B 374 and *Salmonella paratyphi* C DT4.

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 pulverise using kitchen type blender to obtain a fine powder using the method described by [11]. One hundred grams (100 g) of the plant powder was extracted with 70% methanol using reflux method at a temperature of 45°C for 2 hours and the extract was filtered using muslin cloth which was followed by a further filtration using whattman No. 1 filter paper with pore size of 0.7µm to obtain a fine filtrate. The filtrate was then concentrated using RE-6000 rotary evaporator at 50°C. The concentrate was then freeze dried using the LGJ-10 lyophilizer at a temperature of -30°C to a fine powder which was stored in an air-tight amber bottle and kept in the refrigerator for further analysis.

2.3.2. Quantitative Phytochemical Estimation of the Crude Extract

(1). Total Flavonoid

Aluminium chloride colorimetric method [12] was used for flavonoid determination. A 0.5mL (1mg/mL) of the plant crude extract was mixed with 1.5 mL of methanol, 0.1ml of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water and kept at room temperature for 30 min. The absorbance of the reaction mixture was taken at 415nm with a double beam Shimadzu UV spectrophotometer, UV-1800. The calibration curve was prepared by using quercetin solutions at concentrations of 12.5 to 100g/mL in methanol.

(2). Total Phenol

The total phenol content of the crude extracts was determined according to the method described by [13]. A 0.5 mL (1mg/mL) was oxidized with 2.5 mL of 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 minutes at 45°C and the absorbance was taken at 765nm using the double beam Shimadzu UV spectrophotometer, UV-1800. The total phenol content was subsequently calculated using Gallic acid as standard.

(3). Alkaloids

A 0.5g of the crude extract was mixed with 5mL of 96% ethanol -20% H_2SO_4 in ratio (1:1) and filter. 1mL of the filtrate was added to 5mL of 60% H_2SO_4, the mixture was allowed to stand for 5 min and 5mL of 0.5% of formaldehyde solution was added and allowed to stand for 3 hour. The absorbance was taken at a wavelength of 565nm using

Shimadzu UV spectrophotometer, UV-1800 [14]. The concentration of alkaloid in the sample was calculated using the molar extinction coefficient of Vincristine, ε=15136 mol/cm.

(4). Saponins

A 0.5g of the crude extract was mixed with 20mL of 1M HCl and the mixture was boiled for 4 hours and allow to cool. After cooling and filtered, 50mL of petroleum ether was added to the filtrate for ether layer and evaporated to dryness. 5mL of acetone-ethanol (1:1) were added to the residue, 6mL ferrous sulphate reagent and 2mL of Conc. H_2SO_4. The mixture was homogenized and allowed to stand for 10 minutes before the absorbance was taken at 490 nm using Shimadzu UV spectrophotometer, UV-1800 [14].

(5). Tannin

A 0.2g of the extract was weighed into a 50mL beaker, 20mL of 50% methanol was added, covered with para film and placed in a water bath at 80°C for one hour. The mixture was shaken thoroughly and the content was transferred into a 100mL volumetric flask. 20mL of water, 2.5mL Folin-Denis reagent and 10ml of 17% Na_2CO_3 were added and mixed thoroughly. The mixture was allowed to stand for 20 min. Observation for bluish-green colouration was done at the end of range 12.5-100 µg/mL of Tannic acid. The absorbance of tannic acid standard solution as well as sample was taken after colour development on a spectrophotometer at wave length of 760nm using Shimadzu UV spectrophotometer, UV-1800 [15].

2.3.3. Fractionation of the Crude Extracts

15.87g of the crude extract was partitioned with n-hexane, chloroform, ethyl acetate and distilled water in increasing solvent polarity using separating funnel as described by [16]. The fractions were concentrate using the water bath at a temperature of 45°C.

2.3.4. Antimicrobial Susceptibility Screening

(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 [17] by gradually adding normal saline to compare its turbidity to McFarland standard number 0.5 which is equivalent to density of 1.0 x 106 CFU/mL.

(2). Susceptibility Testing of Plant Extracts

Thirty-nine gram (39gm) of the Mueller Hinton agar was dissolved into 1liter of distilled water in a conical flask. The medium was sterilized in the autoclave at 121°C for 15minutes and the media was allowed to cool to holding temperature of 45°C. 20 mL of the sterile Muller Hinton agar was dispensed into sterile petri dishes of diameter 90mm and allowed to set. A sterile cork borer of diameter 6mm 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 200µL of the chloroform, ethyl acetate and aqueous fractions of the extract (30mg/mL) were transferred separately into the wells and 200µL at 1mg/ml of the standard drug (Ciprofloxacin) was 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 370C for 18 hours. The zones of inhibition were then measured to the nearest millimeter using a calibrated veneer caliper. The above method was carried out in triplicates and the mean and standard error of the mean were taken [17].

2.4. Statistical Analysis

The data are presented as Mean ± Standard Error of Mean. 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).

3. Results

Table 1. *Quantitative Phytochemical analysis of 70% methanol of Terminalia microptera leaf extract (mg/g).*

Phytochemicals	Amount
Total Phenol	481.75±0.17
Total Flavonoid	ND
Tannins	70.52±1.66
Alkaloids	541.00±0.61
Saponins	2.13±0.32

Values are expressed in mean ± standard error of mean
ND: Not detected

Table 2. *Percentage yield of partitioned fraction from 70% methanol of Terminalia microptera leaf extract.*

Fractions	Weight (g)	% yield
n-Hexane	-	-
Chloroform	2.65	1.67
Ethylacetate	3.87	24.39
Aqueous	8.28	52.17

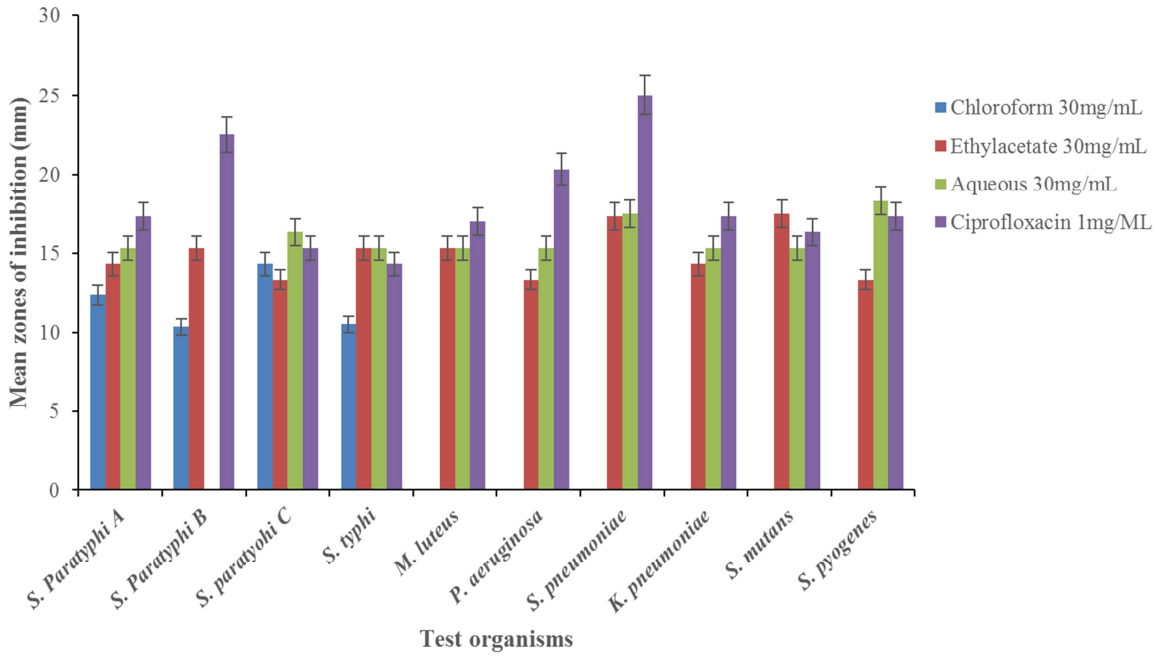

Figure 1. *Mean zones of inhibition of fractions (30mg/mL) on the tested organisms.*

4. Discussions

Qualitative phytochemical screening of seventy percent methanol extracted of *T. microptera* leaf have been reported by [18] which reveals the presence of alkaloid, flavonoids, saponins, cardiac glycosides, anthraquinons, sterols, phlobatanins and terpenes. The quantitative phytochemical analysis indicated high amount of alkaloids (541.00±0.61mg/g), closely followed by total phenol with a concentration of 481.75±0.17mg/g and an appreciable amount of tannins (70.52±1.66mg/g) while saponins was only present in a trace amount (2.13±0.32) (Table 1). The phytochemicals present in plants are responsible for preventing infections or completely treat diseases and have been studied extensively to establish their efficacy and to understand the underlying mechanism of their action. Such studies have included identification and isolation of the chemical components, establishment of their biological potency both by *in vitro* and *in vivo* studies in experimental animals and through epidemiological and clinical-case control studies in man [19].

Alkaloids are natural product that contains heterocyclic nitrogen atoms with basic properties. Alkaloids are naturally synthesized by a large numbers of organisms, including animals, plants, bacteria and fungi [20, 21]. Alkaloids play a significant role in the survival and protection of plant against micro-organisms (antibacterial and antifungal activities), insects and herbivores (feeding deterrens) and also against other plants allelopathically [22]. Alkaloids have many pharmacological activities including antihypertensive effects (some indole alkaloids), antiarrhythmic effect (quinidine, Sparein), antimalarial activity (quinine), and anticancer actions (dimeric indoles, vincristine, vinblastine). Therefore, high amount of alkaloid in this plant is an indication of it various medicinal values.

Phenol or phenolic compounds are the largest category of phytochemicals and the most widely distributed in the plant kingdom. The three most important groups of dietary phenol are flavonoids, phenolic acids, and polyphenols. Phenolic compounds are a large and complex group of chemical constituents found in plants [23]. They are plant secondary metabolites, and they have an important role as defence compounds. Phenols possesses several properties that are beneficial to humans such as its antioxidant properties are important in determining their role as protecting agents against free radical-mediated disease processes. Various biological activities of phenolic acids have been reported. Increases bile secretion, reduces blood cholesterol and lipid levels and antimicrobial activity against some strains of bacteria are some of its biological activities [24].

In medicine, especially in Asian (Japanese and Chinese) natural healing, the tannin-containing plant extracts are used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours [25], and as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals [26]. Recently, tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as AIDS and various cancers [27]. The search for new lead compounds for the development of novel pharmaceuticals has become increasingly important, especially as the biological action of tannin-containing plant extracts has been well documented [28, 29].

Saponins consist of a group of secondary metabolites found widely distributed in the plant kingdom. They form a stable foam in aqueous solutions such as soap, hence the name “saponin”. The physiological role of saponins in plants is not yet fully understood. While there is a number of a publication describing their identification in plants, and their

multiple effects in animal cells and on fungi and bacteria, only a few have addressed their function in plant cells. Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. Saponins may be considered a part of plants defence systems, and as such have been included in a large group of protective molecules found in plants named phytoanticipins or phytoprotectants [19]. Saponin mixtures present in plants and plant products possess diverse biological effects when present in the animal body. Extensive research has been carried out into the membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic properties of saponins and they have also been found to significantly affect growth, feed intake and reproduction in animals [19].

Therefore, the presence of this phytochemicals in good quantity is an indication of its medicinal potency and why it is used by traditional herbalist in treating many ailments [18].

Fractionation of the crude extract yielded three fractions (chloroform, ethyl acetate and aqueous). The highest yield was obtained from the aqueous fractions (52.17%) which was followed by ethyl acetate and chloroform with a yield of 24.39% and 1.67% respectively (Table 2). Natural product or plant extracts are matrix complex mixtures which comprises of several components that are soluble in different solvent based on the polarity of the solvent. The result indicates that the crude extract has no soluble n-hexane portion or may be present in trace amount.

The antimicrobial screening of the crude extracted as reported by [18] indicates that the extract shows a spectrum of activity of 18mm and above against *Streptococcus mutans, Streptococcus pyogenes* and gram negative *Pseudomonas aeruginosa, Klebsiella pneumonia, Micrococcus luteus, Salmonella typhi,* and *Salmonella paratyphi* A, B and C (Figure 1).

Chloroform fraction of this extract was only active on the gram negative organisms (*Salmonella typhi* and *Salmonella paratyphi* A, B and C) with zones of inhibition ranging from 10.00±0.88 – 12.33±0.33mm (Figure 1). The cell walls of gram negative organisms are positively charged and are attracted to an opposite charge [30]. It can be inferred that the chloroform fraction may contain compounds with counter charges that may have bind to the cell walls thereby depriving them from carrying out their normal biological functions.

Ethyl acetate and aqueous fraction were found to be active in most of the tested organisms with zones of inhibition ranging from 11.33±0.67-16.33±0.67mm and 14.33±0.33-16.33±1.00mm respectively (Figure 1). Ethyl acetate and chloroform are secondary solvents and have a close polarity index. Therefore, there is every possibility for the two fractions to contain similar components or constituents and hence a similar zones of inhibition.

The zones of inhibitions recorded in all the fractions were found to be lesser than those recorded in the crude extract as reported by [18]. This may be due to the synergistic effects of the phytochemicals that are present in the crude extract. Secondly, fractionation of this crude extract may have led to decrease in the concentration of the various phytochemicals which may be found in all the fractions thereby decreasing the effect they exert on the organisms.

5. Conclusion

The results of this research work reveals appreciable yield of phytochemical properties in the crude extract of *T. microptera* which partially explains the rationale behind its potency against disease causing microorganisms. Also, all the test organisms were found to be considerably susceptible to the fractions; however, the activity of the fractions might be bacteriostatic in nature as the mean zones of inhibition were seen to be low in comparison to that of the crude extract reported in recent literatures.

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