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PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF THE METHANOL FRACTIONS OF THE LEAF OF *Gmelina arborea* (Verbenaceae)

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ABSTRACT

Leaves of *Gmelina arborea* are used in traditional medicine for the treatment of bacterial infections without scientific validations. The main objective of the research was to carry out preliminary phytochemical and antimicrobial screening of the methanol fractions of the leaf of *Gmelina arborea*. The methanol leaf extract and its fractions were screened for phytochemical and antimicrobial activity using standard procedures. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, phenolic compounds, triterpenes and steroids. Highest zone of inhibition was observed in ethyl acetate fraction with (29mm) against some tested clinical pathogens compared to other fractions screened. MIC ranged between (6.25-12.5mg/ml) was observed in ethyl acetate. MBC of (12.5mg/ml) was observed in ethyl acetate fraction compared to other fractions screened. This study supports the folkloric claims for the use of the plant against some bacterial infections and can be exploited as alternative antimicrobial drug use for the treatment of infectious disease caused by these pathogens.

Keywords: Antimicrobial, *Gmelina arborea*, Methanol fractions, MIC, MBC, and Phytochemicals.

INTRODUCTION

Medicinal plants play a crucial role in health care needs of people around the world especially in developing countries (Bekalo *et al.*, 2009). About 80% of the world population of most developing countries still depends on the use of traditional medicine derived from natural plants (Cunningham, 1993). People living in remote areas depend more extensively on traditional medicine as modern systems are out of reach (Mahonge *et al.*, 2006). Medicinal plants play a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drugs (Prarono, 2002). Approximately 25% of drugs used in modern Pharmacopoeias are derived from plants and many others are synthetic analogues built on prototype compounds isolated from plants. It was estimated that the total number of medicinal plants in international trade is about 2500 species (Schippman *et al.*, 2002).

The medicinal value of plants have assumed important dimension in the past few decades owing mainly to the discovery that extracts from plants contain not only minerals and primary metabolites but also a diverse array of

secondary metabolites with therapeutic values such as anti-inflammatory, antioxidant and antimicrobial potentials (Akinmoladun *et al.*, 2007).

Many plants have been used because of their antimicrobial traits and the antimicrobial properties of plants have been investigated by a number of researchers worldwide. Ethnopharmacologists, botanists, microbiologists, and natural-product chemists are searching the earth for phytochemicals which could be developed for the treatment of infectious diseases (Tanaka *et al.*, 2006) especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective antimicrobial agents. The Antimicrobial Susceptibility Test (AST) is an essential technique in many disciplines of science. It is used in pathology to determine resistance of microbial strains to antimicrobials, and in ethno pharmacology research, it is used to determine the efficacy of novel antimicrobials against microorganisms, essentially those of medical importance. The test is the first step towards new anti-infective drug development. There are various AST methods that are

employed by researchers and these could lead to variations in results obtained (Lampinen, 2005).

Microorganism has evolved numerous defenses against antimicrobial agents, and drug-resistant pathogens are on the rise. This resistance is conferred by Multidrug Resistance Pumps (MDRs), membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials (Stermitz *et al.*, 2000). Secondary metabolites resemble endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters which thus have beneficial medicinal effects on humans due to their recognition in potential target sites (Parekh *et al.*, 2005). Furthermore, multiple drug resistance in human pathogenic microorganism has been developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Levy, 2004). The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments and could help curb the problem of these multi-drug resistant organisms.

Gmelina arborea Roxb. (Verbenaceae) is native to Asia and known by various names, e.g. Yemane, Gamar and Gumhar. This species has been introduced in several countries, particularly in West Africa and especially in Nigeria and Côte d'Ivoire among others. Ethno botanical studies report that the species is widely used to treat many diseases including diarrhea, hypertension and malaria, among others (Tiwari *et al.*, 2008). A recent study of the composition of secondary metabolites of *G. arborea* showed its richness in phenolic compounds, natural antimicrobial and antioxidant substances that plays important role in pharmacology (N'gaman *et al.*, 2009).

Gmelina arborea exhibited significant antimicrobial activity and showed properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents (Prashanth *et al.*, 2006). Thus, *Gmelina arborea* is well anchored in its traditional uses has now found wide-spread acceptance across the world. The objective of the current investigation of this plant is to evaluate phytochemical constituents and to provide antimicrobial justification for the use of the leaf extracts of *Gmelina arborea* against some pathogenic bacteria *in-vitro*.

MATERIALS AND METHODS

Collection of Plant Materials

The plant material was collected from Suleja Niger State, Nigeria in 2014. The leaf was identified at the herbarium of Biological Science Department, Ahmadu Bello University, Zaria,

Nigeria. A voucher specimen No. (DC 8643) was deposited.

Extraction of Plant Materials

The air dried powdered leaves 600mg was extracted with methanol (2.5L) at 45°C for 48 hours using cold maceration techniques. The extract was concentrated to dryness under reduced pressure. The residue obtain was further re-suspended in water (600ml) and partitioned successively with n-hexane (3×500ml), ethyl acetate (3×400ml) and n-butanol (5×400ml). The various fractions were concentrated using rotary evaporator to afford n-hexane (3.52g), chloroform (4.31g), ethyl acetate (3.14g), n-butanol (5.22g) and aqueous (6.33g) respectively. The various fractions of the extract were subjected to phytochemical screening using standard protocols (Sofowora, 2008; Trease and Evans, 2009).

Preliminary Phytochemical Studies of *Gmelina arborea* Extract

Test for Alkaloids (Salehi-Surmaghi *et al.*, 1992). Test for Flavonoids [(a) Shinoda test (Evans, 2009) and (b) Sodium hydroxide test (Evans, 2009 and Sofoworo, 2008)]. Test for Tannins [(a) Ferric chloride test (Sofowora, 2008) and (b) Lead sub-acetate test (Evans, 2009)] Tests for Saponins (a) Frothing test (Sofowora, 2008). Test for Steroids/Triterpenes [(a) Salkowski test: (Sofowora, 2008) and (b) Lieberman-Burchard test (Sofowora, 2008)] Test for Cardenolides [(a) Kella-killiani test and (b) Kedde test (Evans, 2009)]. Test for Anthracenes [Bontrager test (Evans, 2002) were carried out using the extract.

Test for Antimicrobial

Antimicrobial activity of *G. arborea* was investigated against the following clinical isolates comprising of *Staphylococcus epidermidis*, *Bacillus subtilis*, *E. coli*, *Neisseria gonorrhoea*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella dysenteriae* which were obtained from Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Antimicrobial Assay

The extracts of 0.5 g were weighed and dissolved in 10ml of DMSO to obtain concentration of 50mg/ml. this was the initial concentration of the extract used to check the antimicrobial activities of the extract. Mueller Hilton agar was prepared according the manufacturer's instruction, sterilized at 121°C for 15min and the sterilized medium was then poured into sterilized Petri dishes and the plates were allowed to cool and solidified. Diffusion

method was used for screening the extracts. The medium was seeded with 0.1ml of the standard inoculums of the test microbes, the inoculums were spread evenly over the surface of the medium by the tube of the sterile swab. After setting, the use of a numbers of standard cork borer of 6mm in diameters were obtained, a well was cut at the centre of each inoculums medium. The inoculated medium was then incubated at 37°C for 24hours, after each plate was observed for the zone of inhibition of growth. The zone was measured with transparent ruler and the result was recorded in millimeters (Akujobi *et al.*, 2004).

The minimum inhibitory concentrations of the extract were carried out using the broth dilution methods. Mueller Hilton broth was prepared and 10mls was dispensed into test tube, the broth was sterilized at 121°C for 15mins and allowed to cool. Mc-farland turbidity standard number 0.5 was prepared to give turbid solution. Normal saline was prepared at 10mls and was dispensed into sterile test tubes and the test microbes were inoculated and incubated at 37°C for 6hours. Dilution of the test microbes in the normal saline was done until the turbidity marched that of the Mc-farland's scale by visual comparison; at this point the test microbes have concentration of about 1.5×10^8 cfu/ml (Vollokova *et al.*, 2001; Cowad, 1999). Two fold serial dilution of the extract in the sterile broth was made to obtain the concentration of 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml. the initial concentration was obtained by dissolving 0.5g of the extract in the broths, 0.1ml of the standard inoculums of the microbes were then inoculated into different concentration of the extract in the broth. Incubation was made at 37°C for 24hours, after which the test tubes were observed for turbidity (growth). The lowest concentration of the extract in the broth which shows no turbidity was recorded as the minimum inhibitory concentration Nweze *et al.*, 2004). The minimum bactericidal concentration was carried out to check whether the test microbes were killed or only their growth was inhibited. Mueller Hilton agar was prepared and poured into sterilized Petri dishes, this was allowed to cool and solidify. The content of the MIC in the serial dilutions were then subcultured on to the prepared medium. Incubation was made at 37°C for 24hours after which each plate was observed for colony growth. The plate with the lowest concentration of the extract without colony growth was considered the MBC for the bacteria (Ogbulie *et al.*, 2007, Ekpendu *et al.*, 1989).

RESULTS AND DISCUSSION

The preliminary phytochemical studies of the methanol extract of *Gmelina arborea* showed the presence of flavonoids, alkaloids, saponins, cardiac glycosides, carbohydrates, triterpenes and steroids. Many reports suggest that flavonoids of plants belonging to various families exhibit antimicrobial activity against bacterial and fungal pathogens (Kayser and Arndt). Flavonoids, present at high levels in most plants have many biological effects including anti-allergic, anti-inflammatory, antihepatotoxic, anti-ulcer, anti-viral and anti-spasmodic and are of interest in the investigation of disease processes and as potential new drugs (Nag *et al.*, 2004). The presence of alkaloids in this plant is also of great importance to humans because of their medicinal values as significant quantities are used as anti-malarial, analgesic and as stimulants. Alkaloids rich fraction from *Prosopis juliflora* (Fabaceae) was reported to be most sensitive against gram negative *Kleb. Pneumoniae* with a comparable zone of inhibition with that of standard drugs ampicillin (Shachi singh *et al.*, 2011). This therefore gives credence to some of the ethnomedical uses of the plant (Michael, 2002). Many studies have established the usefulness of medicinal plants as a great source for the isolation of active principles for drug formulation (El-Mahmood and Ameh, 2007).

The presence of these phytochemical compounds reported from *Gmelina arborea* are linked to the biological activity such as antimicrobial, anti-inflammatory, immunomodulatory and anti-cancer activity. Research findings indicated that the antimicrobial properties of plants are conferred on them by the presence of secondary metabolites (Edeoga *et al.*, 2005; Enaba *et al.*, 2007; Bishnu *et al.*, 2009.) Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc., have been found *in vitro* to have antimicrobial properties (Danahukar *et al.*, 2000). Phenolic compounds are essentially representing varieties of natural antioxidants, which are used as nutraceuticals and also in control of human pathogenic (Puupponen-Pimiä *et al.*, 2008). Triterpenoidal saponin extracted from aqueous and ethanolic extracts of *Allophylus cobbe* and *Allophylus serratus* was found to have potential antibacterial activity against *B. subtilis* (Chavan and Gaiwad, 2013). Saponin was reported to be active against six strains of *E. coli* compared to standard drugs streptomycin (Michal *et al.*, 2012). The extent of antimicrobial activity of the extracts based on the diameter zones of inhibition has been described as low (12-18

mm), moderate (19-22 mm) and strong activity (23-38 mm) by Ahmad *et al.*, (1999).

Junior and Zani (2000) also reported the categories of zone of inhibitions of antibacterial activity as <9mm zone was considered as inactive; 9-12mm as partially active; while 13-18mm as active and >18mm as very active. This is in lined with the zones of inhibition observed in this study. The highest zones of inhibitions observed were mostly exhibited in ethyl acetate fraction as 29mm against gram negative *Neisseria gonorrhoeae* followed by gram positive *Staph. epidermidis* and *Salmonella typhi* with 28mm against the clinical isolates while, the remaining isolates were inhibited at a zone of inhibitions between 26-27mm but, *VRE*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* have shown resistant against the ethyl acetate fractions. The remaining Pet-ether, Chloroform, n-butanol and Aqueous fraction inhibited the growth of micro-organisms range from 17-26mm whereas, the following gram negative *VRE*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* have shown resistant to all fractions. The zone of inhibitions exhibited by ethyl acetate fraction was closely similar with that of control standard drug with the zone of inhibition range from 23-40mm. The apparent differences in their susceptibility to the extracts might be related to their structural differences in their cell enveloped compositions of gram positive and gram negative bacteria. The gram positive cell wall is simple while that of gram negative is complex consisting of lipoproteins outer membrane and lipopolysaccharides (Jawetz *et al.*, 1978). The outer membrane of gram negative cell envelopes does block the penetration of large molecules and hence the relative resistance of gram negative bacteria to some antimicrobial drugs (Jawetz *et al.*, 1978). The lowest concentration of extracts that did not permit any visible growth was considered as the MIC. The minimum inhibitory concentration was observed in ethyl acetate fraction range from

(6.25 – 12.5) against some both gram positive and gram negative isolates followed by remaining fractions with the MIC of 12.5 indicating minimal activity compared to ethyl acetate fraction this indicates that the chemical constituents of the fractions have therapeutic properties. But resistant was observed in some clinical isolates including *VRE*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* against some fractions. The range of MIC values (6.25-12.5) also demonstrated that the fractions have both static and cidal effect against some tested bacterial isolates (Table 3). The MIC values at (6.25-12.5) also agreed with the findings of El-Mahmoud *et al.*, 2010 on the in-vitro antimicrobial activity of the crude extract of *Gmelina arborea* against some species of pathogenic enterobacteriaceae. The minimum bactericidal concentration in this study range from 25-50mg/ml of some methanol fractions indicate bacteriostatic and bacteriocidal effect of the methanol fractions against some of the clinical isolates. MBC of less than 12.5mg/ml is an indication that the fraction has bacteriocidal effect while MBC of 25-50mg/ml is also an indication of bacteriostatic effect of the fractions; similar result was reported by Doughari *et al.*, 2010 on the in-vitro antimicrobial effect of some *Gmelina arborea* extract against some species of enterobacteriaceae. Ethyl acetate fraction has the minimum bactericidal concentration of 12.5mg/ml against *Staph. aureus*, *Neisseria gonorrhoea*, and *Salmonella typhi*. MBC of 25mg/ml was observed on ethyl acetate fraction against *MRSA*, *B. Subtilis* and *E.coli* while *VRE*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* were resisted against all the fractions. This is a strong indication that *G. arborea* is a medicinal plant that serves as folkloric remedies for various microbial based disease including those associated with gastro intestinal tract infections (Ndukwe *et al.*, 2005, 2007; Doughari *et al.*, 2007).

Table 1: Phytochemical Constituents of the Leaf extracts of *Gmelina arborea*

CONSTITUENTS/TESTS	OBSERVATION	INFERENCE
Alkaloids		
Dragendorff	Orange red colour	Present ++
Mayer	Buff precipitate	Absent ---
Wagner	Dark Brown precipitate	Absent ---
Tannins		
Ferric chloride	Greenish appearance	Absent ---
Lead sub acetate	Whitish precipitate	Absent ---
Flavonoids		
Shinoda	Deep yellow coloration	Present +++
Saponins		
Frothing	Persistent frothing	Present +++
Steroids/Triterpenes		
Salkowski	Red precipitate	Present ++
Libermann burchard	Purple to violet ring	Present ++
Cardiac glycosides		
Kella kiliani	Reddish brown color	Present ++
Legal test	Deep red color	Present ++
Keddi's Test	Violet color	Present +
Carbohydrates		
General test (Molisch)	Red color	Present +++
Sugar test (Barfoed)	Red color	Present ++

Table 2: Zone of Inhibitions(mm) of the fractions against the test Microorganisms (mm)

Test Organism	Aqueous	Chloroform	n-butanol	Pet-ether	Ethyl acetate
MRSA	22	24	20	18	26
VRE	0	0	0	0	0
<i>Staph. epidermidis</i>	24	26	21	18	28
<i>Bacillus subtilis</i>	23	24	20	19	27
<i>E. coli</i>	21	24	20	18	27
<i>Neisseria gonorrhoea</i>	24	26	21	18	29
<i>Klebsiella pneumoniae</i>	0	0	0	0	0
<i>Pseudomonas aeruginosa</i>	0	0	0	0	0
<i>Salmonella typhi</i>	23	25	20	17	28
<i>Shigella dysenteriae</i>	0	0	0	0	0

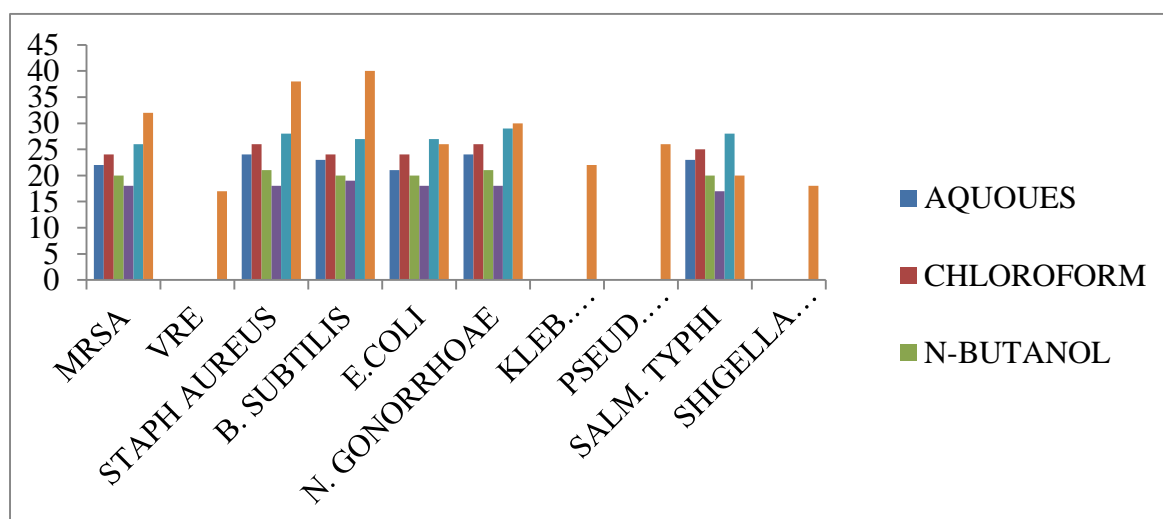


Fig.1: Zones of Inhibition (mm) of Aqueous, Chloroform, n-butanol, Pet-ether and Ethyl acetate Leaf Extracts *Gmelina arborea* Compared with Ciprofloxacin against the Ten Clinical Isolates.

Table 3.3: Minimum Inhibitory Concentration of the various Fractions against the Test Bacterial Isolates

Test Organism	Aqueous fraction(mg/ml)					Chloroform fraction					N- butanol fraction					Pet-ether fraction					Ethyl acetate fraction				
	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125
<i>MRSA</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	*	++	++
<i>VRE</i>	NT					NT					NT					NT									
<i>Staph. epidermidis</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	-	*	+
<i>Bacillus subtilis</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	-	*	+
<i>E. coli</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	-	*	+
<i>Neisseria gonorrhoea</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	-	*	+
<i>Klebsiella pneumoniae</i>	NT					NT					NT					NT									
<i>Pseudomonas aeruginosa</i>	NT					NT					NT					NT									
<i>Salmonella typhi</i>	-	-	*	+	++	-	-	*	+	++	-	-	*	+	++	-	-	*	++	+++	-	-	-	*	+
<i>Shigella dysenteriae</i>	NT					NT					NT					NT									

Key: - = No Turbidity (No Turbidity), * = MIC, + = Turbid (Light growth), ++ = Moderate Turbidity, +++ = High Turbidity

Table 3.4: Minimum Bactericidal Concentration of the fractions against the Test Microbes

Test Organism	Aqueous fraction(mm/ml)					Chloroform fraction					N- butanol fraction					Pet-ether fraction					Ethyl acetate fraction				
	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125	50	25	12.5	6.25	3.125
<i>MRSA</i>	-	*	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	*	+	++	++
<i>VRE</i>	NT					NT					NT					NT									
<i>Staph. Epidermidis</i>	-	*	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	-	*	+	++
<i>Bacillus subtilis</i>	-	*	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	*	+	++	++
<i>E. coli</i>	*	+	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	*	+	++	++
<i>Neisseria gonorrhoea</i>	-	*	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	-	*	+	++
<i>Klebsiella pneumoniae</i>	NT					NT					NT					NT									
<i>Pseudomonas aeruginosa</i>	NT					NT					NT					NT									
<i>Salmonella typhi</i>	-	*	+	++	+++	-	*	+	++	+++	*	-	+	+++	++++	*	-	+	+++	++++	-	-	*	+	++
<i>Shigella dysenteriae</i>	NT					NT					NT					NT									

Key: - = No Tubidity, * = MBC, + = Scanty Colonies growth, ++ = Moderate Colonies growth, +++ = Heavy Colonies growth

CONCLUSION

Gmelina arborea possess some metabolites and demonstrated antimicrobial activity against some

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