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PHYTOCHEMICAL AND ANTIBACTERIAL ACTIVITIES OF *Terminalia superba* Engl. and Diels LEAF AND STEM BARK EXTRACTS

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ABSTRACT

Drug resistance by clinically important pathogens is now a worldwide problem with far-reaching consequences especially considering that the emergence of drug resistance is now outpacing the development of new drugs. This study was aimed at evaluating the antibacterial potential of the leaves and stem bark of *Terminalia superba* in a bid to identify potential sources of cheap antimicrobial agents. Extraction was done using solvent partition co-efficient method with four different solvents. The antibacterial susceptibility studies were done using the Agar well diffusion techniques. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the most active extracts were determined using the broth dilution method. The results of the phytochemical screening revealed the presence of Flavonoids, Tannins, Saponins, and Terpenoids. The different fractions of the leaves and stem bark had antibacterial activities, which was found to be more on the Gram positive bacteria *Staphylococcus aureus* than on the gram negative bacteria *Escherichia coli*. The Methanolic fractions had the highest activity, followed by that of Ethyl acetate, Aqueous and then the Hexane fractions. The results of this study show that *Terminalia superba* contains therapeutically useful compounds justifying the use of the plant in traditional medicine.

Keywords: Phytochemical, Antibacterial, *Terminalia superba*, Leaf, Stem.

Introduction

Nature has bestowed on humans a very rich botanical wealth and a large number of diverse types of plants that are growing all over the world. Plants remain the most abundant natural primary source of active drugs and are invaluable in the ethnomedical treatment of diverse ailments (Olasehinde *et al*., 2012). Medicinal plants are generally sources of various phytochemicals, some of which are usually responsible for their biological activities. Many people rely on herbal medicine as their primary source of health care. Many people rely on herbal medicine as their primary source of health care. In Africa, millions of people depend on traditional medicine (Kelmanson *et al*., 2000; Adewummi *et al*., 2001). Almost 80% of the population in Africa still relies on traditional medicine to cure infections of early childhood, including malaria. Herbal medicines are believed to have stood the test of time because of their

general safety, efficacy, cultural acceptability and lesser side effects. Being part of physiological functions in living flora, the chemical constituents present in herbal medicines are believed to have better compatibility with the human body (Kamboj, 2000).

Even though pharmaceutical industries have produced a number of antibiotics to combat microbial infections in the last three decades, resistance to these drugs by microorganisms has increased since bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents. The problem of microbial resistance is growing and the outlook for the use of antimicrobial agents in the future is still uncertain. Due to environmental factors and inadequate public health measures in many developing countries new tropical infections as well as infections that were previously isolated to remote locations, are becoming more prevalent in several areas of Africa especially Nigeria (Bravo *et al*.,2003). The high cost of pharmaceutical medication and the problem of drug resistance contributes to the continuous search for natural and cheaper alternative medicines. Medicinal plants are rich in compounds which may serve as alternative, cheap and safe antimicrobials for the treatment of common ailments. Synthetic antimicrobial agents such as antibiotics and antifungal drugs are widely used to cure infections, but their indiscriminate use causes antimicrobial drug resistance, necessitating the use of medicinal plants as the alternative therapeutic agents (Chanda and Baravalia, 2010).

Plant-based antimicrobials represent a vast array of untapped sources for medicines. In many cases they are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Mahady, 2005).

Medicinal plants are rich in compounds which may be potential natural drugs and which may serve as alternative, cheap and safe antimicrobials for the treatment of common ailments. The present report is focused on *Terminalia superba* (*Combretaceae*), a tree of about 30-50m high. It is a member of the genus *Terminalia* that comprise of about 100 species distributed in tropical regions of the world. *T. superba* is commonly known as Shingle wood, corina or limba in English. In Nigeria it is commonly known as white Afara (Yoruba) and Ujuju Ocha in Igbo and is locally used in the treatment of various ailments, including diabetes mellitus, gastroenteritis, female infertility and abdominal pains (Adjanohoun *et al*., 1996). Therefore, this research was aimed at evaluating the antibacterial activities of the leaves and stem bark extracts of *Terminalia superba* on some pathogenic bacteria.

Materials and Methods

Sample Collection and Identification

The *Terminalia superba* plant samples were taken to the herbarium unit within the college where it was identified by a Plant Taxonomist.

Preparation of Plant Materials

The plant leaf and stem bark samples were air dried at room temperature (25^{o}C) for two weeks and then pulverized into fine powder using a mortar and pestle. The powders were

sieved and stored in air tight bottles until when needed.

Extraction of the Plant Material

The fractionation of the various plant parts was done using the Harborne method of solvent partition co-efficient. The extraction was carried out based on solvent polarity using four different solvents n-hexane, ethyl acetate, methanol and distilled water. It was carried out in their graded form in the following order.

Extraction using n-Hexane

200gm of the powdered leaf sample and 150gm of the stem bark sample were weighed using a top loading balance and it was then transferred into a large extracting flask (bottles) the content was soaked with 1500ml of hexane and allowed to stand for three (3) days at room temperature with continuous shaking and stirring with a sterile glass rod. The suspension was then filtered with a sterile muslin cloth and then filtered again using sterile Whatman No.1 filter paper inserted in a funnel. The plant residue was subjected to several parts of rinsing and filteration to attain an exhaustive level of extraction. The final residue was air dried and re packed for extraction with ethyle acetate.

Ethyle Acetate Extraction

The same procedure above was adopted for the extraction with ethyle acetate after which the methanol extract was used after total drying of the ethyle acetate residue.

Methanol Extraction

The same procedure as above was adopted using methanol as the extracting solvent on the residue of the ethyle acetate.

Water extraction

Distilled water was finally used to extract the methanol residue above after air drying, the packed water content was allowed to stand overnight for two (2) days after which it was filtered and the filtrate dried in a water bath and a drying cabinet. The collective filtrates of n-hexane, ethyle acetate and methanol were concentrated to dryness on a rotary evaporator and later in a drying cabinet to obtain the hexane fraction, ethyle acetate fraction and methanolic fractions while that of water was concentrated on a water bath and a drying cabinet to obtain the aqueous fraction. The various fractions were then tested for their phytochemical constituents. The extracts were stored in a refrigerator at 4^{o}C until further use.

Yield Percentage of Solvent Extracts

After drying, the yield of each extraction was measured separately and the extraction was efficiently quantified by determining the weight of each of the extracts and the yield percentage was then calculated as $\frac{\text{Dry weight}}{\text{Dry material}} x\ 100$ (Parekh and Chanda, 2007)

Phytochemical Determination

The plant fractions were screened for their phytochemical constituents to determine the presence of alkaloids, saponins, tannins, flavonoids, carbohydrates, steroids, anthraquinones, cardiac glycosides and terpenoids using standard phytochemical screening procedures.

Test for Alkaloids

About 0.5g of each extract was stirred with 3ml of 1% aqueous hydrochloric acid on a steam bath; 1ml each of the filtrate was treated with a few drops of Mayers reagent, Dragendorff's reagent and Picric solution.

Precipitation with either of this reagents was taken as preliminary evidence for the presence of alkaloid in the extract. (Sofowora, 2008)

Test for Saponins

About 0.5g of each plant extract was shaken with water in a test tube. Frothing which persist on warming was taken as preliminary evidence for the presence of saponins.

Test for Tannins

About 0.5g of plant extract was stirred with 1ml of distilled water and filtered, ferric chloride was added to the filtrate. A blue-black, green, or blue green precipitate indicated the presence of tannins.

Test for Authraquinones

Borntrager's test was used for the detection of anthraquinones, 0.5g of each of the extracts was taken into a dry test tube and 5ml of chloroform was added and shook for 5 minutes. The extract was filtered, and the filtrate shaken with an equal volume of 100% ammonia solution. A pink, violet or red colour in the ammoniacal layer (lower layer) indicated the presence of free authraquinones.

Keller Killiani Test for Cardiac Glycoside

100mg of the extract was dissolved in 70% alcohol and filtered. About 3 drops of lead sub- acetate was introduced into the filtrate and filtered. The filtrate was extracted with 10mls of choloform in a separating funnel and concentrated to dryness. The resulting residue was dissolved in 1ml of glacial acetic acid containing one drop of Ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring obtained at the interphase indicates the presence of a deoxysugar characteristic of cardenolides.

Salkowski test for steroidal ring

About 100mg of the extract was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interphase indicates the presence of steroidal ring.

Lieberman's Test for Steroid and Terpenes

A little quantity of each extract was dissolved in chloroform, and 1ml of acetic anhydride was added, then two drops of concentrated Sulphuric acid was added. A pink colour which changes to bluish green on standing is indicative of the presence of steroid and terpenes.

Test for Flavonoids

2gm of the powdered leaves and stem bark samples was completely detanned with acetone. The residue was extracted in warm water after evaporating the acetone on a water bath. The mixture was filtered while hot. The filtrate was cooled and used for the following tests.

Lead Sub- Acetate Test for Flavonoids

5ml of the detanned water extracted was added to lead acetate solution. A yellow coloured precipitate indicates the presence of flavonoids.

Sodium Hydroxide Test for Flavonoids

5ml of 20% sodium hydroxide was added to equal volume of the detanned water extract. A

yellow solution indicates the presence of flavonoids.

Test for Carbohydrates

100mg of each extract was dissolved in 3ml of distilled water and mixed with a few drops of Molisch reagent (10% solution of napthol in alcohol) then 1ml of concentrated sulphuric acid was carefully added down the side of the inclined tube so that the acid formed a layer beneath the solution. A white colour at the base indicated the presence of carbohydrates.

Reconstitution of Stock Solution

1gram of the plant extract was dissolved in 2mls of 10% DMSO (Dimethyl Sulphur oxide) to give a stock solution of 500mg/ml, a double fold serial dilution was carried out by taking 1mls of the stock solution and introducing it into another 1mls of 10% DMSO to further breakdown the concentrations into three more concentrations which were 250mg/ml, 125mg/ml and 62.5mg/ml.

Purity Test of Plant Extracts

A purity test was carried out by streaking a loopful of the reconstituted plant extracts on prepared nutrient agar plates and incubating them in an incubator for 3-7 days at 27 ^{o}C for possible growth, the absence of any growth indicates purity and viability of the extracts. (Khan *et al*., 2006)

Source of Microorganisms.

Standard clinical isolates of the bacteria *Escherichia coli* and *Staphylococcus aureus*, were obtained from the Veterinary Research Institute Vom. The organisms were collected in a suspension of nutrient broth (NB). The organisms were subcultured on nutrient agar (NA) to obtain pure cultures and then it was viewed under the microscope for identification.

Antibacterial Susceptibility Testing

Agar Well Diffusion Techniques

The antimicrobial susceptibility test was performed with the clinical isolate of the bacteria *Escherichia coli* and *Staphylococcus aureus* using the agar well diffusion technique as described by (Nair *et al*., 2005). The bacteria inoculum was prepared from subculture as follows; four to five colonies of the isolate were emulsified in sterile nutrient broth and turbidity adjusted to 0.5 McFarland standard. A sterile cotton swap was dipped into the standardized bacterial suspension and used to evenly inoculate the nutrient agar plates. The plates were allowed to stay for 5 minutes before wells of about 6mm in diameter were aseptically punched with a sterile cork borer (4 holes per plates) and the wells were filled with 100 micro liters of the different concentrations of the plant extracts. The plates were left for 30 minutes before incubation in order for the extracts to diffuse into the agar. The plates were incubated at 37 ^{o}C for 24 hours and the zones of inhibition were measured to the nearest millimeter (mm).

Determination of Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC).

The minimum inhibitory concentration which is the concentration giving the least inhibitory activity was determined using the broth dilution method, Standardized inocula of 1 ml of broth containing the organism was introduced into a test tube containing 5mls of sterile broth, 200uls of the reconstituted extract at various concentrations was

introduced into the test tubes and it was incubated at 37 ^{o}C for 24 to 48 hours and observed for growth in the form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered as the MBC's value.

Data collection and Statistical Analysis

***In vitro* antifungal assay**

The experiment was carried out in triplicates and the data obtained was subjected to analysis of variance (ANOVA) which was used to determine the significance in the zone of inhibition, the difference between the antimicrobial activities of both the leaf and stem bark extracts and the susceptibility of the test organism. The least significant difference of 0.05 was used to compare the means.

Results and Discussion

Phytochemical Screening

The phytochemical determination (Investigation) of the different fractions of the leaf and stem bark extracts of *Terminalia superba* revealed the presence of some phytochemical compounds which are known to exhibit medicinal as well as physiological properties. These compounds include flavonoids, steroids, tannins, saponins, terpenoids, Anthraquinones, and carbohydrates. Alkaloids and cardiac glycosides where absent, while tannins and Flavonoids were present in all fractions in abundance, saponins and carbohydrates were present in most of the different fractions, while steroids, anthraquinones and terpenoids were present in some of the fractions of *Terminalia superba*. The results are summarized in table 1

Table 1: Fractions of *Terminalia superba*

Constituents	Hexane fraction leaf	Hexane fraction stem	Ethyle Acetate fraction leaf	Ethyle Acetate fraction stem	Methanol Fraction leaf	Methanol Fraction stem	Water Fraction leaf	Water fraction stem
Alkaloids	-	-	-	-	-	-	-	-
Saponins	-	+	+	++	++	++	++	++
Tannins	+	++	+	+++	+++	+++	+++	+++
Flavonoids	+	+	+++	+++	+++	+++	++	+++
Carbohyrdrates	+	+	+	-	++	+++	++	++
Steroids	-	++	+++	+	-	+	-	-
Anthraquinones	-	+	+	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-	-	-
Terpenoids	+++	-	++	-	+	-	-	-

KEY: + = presence ++= more present
+++= Highly present - = absence

Percentage yield

The methanolic fraction of the stem bark of *Terminalia superba* had the highest yield of 21.6% followed by methanol leaf fraction of 12.7% out of 200gm and 150gm of powdered plant material. While ethyl acetate fraction leaf fraction gave the lowest yield of 0.9gm

(0.6%) out of 150gm of dry plant powder. The results are summarized in table 2.

Purity (Sterility test)

The percentage purity was determined. It was observed that no growth occurred when the reconstituted extract was streaked on PDA (Potato Dextrose Agar) plates with the plant fractions, thereby establishing the sterility of the plant fractions

Table 2: Percentage yield of the various fractions of *Terminalia superba*

Solvent	Weight of dry plant powder (gm)	Weight of dry extracts. (gm)	Yield Percentage (%)
Hexane fraction leaf	150	13.5	9
Hexane fraction stem	200	8.6	4.3
Ethyl acetate fraction leaf	150	0.9	0.6
Ethyl acetate fraction stem	200	3.7	1.85
Methanol fraction leaf	150	19.1	12.73
Methanol fraction stem	200	43.2	21.6
Water fraction leaf	150	5.7	3.8
Water fraction stem	200	8.2	4.1

Antibacterial Activities and Analysis of Variance

At p=0.05 there was a significant difference in the antibacterial activity of the different fractions of *T.superba* on *E.coli*, and also there was a significant difference in the antibacterial activity of the different fractions of *T.superba* on *S.aureus*. Values are presented as mean ± standard deviation. Ranking was done across the fractions and values with the same superscripts are not significant**.** The results are summarized in Table 3 and 4 below. Significant antimicrobial effects, expressed as MIC of each plant extract against test microorganism is as summarized in Table 5 and 6. The data revealed variability in the MIC among plant extracts.

Table 3: The Antibacterial Activities of the Different Fractions of the Leaf and Stem Bark of *Terminalia superba* on *Escherichia coli*

	Concentration			
Extract	62.5 mg/µl	125 mg/µl	250 mg/µl	500 mg/µl
WLS	2.00 ± 0.40^{c}	3.07 ± 0.3^{e}	6.43 ± 0.45^{f}	7.83 ± 0.91^{g}
MLS	3.77 ± 0.50^{c}	5.83 ± 0.57^{d}	9.60 ± 0.36^{e}	13.00 ± 0.40^{e}
EaLS	6.00 ± 0.51^{b}	8.77 ± 0.65^{bc}	13.10 ± 1.10^{c}	15.60 ± 0.36^{c}
HLS	3.67 ± 0.58^{c}	5.00 ± 0.40^{d}	8.53 ± 0.64^{e}	14.10 ± 0.85^{d}
WSS	3.50 ± 0.36^{c}	5.80 ± 0.27^{d}	8.50 ± 0.82^{e}	10.43 ± 0.25^{f}
MSS	6.23 ± 0.55^{b}	9.20 ± 0.53^{b}	14.27 ± 0.67^{b}	17.17 ± 0.57^{b}
EaSS	9.00 ± 1.00^{a}	11.67 ± 1.53^{a}	16.00 ± 1.00^{a}	19.37 ± 0.81^{a}
HSS	5.23 ± 0.32^{b}	7.53 ± 0.50^{c}	10.80 ± 0.53^{d}	14.90 ± 0.66^{cd}
LSD	1.10			
P-value	<0.0001			

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Table 4: The Antibacterial Activities of the Different Fractions of the Leaf and Stem Bark of *Terminalia superba* on *Staphylococcus aureus*

Extract	Concentration 62.5 mg/µl	125 mg/µl	250 mg/µl	500 mg/µl
WLS	4.90 ± 0.53^{f}	5.93 ± 0.38^{f}	7. 63 ± 0.65^{f}	9. 83 ± 0.75^{f}
MLS	7. 10 ± 0.85^{d}	10. 53 ± 0.57^{d}	13. 60 ± 0.60^{d}	16.37 ± 0.71^{d}
EaLS	10.90 ± 0.10^{b}	13. 37 ± 0.91^{c}	16.63 ± 1.74^{c}	20. 67 ± 1.16^{c}
HLS	5. 17 ± 0. 38^{e}	7.17 ± 0.38^{e}	11. 07 ± 0. 21^{e}	13.03 ± 0.15^{e}
WSS	5. 87 ± 0. 59^{e}	6. 90 ± 0.70^{e}	10. 10 ± 0.87^{e}	12. 50 ± 0. 61^{e}
MSS	11. 50 ± 0. 56^{b}	15. 60 ± 0. 60^{b}	18. 37 ± 0. 80^{b}	20. 87 ± 0. 40^{b}
EaSS	14.20 ± 1.06^{a}	16.13 ± 1.21^{a}	19. 73 ± 0.47^{a}	23.73 ± 0.25^{a}
HSS	7. 03 ± 0.25^{d}	10. 53 ± 0.60^{d}	13. 43 ± 1.40^{d}	15. 83 ± 0.65^{d}
LSD	1.22			
P-value	0.0001			

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Table 5: The Mic and Mbc of the Different Fractions of the Leaf and Stem Bark of *Terminalia superba* on *Escherchia coli*

Concentration / Extract	62.5 mg/µl	125 mg/µl	250 mg/µl	500 mg/µl
WLS	+	+	+	+
MLS	+	+	+	_
EaLS	+	+	_	_
HLS	+	+	+	_
WSS	+	+	+	_
MSS	+	+	_	_
EaSS	+	_	_	_
HSS	+	+	_	_

Key: + present - Not present

Table 6: The Mic and Mbc of the Different Fractions of the Leaves and Stem of *Terminalia superba* on *Staphylococcus aureus*

Concentration / Extract	62.5 mg/µl	125 mg/µl	250 mg/µl	500 mg/µl
WLS	+	+	+	_
MLS	+	+	_	_
EaLS	+	_	_	_
HLS	+	+	_	_
WSS	+	+	+	_

MSS	_	_	_	_
EaSS	_	_	_	_
HSS	+	+	_	_

Key: + present - Not present

Discussion

The increase in resistance to conventional antibiotics by microorganisms has necessitated the search for new, efficient and cost effective ways for the control of infectious diseases (Samie *et al*., 2005; Ndip *et al*., 2007). Therefore, this study evaluated the antimicrobial activity of the leaves and stem bark extracts of *Terminalia superba* on *Staphylococcus aureus* and *Escherichia coli*.

The result of the extraction observed that the methanol fraction gave the highest percentage of 21.6% and 12.7% for stem bark and leaf extract respectively. Followed by n-hexane with 9% (Stem bark) and 4.3% (leaf), then Aqueous extracts 4.1% (stem bark) and 3.8% (leaves). Ethylacetate had the lowest percentage yield of 1.85% and 0.6% for stem bark and leaf extracts respectively. These findings concur with the work of others. (Asres *et al.*, 2001; Masoko and Eloff, 2006; Eloff *et al.*, 2008) who also found out that methanol and acetone yield more compound of *Terminalia superba, Combretum molle and Combretum wooddi*. And this agrees with findings that methanolic extracts always have the highest percentage yield on the *Combretaceae* family.

Angeh *et al.*, (2007) worked on different species of plants in the family *Combretaceae* and found out that they yield differently within species but methanol yield was highest in all the specie of the family *Combretaceae*. Methanol is a very volatile solvent and most secondary methabolites are soluble in methanol as such this might be responsible for the high percentage yield recorded for the methanolic fractions. Most plant metabolites are usually soluble in polar solvents and this could explain why methanol had the highest percentage yield.

The phytochemical results indicated that the different fractions contained the presence of secondary metabolites such as Tannins, Saponins, Flavonoids, Steroids, Terpenoids, but Alkaloids were absent in all the extracts. This agrees with the previous findings of others. (Dongmo *et al.*, 2006). (Kouakou *et al.*, 2013: Goze *et al.*, 2014, Ahon *et al.*, 2011). Tannins, Saponins and flavoniods were present in abundance which was also reported by Masoko and Eloff (2006). The environment, period of harvest of organs, stocking conditions of organs and extract solvents may influence the synthesis and expression of phytochemical components in the plant. (Sauvion *et al*., 2013)

The antibacterial activity of the Methanolic, Ethyl acetate, n-Hexane and Aqeuous extracts of the leaf and stem of *Terminalia superba* had a broad spectrum activity which was more on the Gram positive organism *Staphylococcus aureus* than on the Gram negative organism *Escherichia coli*. The results of this research reveals that *S.aureus* was the most susceptible to the different fractions which had zones of inhibitions between 4.90±0.53 to 23.73±0.25 for the leaves and stem bark extracts while *E.coli* had zones of inhibitions between 2.00±0.40 to 19.37±0.81. Previous work done have

demonstrated potent antimicrobial activities of this plant against Gram positive bacteria. (Klamason *et al*., 2007; Kumar *et al*., 2010). Most plant extracts have been reported to be more active against Gram positive than on Gram negative, bacteria; this has been attributed to the fact that Gram-negative bacteria contain an outer membrane with a lipopolysaccharide layer which makes them impermeable to certain antibiotics and bactericidal compounds (Fennel *et al*., 2004). The data represent mean of three replicates ± standard deviation (SD). The results were subjected to analysis of variance, differences between means were considered significant at p-value < 0.05. The results of the MIC values in this study supports previous findings in literature that the antimicrobial activities have a direct relation to increasing the extracts concentration (%) (Bhalodia and Shukla 2011) as such the results varied in the MIC among plant extracts which ranged from 62.5 mg/µl - 500 mg/µl

Conclusion

The results of this research reveals that the *in-vitro* activity of the different fractions of the leaves and stem bark of *Terminalia superba* show the presence of anti-bacterial activity and, further supports it used in traditional medicine. The plant may provide a novel and lead compound for the synthesis of new drugs. Further studies should be carried out to isolate and characterize the active compounds which are essential.

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