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ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL CONSTITUENTS OF AFRICAN BIRCH (*Anogeissus leiocarpus*) STEM BARK EXTRACTS

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ABSTRACT
The genus Anogeissus (Combretaceae) is widely distributed in most tropical and subtropical countries of the world and has long been used in traditional medicine to treat a broad spectrum of disorders. The stem-bark powder of the plant was extracted with butanol, hexane and water using percolation and soxhlet extraction methods. The extract fractions were screened for the presence and estimation of secondary metabolites using standard procedures. They were further tested for antibacterial activity against clinical bacterial isolates of respiratory tract infections including Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella ozaenae, Escherichia coli and Pantoea agglomerans using disc diffusion and Epsilometer test (E- test) techniques. The results of phytochemical screening indicated the presence of secondary metabolites including alkaloids, cardiac glycosides, reducing sugars, tannins, saponins, flavonoids, amino acids, anthraquinones, carbohydrates, steroids, triterpenes, monosaccharides and glycosides in the fractions of the extract. Bioassay test results showed that Klebsiella spp., P. agglomerans and E. coli were sensitive to aqueous and butanol extract fractions of the plant with highest sensitivity to aqueous fraction against E. coli (17-23 mm) using disc diffusion test and also having MIC and MBC values of 100 and 200 mg/ml, respectively. The plant extract fractions were found to show inhibitory activity against the test isolates which may be related to the presence of secondary metabolites, some of which are reported to be responsible for antimicrobial properties. The results suggest that A.leiocarpus stem bark has the potential for the production of drugs against resistant bacteria.
Key words: Anogeissus leiocarpus, Secondary metabolites, Antibacterial, Bacteria.

INTRODUCTION

All over the world, medicinal plants serve as a potent agent in the treatment of diseases, this may be due to the presence of diverse group of phytoconstituents such as tannins, saponins and cardiac glycosides among others (Issa *et al*., 2021; (Odeh *et al.,* 2014).Plants remain the most common source of antimicrobial agents, their usage as traditional health remedies arecommon in Asia, Latin America and Africa (Bibitha *et al*., 2002; Maghrani *et al*., 2005). In recent years, pharmaceutical companies have spent a lot of money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population (Yusha'u et al., 2014). *Anogeissus leiocarpus* is a tree widely distributed in northern Nigeria. The bark and seed of the tree is used for the treatment and prevention of worm infestation in equine species (Ahmad and Wudil, 2013). Traditional healers in the north eastern part of Nigeria also believe that the bark of the plant is very effective in the treatment of African trypanosomiasis (Mukhtar *et al.,* 2017). *Anogeissus leiocarpus* is a deciduous tree species that can grow up to 15–18 m of height and measure up to 1 m diameter (Ahmad, 2014). Bark greyish and scaly, branches often drooping and slender, leaves alternate, ovate–lanceolate in shape, 2-8 cm long and 1.3-5 cm across (Ouedraogo *et al*., 2013). The leaves are acute at the apex and attenuate at the base, pubescent beneath. Inflorescence globose heads, 2cm across, yellow; the flowers are bisexual, petals absent, while the fruits are globose, cone like heads; each fruit is broadly winged, dark grey, 3cm across (Ouedraogo *et al*., 2013).

It can reproduce by seeds as well as vegetative propagation (Ouedraogo *et al*., 2013; El Ghazali *et al.,* 2003). *Anogeissus leiocarpus* is typical element of woodlands and savannas of the Sudanian regional centre of endemism (Ahmad, 2014). It has large ecological distribution ranging from the region of Sahara up to the out layer humid tropical forests. In West Africa, from Senegal to Nigeria, Cameroon and extends to Ethiopia and East Africa. It grows in dry forests and gallery forests (Ouedraogo *et al*., 2013; Hennenberg, 2005). Many traditional uses have been reported for the plant. In Sudanese traditional medicine the decoction of the bark is used against cough (El Ghazali *et al.,* 2003). Rural populations of Nigeria use sticks for dental hygiene, the end of the sticks are chewed into fibrous brush which is rubbed against teeth and gum (Rotimi, 1988). Ivory Coast traditional practitioners use the plant for parasitic disease such as Malaria, Trypansomiasis, Helminthiasis and dysenteric syndrome (Okpekon, 2004). In Togolese traditional medicine, it is used against fungal infections such as dermatitis and Mycosis, also the decoction of leaves is used against3stomach infections (Batawila *et al*., 2005). The plant is also used for the treatment of diabetic, ulcers, general body pain, blood clots, asthma, coughing and tuberculosis (Victor, 2013). Many works reported the plant to possess a vast number of pharmacological activities including antiplasmodial (Mann *et al*. 2014), antioxidant (Ahmad, 2014), antibacterial (Aliyu and Sani, 2011), antidiabetic (Mann *et al*. 2014), leishmanicidal (Ahmad, 2014), antimalarial (Ahmad, 2014), anthelmintic (Ahmad, 2014), antifungal (Mann *et al*. 2008) and trypanocidal activities (Ahmad, 2014; Bizimana, 1994 and Mann *et al*. 2014).This study aimed at determining the phytochemical constituents of stem bark of African Birch (*Anogeissus leiocarpus*) as well as investigate the antibacterial activity of the plant against some clinical bacterial isolates.

MATERIALS AND METHODS

The bark of African birch (*Anogeissus leiocarpus*) plant was picked directly from the tree in a local farm at Kureken Sani ward of Kumbotso Local government in Kano. The stem bark of the plant was confirmed at the herbarium section of plant science department, Bayero University, Kano, with herbarium accession number BUKHAN 0029. The plant parts were dried at room temperature and then grounded into powder with pestle and mortar as described by Mukhtar and Tukur (1999).

Extraction of plant materials

The powdered plant materials (500 grams) were subjected to different extraction methods using 3 different solvents. These include maceration and Soxhlet extraction. The aqueous extract of powdered African birch stem bark was made by maceration in distilled water. About 50g of each powdered plant were taken and mixed in 500ml of distilled water. This was done as described by Fatope *et al.,* (1993). The butanol and hexane fraction were extracted using Soxhlet Extraction apparatus as described by Lekgari, (2010).

Phytochemical Screening

Phytochemical screening for major bioactive constituents such as alkaloids, flavonoids, triterpenes, tannins, saponins, carbohydrates, amino acids, reducing sugars, glycosides, cardiac glycosides, monosaccharides, steroids and anthraquinones were determined using standard phytochemical test methods (Cuilci, 1994; Sofowora, 2006; Trease and Evans, 1978).

Test Organisms

The test organisms were Gram negative enterobacteriaceae isolates of respiratory tract infections obtained from pathology Departments (Microbiology laboratories) of three tertiary healthcare institutions which include Murtala Muhammad Specialist Hospital, Muhammad Abdullahi Wase Teaching Hospital and Sir Muhammad Sunusi Specialist Hospital, all in Kano metropolis. The isolates were obtained from sputum of patients with respiratory tract infections (RTIs). The isolates after collection were then subjected to purification (subculture), Gram staining and biochemical test using Microgen GN-ID A identification system (MID-64CE), for re-identification.

Standardization of inoculums

Few colonies of the overnight growth of confirmed isolates to be tested were dispensed in sterile normal saline to match the 0.5 McFarland standards for sensitivity tests as described by Clinical laboratory standard institute (Clinical Laboratory Standard Institute, 2010).

Antibacterial Sensitivity Test (Bioassay)

Agar well Diffusion Method

The agar well diffusion method previously described by Lima *et al*.(1993) was used. Mueller Hinton Agar was prepared as specified by the manufacturer. The media was autoclaved and poured aseptically into sterile Petri dishes and was allowed to gel. A loopful of the standardized bacterial suspension was streaked evenly on each agar plate. Stock solution (400mg/ml) of the stem bark extract of *Anogeissus leiocarpus* was separately prepared to obtain the working concentrations of 200mg/ml, 100mg/ml, 50mg/ml, and 25mg/ml,

respectively. Then, 0.1 ml of each crude extracts was added into four wells (6mm diameter) which were bored with a sterile cork borer in each plate. DMSO (0.1 ml) was inoculated on the control plate to serve as negative control and 0.1ml of ceftriaxone dilution (30µg/ml) was also added to serve as positive control. The plates were allowed to stand for 30 minutes on a flat surface for pre- diffusion of the extract, and were then incubated at 37^0C for 24 hours. The antibacterial activity of the extracts was determined after overnight incubation by measuring the zones of inhibition and the results were recorded in millimeter (mm).

Discs preparation for Minimum Inhibitory Concentration (MIC)

Stock solution of the plant extracts recovered was prepared. Five different concentrations of each of the plant extract were prepared viz; 25mg/ml, 50mg/ml, 100mg/ml, 200mg/ml and 400mg/ml, which finally yielded disc potencies of 0.25mg/disc, 0.5mg/disc, 1mg/disc, 2mg/disc and 4mg/disc, respectively. This was followed by introducing 100 sterile paper discs into each concentration which were allowed to absorb the solution and kept for further analysis.

Minimum Inhibitory Concentration (MIC)

Extract concentrations that showed activity against the test bacterial isolates were evaluated for MIC by double fold dilution using improvised Epsilometer – test (E- test) method with filter paper discs. The culture of the standardised bacterial suspension was inoculated on a Mueller Hinton agar using a sterile cotton swab. The E - test set up (filter paper discs with different concentration of the extract) was then placed on to the inoculated agar plate, after placing each of the discs with different concentrations; it was allowed to be absorbed into the medium. It was then incubated at 37ºC for 24 hours. After incubation, bacterial growth becomes visible on the plate, and a symmetrical zone of inhibition ellipse along the arranged discs was observed. The MIC value was read from the point where the ellipse edge intersects as seen on the plate, i. e. the MIC value was read at the point of complete inhibition of all growth. The least concentration with no detectable bacterial growth was considered as the minimum inhibitory concentration (Akinyemi *et al*., 2005).

Minimum Bactericidal Concentration (MBC)

Sterile Mueller Hinton agar plates were inoculated with a loop full of sample from the MIC plate that shows least bacterial growth. Sterile agar plate was streaked only with the test bacterial isolates to serve as control. Plates inoculated with the bacteria were incubated at 37ºC for 24 hours. The lowest concentration at which no growth was observed on the medium was taken as the Minimum Bactericidal Concentration (Akinyemi *et al*., 2005).

RESULTS AND DISCUSSION

The result for the extraction of *Anogeissus leiocarpus* stem bark yield higher extract for the aqueous extract (14g) while n-Butanol extract yielded 1.9g and n- hexane extract yielded 1.5g, respectively, with variation in color and texture of the extracts (table 1). On subjecting fractions of the plant extracts to quantitative as well as qualitative phytochemical tests, the results for qualitative analysis showed that aqueous extracts of the plants contain some secondary metabolites including saponins, reducing sugars, carbohydrates, flavonoids, Tannins, amino acids, steroids, cardiac glycosides, triterpenes and alkaloids, respectively; Anthraquinones were present only in aqueous extract of *Anogeissus leiocarpus*. Butanol extract fraction of the plant also contain alkaloids, carbohydrates, flavonoids, tannins, amino acids, steroids, triterpenes, while reducing sugars, glycosides and anthraquinones were not present in the butanol extract fraction of the plants; monosaccharides were found to be contained in the stem bark butanol extract (Table 2). This may be due to the fact that water dissolves most of the substances than any other liquid. However, both water and butanol extracts contain more secondary metabolites than hexane extract which may be related to the polarity of both solvent and the constituents of the extracts. The results of this study showed that water extracted more components than butanol and hexane (which is having the least percentage of extract), which may be associated with the polarity of the components making them more soluble in more polar (butanol and water) than least polar solvent (hexane) that may be responsible for the variation in physical properties of the extracts respectively. The report from this findings agrees with the work of Barku and Abban (2013), which reported that *A. leiocarpus* extracts contains all the secondary metabolites that were detected in this studies including tannins, saponins, flavonoids, steroids, amino acids and reducing sugars. Even though, carbonyls were reported to be present which were not detected in this studies, similar report has been made by Mann *et al.* (2010) and Kabore *et al*. (2010). Their work revealed the presence of alkaloids, glycosides, phenols, steroids, tannins, saponins, flavonoids and anthraquinones.

In similar studies conducted by Aliyu and Sani (2011), alkaloids, tannins, saponins, flavonoids and glycosides were found to be present in aqueous and ethanol stem bark extracts of *A. leiocarpus*. Some of these metabolites were reported to be responsible for antimicrobial activity associated with some ethno-medicinal plants (Singh and Bhat, 2003).

Table 1: Physical Parameters of *Anogeisuss leiocarpus* stem bark extract

Physical parameters	Aqueous fraction	Butanol fraction	Hexane fraction
Colour of extract	Brown	Dark brown	Dark brown
Texture	Powdered granules/ crystalline	Gummy	Oily
Weight of extract (g)	14	1.7	1.5
Percentage yield (%)	2.8	0.6	0.5

Table 2: Qualitative Phytochemical Constituents Of *Anogeissus leiocarpus* Stem Bark Extracts

Tests	Aqueous fraction	Butanol fraction	Hexane fraction
Alkaloids	+	+	+
Amino acids	+	+	+
Cardiac glycosides (KellarKillani test)	+	+	+
Saponins	+	+	-
Tannins	+	+	-
Carbohydrates (Molisch's test)	+	+	+
Reducing sugars	+	-	-
Triterpenes	+	+	+
Monosaccharide (Barfoed's test)	-	+	-
Glycosides (Phenolic aglycones)	+	-	-
Anthraquinones Borntrager's test	+	-	-
Flavonoids	+	+	-
Steroids	+	+	+

KEY: (+): indicates the presence of phytochemical constituents
(-): indicates the absence of phytochemical constituents

The extracts were screened for phytochemical constituents where the secondary metabolites detected in the plants were quantified. The highest concentration of flavonoids was estimated to be found in the hexane extract fraction (61.957 ± 0.400) with aqueous extract fraction having the lowest flavonoids estimation (47.710 ± 1.860), butanol extract fraction was having highest estimation of alkaloids (118.670 ± 0.882) and aqueous extract fraction was found to have the lowest estimation (29.889 ± 0.347), aqueous stem bark was having the highest estimation of tannins (6.975 ± 0.021) while butanol fraction was having the least estimation (5.173 ± 0.302) respectively. Saponins (0.818 ± 0.001) was only estimated in aqueous extract fraction, and cardiac glycosides (3.539%± 0.003) were observed to be higher in aqueous extract while the lowest were obtained in hexane extract fraction (0.173% ± 0.003) respectively. Also steroids were reported to be in higher quantity in hexane stem bark extract (0.995 µg/ml ± 0.01) whereas aqueous stem bark extract was found to be with the lowest concentration of steroids (0.005 µg/ml ± 0.004) (Table 3). The high contents of phenolic compounds estimated indicated that these compounds contribute to the antimicrobial activity of the plant. This indicated a broad range of activities, which may help in the protection against chronic diseases. These biological active compounds also known as secondary metabolites constitute an important source of antimicrobials and many pharmaceutical drugs. These metabolites also help in the antimicrobial activities of the plant through different mechanisms. The results of the present study is not in line with the work of Barku and Abban (2013), which reveals Quantitative estimation of bioactive phytoconstituents and showed that the plant contains alkaloids (152.0 ± 0.1 mg/g), phenolics (1294.81 ± 3.0 mg/g), flavonoids (330.7 ± 3.0 mg/g) in the methanol extract and alkaloids (80.20 ± 0.0 mg/g), phenolics (616.5 ± 4.4 mg/g), flavonoids (202.5 ± 4.0 mg/g) in the

ethyl acetate extract, respectively. This might be as a result of different solvent that is being used for the extraction. This may be due to the fact that some solvents used for the extraction were unable to dissolve appreciable amount of the metabolite to be detected by phytochemical screening procedure employed.

Table 3: Quantitative Phytochemical Analysis of *Anogeissus leiocarpus* Stem Bark Extracts

Plants extracts	Alkaloids (µg/mL)	Flavonoids (µg/mL)	Saponins (µg/mL)	Taninns (µg/ml)	Cardiac glycosides (%)	Steroids (µg/mL)
Aqueous stem	29.889 ± 0.347[b]	47.710 ± 1.860[f]	0.818 ± 0.001[c]	6.975 ± 0.021[c]	3.539 ± 0.003[b]	0.005 ± 0.004[d]
N-Butanol stem	118.670 ± 0.882[d]	54.269 ± 12.174[h]	_____	5.173 ± 0.302[e]	1.165 ± 0.01[e]	0.222 ± 0.015[e]
N-Hexane stem	_____	61.957 ± 0.400[a]	_____	_____	0.173 ± 0.003[g]	0.995 ± 0.015[g]

Values are represented as mean ± standard deviation of triplicates. Values with different superscript along the same column are significantly different ($p<0.05$).

Antibacterial activity of the plant extracts using agar well diffusion method indicated that the test organisms were more sensitive to the aqueous extracts and also to a small extent sensitive to butanol extract fraction of the stem bark of *Anogeissus leiocarpus*, while there is very little or no activity observed in hexane extract fraction against the test organisms (Table 4). This could be attributed to the fact that these organisms are highly resistant organisms and more so, based on the reviewed literature, the literature showing antibacterial activity of these extract against multi- drug resistant Extended Spectrum Beta Lactamases (ESBLs) producing Gram negative enterobacteriaceae is scarce. Sensitivity of the ESBLs producing bacteria to the aqueous extracts of *Anogeissus leiocarpus* varies from one organism to the other. These findings is in line with the study of Barku and Abban (2013), which reveals that the zones of inhibition produced by the test organisms indicated their susceptibility to the extracts and the zones of inhibition were observed to be varied from one organisms to another, where the extract has activity on the Gram negative bacterial strains more than the Gram positive strains which include *Klebsiella pneumonia*, *Citrobacter spp* and *E. coli.* The activity of the plant extracts reported in this research may be related to the presence of some secondary metabolites like alkaloids and tannins whose antimicrobial properties were well documented (Tschehe, 1971). The result of the present study is also in line with the report by Ikhram *et al*. (2015), which revealed that all the test organisms including *E.coli, Klebsiella* and *Salmonella* (Gram negative enterobacteria) were found to be sensitive towards the extracts and fractions of *A. leiocarpus*. The fractions of bark extracts appeared to possess high activity among the test organisms with *E.coli* having zone diameter of 17mm, *Klebsiella* spp 15mm and *Salmonella* spp 15mm respectively. Thus, the results of this study had demonstrated some antimicrobial properties of *A. leiocapus* stem bark that may be useful in further ethno medicinal and pharmacological aspect for future research.

The minimum inhibitory concentration of the extracts shows that 25mg/ml was the lowest concentration of the extract that was tested against the test organisms, where the MIC is reached at 50mg/ml concentrations and above against the test organisms. (Table 5).

Table 4: Range of Antibacterial Activity of *Anogeissus leiocarpus* Stem Bark Extracts Against The Test Organisms.

	***Anogeissus leiocarpus* extracts Zones of inhibition (mm)**		
Isolates	**Aqueous**	**Butanol**	**Hexane**
E. coli (n=4)	17-23	12-18	0
Klebsiella spp (n=6)	10-14	10-14	8-9
P. agglomerans (n=19)	9-13	8-14	0-7

KEY: Concentrations (mg/ml) - 25, 50, 100, 200
Well size: 6mm
Control: Ceftriaxone disc (30µg/disc) = 36mm

Table 5: Range Of Antibacterial Activity Of Aqueous Extract of *Anogeissusleiocarpus*on The Bacterial Isolates

Bacterial Isolates	MIC (mg/ml)	MBC (mg/ml)
E. coli (n=4)	100-200	**
Klebsiella pneumoniae (n=3)	50-100	**
Klebsiella oxytoca (n=2)	50-100	**
Klebsiella ozaenae (n=1)	50-100	**
P. agglomerans (n=19)	50-200	**

KEY: MIC- Minimum Inhibitory Concentration MBC- Minimum Bactericidal concentration
** - MIC or MBC is greater than 400mg/ml (which is the highest concentration).

CONCLUSION

The study indicates that *Anogeissus leiocarpus* stem bark possesses some secondary metabolites including Alkaloids, tannins, saponins, flavonoids, reducing sugars, amino acids, carbohydrates, monosaccharide, triterpenes, anthraquinones, cardiac glycosides and steroids which might be the possible reason of having a significant antibacterial activity against some of the multi drug resistant bacterial strains used in the study. The stem bark of *A. leiocarpus* therefore provides possible alternative and easily affordable sources of antimicrobial agents for the treatment of many diseases associated with the test organisms. The MIC range was determined at the concentration of 50-200mg/ml for the test isolates, whereas the MBC is greater than the highest concentration (400mg/ml) for all the test isolates.

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