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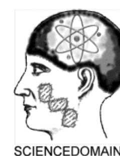

Phytochemical Studies, *In-vitro* Antibacterial Activities and Antioxidant Properties of the Methanolic and Ethyl Acetate Extracts of the Leaves of *Anogeissus leiocarpus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author BYAV designed the study, determined the antioxidant activity, phenolic and flavonoid content, performed the statistical analysis, and wrote the first draft of the manuscript. Author AG determined the phytoconstituents and antibacterial activity. Both authors managed the analyses of the study, the literature searches and read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To conduct a study on the antibacterial activity, phytochemical constituents and antioxidant properties that identify the medicinal potency of medicinal plants on the methanolic and ethyl acetate extracts of the leaves of *Anogeissus leiocarpus*. Antibacterial activity was determined by well diffusion method against four bacterial strains commonly associated with wound infections.
Study Design: Standard Phytochemical quantitative and qualitative test, DPPH radical scavenging activity and well diffusion method.
Place and Duration of Study: Department of Chemistry (Natural Products Laboratory), University of Cape Coast and Department of Microbiology, Korle-Bu Teaching Hospital, University of Ghana, between March 2012 and January 2013.
Methodology: Standard chemical procedures were used to identify phytoconstituents present in the plant extracts. DPPH radical scavenging assay and well diffusion method were used to determine antioxidant and antibacterial activity respectively. UV-spectrophotometry was employed to further quantify the phenolic and flavonoid content.

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Results: The extracts showed pronounced activity against all the bacterial strains tested. Ethyl acetate exhibited the maximum zone of inhibition against *Klebsiella pneumonia* while the Methanol extract exhibited the maximum zone of inhibition against *Citrobacter sp*. All the extracts showed higher sensitivity against Gram negative bacterial strains than the Gram positive bacterial strain used in this study. Phytochemical study was performed by using standard methods. Quantitative estimation of bioactive phytoconstituents 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. In similar fashion, the results from the scavenging activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) and the Ferric reducing antioxidant power (FRAP) assay for both extracts were high and concentration dependent.
Conclusion: The study clearly indicates that *A. leiocarpus* possesses significant antioxidant activity and antibacterial activity against the tested wound infectious bacterial strains. Hence constituents of the plant could be studied in the aim to be used in chemotherapy.

Keywords: Antibacterial activity; phytoconstituents; well diffusion; antioxidant activity.

1. INTRODUCTION

The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raise serious concern of health delivery and accessibility due to untreatable bacterial infections. There is therefore the needed urgency to the search for new antimicrobial. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents [1].

Since time immemorial, plants have been used in preparation of drugs thus, act as sources of medicine. Various species of plants have been used and consumed due to the presence of high antioxidant and antibacterial phytoconstituents. The extracts of medicinal plants and natural products therefore have become a great source of antioxidant and anti-ageing properties [2]. Many human diseases are caused by oxidative stress that results from imbalance between the formation and neutralization of pro-oxidants [3].

To be able to identify the medicinal potency of any plant, the *in vitro* antibacterial activity and antioxidant activity assay must be carried out on the selected plants as the first step. The present study deals with initial phytochemical screening, evaluation of the antioxidant activity and the antibacterial activity of leaf extracts of *A. leiocarpus.*

Anogeissus leiocarpus (DC) Guill and Perr belongs to the Combretaceae family (Common name: Axle-wood tree). *A. leiocarpus* is used medically for the treatment of ascaricide, gonorrhoea, general body pain, blood clots, asthma, coughing and tuberculosis [4]. The plant is also used in Nigeria as an antimicrobial agent against bacterial infections. The leaves of the plant are used externally as a decoction in the eastern part of Nigeria for the treatment of skin diseases and the itch of psoriasis. The powdered bark is applied to wounds, sores, boils, cysts and diabetic ulcers with good results [4]. An ethnobotanical survey carried out by these researchers at Kpando municipality in the Volta Region of Ghana, revealed that many of the indigenous people use the fresh leaves of *A. leiocarpus* to heal wounds. A preliminary study was therefore designed to evaluate the antibacterial

activity of leaf extracts of *A. leiocarpus* on some common bacterial strains associated with wound infection. Antioxidants are believed to have positive correlation with wound healing. Hence the study also examined the antioxidant activity of the leaf extracts.

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of *A. leiocarpus* were collected from Kpando in the Volta Region of Ghana during the month of March- 2012. The plant material was properly identified by Mr. Agyarkwa and Mr. Otoo, the Curators at the Herbarium of School of Biological Sciences, University of Cape Coast, Ghana. A voucher specimen was deposited in University of Cape Coast Herbarium.

2.2 Preparation of Extract

The collected leaves were cleaned and dried under shade at room temperature. The dried materials were ground into fine powder using a mechanical blender. Dry leaf powder (106.9 g) was packed in Soxhlet apparatus and extracted successively with Ethyl acetate and methanol. The extracts were separately filtered through Whatmann No. 1 filter paper and the solvent was removed under reduced pressure at 35-45ºC using rotavapor. The dried extracts were weighed to calculate the yield of methanol (10.50 g, 9.5%) and ethyl acetate (6.61 g, 6.2%) and then stored at 4ºC in storage vials for experimental use.

2.3 Antibacterial Activity

2.3.1 Test microorganisms

Microbial cultures of four different strains of both Gram positive and Gram negative bacteria commonly associated with wound infections were used for the determination of antibacterial activity. Three standard bacterial strains viz. *Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC4352)* and one clinical isolate *Citrobacter sp.* From Korle-Bu Teaching Hospital Central Laboratory, Accra (Ghana).

2.3.2 Antimicrobial activity assay

Well diffusion method using Müeller-Hinton agar plates were used to demonstrate the antimicrobial properties of the crude extracts [5]. A suspension of the bacteria compared to 0.5 Macfarland standard was seeded on the Mueller-Hinton agar plates. Wells of 6 mm in diameter and 2 cm apart were punctured in the culture media using sterile cork borers. 80 µl of the crude extracts was administered to fullness in each well and the plates were incubated overnight at 37ºC. Growth was determined by measuring the diameter of the zone of inhibition. The solvents were used as the negative controls whiles 10 µg ampicillin discs (Oxoid), 30 µg cefotaxime, 30 µg chloramphenicol were used as the positive controls. The control zones of the solvents were deducted from the zones of inhibition created by the crude extracts. The experiments were carried out in triplicates and results were calculated as mean ± SD.

2.4 Scavenging Activity against 1,1-Diphenyl-2-Picryl Hydrazyl Radical (DPPH)

The crude extracts of different solvents (ethyl acetate and methanol) of the plant were screened for DPPH radical Scavenging activity. DPPH radical scavenging activity was measured according to the method of Braca et al. [6], Coutinho et al. [7] and Rajeswara et al. [8]. Extract solutions were prepared by dissolving 0.05 g of dry extract in 50 ml of methanol. An aliquot of 2ml of 0.004% DPPH solution in methanol and 1ml of plant extract in methanol at various concentrations (200, 400 and 800 ppm) were mixed and incubated at 25℃ for 30 min. and absorbance of the test mixture was read at 517 nm using a spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd.) against a DPPH control containing only 1 ml of methanol in place of the extract. The DPPH solution in methanol was prepared daily before the absorbance measurements. DPPH is a purple coloured stable free radical. When reduced it gives the yellow colored Diphenyl picryl hydrazine. All experiments were performed thrice and the results were averaged. Ascorbic acid was used as a standard [9]. Percent inhibition was calculated using the following expression:

$$\% \text{ Inhibition} = (A_{blank} – A_{sample} / A_{blank}) \times 100$$

Where A_{blank} and A_{sample} stand for absorption of the blank sample and absorption of tested extract solution respectively.

2.5 Ferric Reducing Antioxidant Power Assay

The reducing antioxidant power of both ethyl acetate and methanol extracts was determined by the method of Oyaizu [10]. Different concentrations of plant extracts (250 – 1000 ppm) in 1 ml of distilled water were mixed with phosphate buffer (3.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd). Increased absorbance of the reaction mixture indicates increase in reducing power. Ascorbic acid was used as standard.

2.6 Phytochemical Study

Phytochemical screening for major bioactive constituents like alkaloids, phenolics, flavonoids, tannins, carbohydrates and lipids were determined by using standard phytochemical methods [11,12,13].

2.7 Determination of Total Phenolics

Total phenolic content was estimated using Folin-Ciocalteu reagent based assay as previously described [14] with little modification. To one ml of each extract (100μg/ml) in methanol, 5ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4 ml (75 g/l) of Na_2CO_3 were added. The mixture was allowed to stand at 20C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1 ml aliquots of 20, 40, 60, 80, 100 μg/ml methanolic gallic acid solutions were used as standard for

calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE mg/g dry weight of extract), and the values were presented as mean ± SD of triplicate analysis.

2.8 Determination of Total Flavonoids

Total flavonoid content was determined by aluminum chloride colorimetric method [15]. This method is based on the formation of a complex flavonoid–aluminium, having the absorbance maximum at 510 nm. Extract solution (0.25 ml, 1 mg/ml) of each plant extract was added to 1.25 ml of distilled water. Sodium nitrite solution (0.075 ml, 5%) was then added to the mixture followed by incubation for 5 minutes after which 0.15 ml of 10% aluminium chloride was added. The mixture was allowed to stand for 6min at room temperature before 0.5 ml of 1 M sodium hydroxide was finally added and the mixture diluted with 0.275 ml distilled water. The absorbance of the reaction mixture was measured at 510 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoid contents were expressed as mg quercetin equivalent (QE)/g dry weight (D.W.).

2.9 Alkaloid Estimation

2.5 g of the plant powder was extracted using 100 ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25 ml. Concentrated ammonium chloride was added stepwise for precipitation. The whole solution was kept as such so that precipitate will settle. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [1].

3. RESULTS AND DISCUSSION

Preliminary phytochemical screening of the *A. leiocarpus* leaf extracts revealed the presence of different phytoconstituents as shown in Table 1. Different varieties of phytochemicals have been found to be present in both extracts 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 microbiocides, pesticides and many pharmaceutical drugs [4,16]. These metabolites help in the antimicrobial activities of the plant through different mechanisms. The methanol extract was found to contain larger amount of these metabolites than the ethyl acetate extract (Table 2). The polarity of methanol could account for this. It is also worthy to note that the result agrees vividly with literature [4]. However, our study identified anthranquinones, glycosides and carbonyls as additional phytoconstituents present in the leaf of *A. leiocarpus*.

Table 1. Preliminary phytochemical screening of *A. leiocarpus* leaf extract

Phytoconstituent	Test performed	Result	
		Methanol	Ethyl acetate
Alkaloids	Dragendorf, Mayer, Wagner Test	+	+
Flavonoids	Alkaline Reagent and Shinoda Test	+	+
Steroid	Liebermann Burchard And Salkowski Test	+	+
Triterpenoid	Liebermann Burchard and Salkowski Test	+	-
Tannins	Ferric Chloride Test	+	+
Saponins	Foam Test	+	+
Glycosides	Killer-Killiani Test	+	_
Anthraquinones	Brontrager's Test	+	_
Carbonyls	2,4-Dinitrophenyl Hydrazine Test	+	_

** (+): Indicates the presence of chemical constituents. (-): Indicates the absence of chemical constituents*

Table 2. Quantitative estimation of phytoconstituents present in methanol and ethyl acetate extracts of *A. leiocarpus*

Phytoconstituents	Quantity (mg/g)	
	Methanol	Ethyl acetate
Alkaloids	152.0 ± 0.1	80.2 ± 0.0
Phenolics	1294.8 ± 3.0	616.5 ± 4.4
Flavonoids	330.7 ± 3.0	202.5 ± 4.0

The zones of inhibition produced by the test organisms indicated their susceptibility to the plant extracts; it was observed that the zones of inhibition varied slightly from one organism to another and from one solvent extract to the other. Ethyl acetate exhibited the maximum zone of inhibition against *Klebsiella pneumonia* while the methanol extract exhibited the maximum zone of inhibition against *Citrobacter sp.* (Table 3). All the extracts showed higher sensitivity against Gram negative bacterial strains than the Gram positive bacterial strain which is usually not the case, for gram negative bacteria are generally resistant. The methanol extract significantly inhibited the growth of *Citrobacter sp.* more than the rest of the organisms while ethyl acetate significantly inhibited the growth of *Klebesiella pneumonia* and *Citrobacter sp.* compared to the rest. However, Ethyl acetate presented a better antibacterial activity than the methanol extract even though methanol extract exhibited a larger proportion of the essential phytoconstituents. Comparing the results obtained with that cited in literature [4], *A. leiocarpus* leaf extract from Ghana seemed to be more potent in antibacterial activity than the leaf extract from Nigeria. Meanwhile, the plant extracts were of lower activity than the respective standard antibiotics used.

Table 3. Antibacterial activities of the various plant extracts

Organism	Zone of inhibition (mm) of plants extracts (1.0 mg/ml)		Zone of inhibition (mm) of standard antibiotics		
	Methanol	Ethyl acetate	10µg AM	30µg CE	30µg CH
Staphylococcus aureus	8.79 ± 0.0^{A}	8.79 ± 0.1^{C}	30.00±0.1		
Klebsiella pneumonia	8.79 ± 0.2^{A}	11.27 ± 0.2^{D}			
Citrobacter sp.	10.9 ± 0.2^{B}	9.65 ± 0.1^{E}	12.00±0.1	29.00 ±0.1	
Escherichia coli	9.03 ± 0.2^{A}	8.79 ± 0.2^{C}			19 ± 0.0

**Values are expressed as the mean ± standard deviation (n = 3). Means with different superscript letters within a column are significantly different (p < 0.05).*

**Inhibition zones 15 mm was declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities. AM is Ampicillin, CE is Cefotaxime and CH stands for Chloramphenicol.*

Many chemical reactions and metabolic processes can induce the production of reactive oxygen species (ROS) which includes free radicals in the cells. Free radicals can initiate the oxidation of bio molecules, such as protein, lipid, amino acids and DNA which will lead to cell injury and can induce numerous diseases including cardiovascular disease, ageing, cancer, atherosclerosis and a variety of other disorders [17]. Phenolics have been known to possess a capacity to scavenge free radicals to act as antioxidants. Studies have shown that phenolics play an important preventive role in the development of cancer, heart diseases and ageing related diseases [18,19]. Total phenolic content of the leaf extracts were found to be 1294.81± 3.0 mg/g and 616.5± 4.4 mg/g for methanol and ethyl acetate respectively. Flavonoid content of 330.7 ± 2.5 mg/g and 202.5± 3.6 mg/g were similarly recorded for methanol and ethyl acetate respectively (Table 2). The high contents of phenolic compounds indicated that these compounds contribute to the antioxidant activity. The results from the scavenging activity against DPPH and the FRAP assay supported this view (Table 4 and Fig. 1). The percentage inhibitions of all extracts were high and concentration dependent. Similarly, the ferric reducing antioxidant power increases with increasing concentration indicating strong antioxidant properties of the extracts. *A. leiocarpus* can therefore be regarded as promising natural plant sources of antioxidants with high value.

Table 4. DPPH free radical scavenging ability of methanol and ethyl acetate extracts of *A. leiocarpus*

Drug	Percentage inhibition (*I* %)		
	200 ppm	400 ppm	800 ppm
Ascorbic acid	92.75 ± 0.2	93.61± 1.4	96.78 ± 2.0
Methanol extract	88.85± 0.1	93.56 ± 0.1	94.19 ± 0.1
Ethyl acetate	91.96 ± 2.0	91.89± 0.7	92.43 ± 0.7

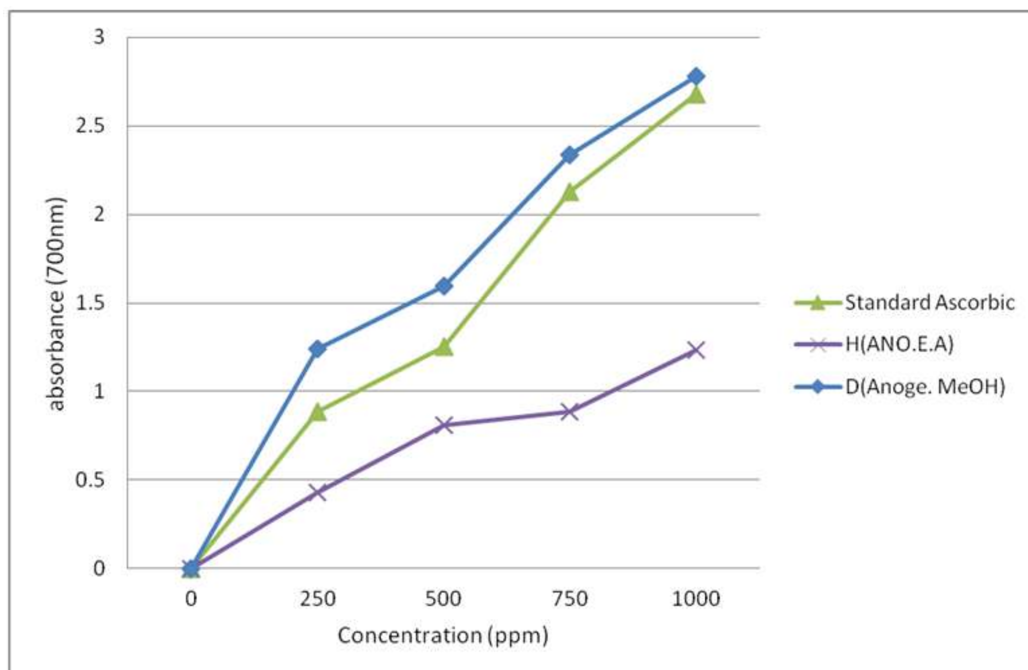

Fig. 1. Ferric reducing power of methanol and ethyl acetate extracts of *A. leiocarpus* compared with ascorbic acid

4. CONCLUSION

The study clearly indicates that *a. leiocarpus* possesses significant antioxidant activity and antibacterial activity against diverse bacterial strains. The leaves of *A. leiocarpus* therefore provide possible alternative and easily affordable sources of antimicrobial agents for the treatment of many diseases associated with the test organisms. Hence constituents of the plant could be studied in the aim to be used in chemotherapy.

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COMPETING INTERESTS

No competing interest.

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