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RESEARCH ARTICLE

ANTIMICROBIAL AND THE TOTAL ANTIOXIDANT ACTIVITIES OF THE METHANOLIC EXTRACT OF ALLANBLACKIA FLORIBUNDA FRUIT

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ABSTRACT

The role of natural products, especially medicinal plants in the treatment of various ailments cannot be overemphasized, several plants have being investigated for their medicinal values. Different parts of *Allanblackia floribunda* tree is being used locally for the treatment of diseases, in this study, the antimicrobial activities of the methanol extracts of the seed, pulp and peel of *Allanblackia floribunda* fruit were tested against Ten (10) strains of bacteria including six (6) Gram-negative isolates; *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella* sp. and *Pseudomonas aeruginosa*, and four (4) strains of Gram-positive; *Staphylococcus aureus*, *Bacilluscereus*, *Micrococcus luteus* and *Streptococcus pneumonia.* Different concentrations of the methanol extracts of each part of the fruit exhibited different antibacterial actions in a dose dependent manner against most of the tested organism.In order to investigate the phenolic content of the pulp, peel and seed of *Allanblackia floribunda*, the total polyphenol content of the methanol extracts of each part was determined. The total polyphenol content of the methanol extract of the peel (251.3 mg/g) was significantly ($p<0.05$) higher than the pulp (78.92mg/g) and both are significantly higher than the methanol extract of the seed (5.56mg/g). The polyphenol content of *Allanblackia floribunda* fruits reduces as we go from the peel to pulp and the seed. This results correlated well with the ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity and oxygen radical absorbance capacity (ORAC) assay carried out on the three parts. This indicate that the antioxidant capacity is related to an additive effect of various polyphenols in the extract.

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INTRODUCTION

The use of plants for therapeutic purpose has long been in existences due to the cultural and traditional acceptability in the treatment of varying degree of ailment in different localities (Mulliken, 2000). It is believed that the remedy for any ailment is residue in one plant or in the combination of plants. These has arose the interest of researchers to the phytochemical constituents present in each plant so as to establish their biological specificity demonstrated them in relation to their chemical constituents. Plants are the major source of complex and highly structurally diverse chemical compounds known as phytochemicals (Schippmann *et al*., 2002). Many of the secondary metabolites may not be of great importance to the plant, however, regardless of their utility to the parent organism, their value to man as drugs, herbs, flavourings, poisons, dye and so on is undisputed, which had led to the global acceptability of natural products in the treatment of diseases (Borris, 1996).

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The use of herbs to treat disease is almost very common among non-industrialized societies, as such; many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies. The study done on medicinal plants and vegetables strongly supports the idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems (Cao, 1996). The pharmaceutical industry has remained interested in the traditional uses of medicinal plants in its drug discovery efforts, out of the 1073 small-molecule drugs that were approved in the period 1981 to 2010; over half were either directly derived from or inspired by natural substances (Smith-Hall *et al*., 2012). In addition, the investigation of antimicrobial and antifungal agents of divers plants have been carried out (Cowan, 1999; Osato *et al*., 1993), but no work have been reported on the antimicrobial investigation of *Allanblackia* fruit. *Allanblackia floribunda* (*Clusiaceae*) Oliv, is commonly known as tallow tree which is been produced by seeds (Orwa *et al*., 2009). They are limited in occurrence to tropical Africa, but it is centred mostly in the lowland rainforest (Ajibesin *et al*., 2008). The extracts from different parts of the tree is use for diverse medicinal purposes which includes the use as treatment for cough (Betti, 2004), the

stem bark is used for the treatment of toothache, diarrhoea and as pain reliever (Abiww, 1990), while all parts of the plants are used traditionally in the treatment of small pox, chicken pox and measles indicating possible antiviral activity (Ayoola *et al*., 2009). In Gabon, the bark of *Allanblackia floribunda* is pounded and rubbed on the body to relief painful conditions (Orwa *et al*., 2009; Betti, 2004). The anti-inflammatory and the hypoglycaemic properties of the plants was confirmed in a study carried out by (Betti, 2004; Ayoola *et al*., 2009). According to scifinder and the dictionary of natural products database, there exist scanty information on the biological characterization of *Allanblackia floribunda* fruit but other member of the Clusiaceae family potentially demonstrated antimicrobial activities. Extracts from *Gaciniasmeathmannii, G.lucidaVesque, Kulmeyeravariabilis, Colophyllumbrasiliense* (*Clusiaceae*) showed good inhibitory when tested for their antimicrobial activities against some gram positive and gram negative bacteria (Kuete *et al*., 2007; Itebert *et al*., 2011; Coqueiro *et al*., 2013; Pretto *et al*., 2004). The leaves, stem bark and root of *Allanblackia floribunda* gave good inhibitory effects against gram positive and gram negative bacterial except *A.flavus* (Ajibesin *et al*., 2008).

The antitumor, radical scavenging, antibacterial and antifungal activities of the root bark extract of *Allanblackia floribunda* was investigated and the extract showed a moderate tumor reducing activity while the DPPH radical scavenging test showed that it is able to scavenge more than 50% of the free radicals (Kuete *et al*., 2011). Antioxidant and immunomodulatory properties of polysaccharides from *Allanblackia floribunda* stem bark and the result obtained from this study support the ethno medicinal use of the plant for this purpose (Boudjeko *et al*., 2015). This study is focused at evaluating the seed, peel and pulp of *Allanblackia floribunda* for the antimicrobial activities against Ten (10) strains of bacteria including six (6) Gram-negative isolates; *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella* sp. and *Pseudomonas aeruginosa*, and four (4) strains of Gram-positive; *Staphylococcus aureus*, *Bacilluscereus*, *Micrococcus luteus* and *Streptococcus pneumonia*, in order to validate the ethno-botanical use of the plant for microbial diseases. The total polyphenol, flavonoid/proanthocyanidin, flavonol content was investigated, so also the antioxidant activity using the oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and the trolox equivalent antioxidant capacity (TEAC) assay.

MATERIALS AND METHODS

Sample Collection and Preparation: The matured fruit of *Allanblackia Floribunda* was collected from a forest at IgbaraOdo Ekiti in Ekiti State Nigeria in large quantity and was identified in the herbarium unit of the Department of Plant Science, Ekiti State University. The fruits were collected during the raining season. The seed, peel and pulp of the fruit were properly separated and air dried at ambient temperature. The dried samples were milled into powder form for easy extraction.

Extraction: 50 g of the seed, pulp and peel was macerated with methanol separately. The methanol extracts obtained were concentrated to dryness using a vacuum extractor at 40 ºC. The crude extracts of the three parts were then subjected to further analysis.

Sterilization of materials used and disinfection of working area: All glass ware were washed with detergent and rinsed thoroughly with distilled water, air dried and sterilized by dry heat in a hot air oven at a temperature of 160 ^{o}C for 1 hour. Pipettes were plugged with absorbent cotton wool and wrapped with aluminum foil paper and sterilized to prevent external contamination. All media were sterilized in the autoclave at a temperature of 121 ^{o}C for 15 minutes. All non-reusable materials (such as needle and syringe) were appropriately disposed in the bin after sterilization by moist heat. Inoculating loop was sterilized in Bunsen burners' flame until red hot and allowed to cool, before and after every use. The cork borer was also dipped into absolute alcohol before flamed and allowed to cool. With the use of cotton wool soaked in 70 % alcohol and disinfectant, the work bench surface was properly swabbed before and after daily work to prevent all forms of contamination.

Preparation of media: All media used in this research work were prepared and sterilized according to the manufacturers' instructions.

Nutrient agar: 28 g of dehydrated powder of nutrient agar (Biotech, India) was dissolved in one litre (1000 mL) of distilled water in a conical flask, mixed together and corked properly. It was then homogenized over hot plate and later sterilized using the autoclave at a temperature of 121 ^{o}C for 15 minutes. 5 ml of homogenized nutrient agar was aseptically distributed into Bijou bottles and sterilized by autoclaving after which were left to solidify in slanted form. These were used to maintain the stock culture of isolates used in the experiment.

Nutrient broth: 13 g of bacteriological peptone water powder was dissolved in one litre (1000 mL) of distilled water and thoroughly mixed. It was sterilized by autoclaving at 121 ^{o}C for 15 minutes.

Mueller-Hinton Agar: 38 g of the dehydrated powder of Mueller-Hinton agar (Biotech, India) was dissolved in one litre (1000 mL) of distilled water and mixed thoroughly. It was warmed to aid adequate distribution (homogenization) and sterilized by autoclaving at holding pressure and temperature of 121 ^{o}C for 15 minutes.

Antibacterial screening: The potency of the plant extract as inhibitory agent to microbial growth was determined using modified method of agar well diffusion described by (James, 2009).

Test bacteria: Ten (10) strains of bacteria including six (6) Gram-negative isolates; *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella* sp. and *Pseudomonas aeruginosa*, and four (4) strains of Gram-positive; *Staphylococcus aureus*, *Bacilluscereus*, *Micrococcus luteus* and *Streptococcus pneumonia*,were subjected to susceptibility test in the course of this research. All these organisms were obtained from the culture bank of the Microbiology laboratory of the Department of Microbiology, Ekiti State University, Ado-Ekiti.

Reconstitution of crude extract: The crude extract was reconstituted with Dimethyl sulfoxide (DMSO) in order to make a stock solution using volumetric flasks, after which were sterilized by filtration using 0.45µm aqua membrane nylon filter disk (Becton, Dickinson Company).

Reconstituted and filtered extracts serving as stock solutions were stored in the freezer of a regular refrigerator (Haier Thermocool, Deluxe series HRF-350N) set at 4°C (Adeniran, 2013). Working solutions were made in different concentrations (w/v) from the stock solution.

Agar diffusion method for antibacterial assay: Twenty millilitre (20 mL) of sterile Mueller-Hinton agar (MHA) were poured and allowed to set before 100 µL of bacterial suspension (with standard density) for each bacterial strain were spread on to the surfaces, using sterile swab. With the aid of a 6 mm diameter cork borer, five (5) cups were bored separately at equidistance on the agar. The borer was sure sterilized before and after every use. Each cup was respectively filled with 0.2 ml of the extract at different concentrations. The plates were kept on working bench at room temperature for 1 hour (to allow extract diffusion) before taken for incubation at 37°C for 24 hours. Zone of inhibition were measured to determine the effectiveness of the extract against each organism and results were expressed in millimetres (Moses *et al*., 2013).

The Polyphenol and Flavonoid Content of the Extracts: The total polyphenol content of the methanol extract of the peel, pulp and seed of *Allanblackia floribunda* fruit was determined (Singleton *et al*., 1999). A volume of 25µl of the extracts was incubated in a clear 96-well flat bottom plate for 5min with 125µl freshly prepared 0.2N FolinCiocalteu's phenol reagent where after 100µl 7.5% sodium carbonate was added and incubated for 2h. The absorbance at 765nm was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and total polyphenols calculated using the standard gallic acid in 10% ethanol. Results were expressed as mg gallic acid equivalents per gram extract. The flavonoid content: flavanol and flavonolof the methanol extracts was determined colourimetrically at 640nm and spectrophotometrically at 360nm. For the flavanol assay, a freshly prepared 0.05% DMACA solution was made by dissolving DMACA solution in 8% HCl prepared in methanol. 50µl of the extracts were incubated clear in a 96-well flat bottom plate with 250µl DMACA solution for 30min. for the flavonol assay, a solution of 0.1% HCl prepared in 95% ethanol was made. The extracts (12.5µl) were incubated in a clear 96-well flat bottom plate with 12.5µl 0.1% of HCl-ethanol solution and 225µl 2% HCl for 30min. The absorbance was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and flavanols and flavonols were calculated using standard catechin in methanol and 95% ethanol respectively. The results were expressed as mg standard equivalents per gram of the extract.

The Total Antioxidant Capacity of the Extracts: The oxygen radical absorbance capacity (ORAC) was determined according to the fluorometric method described by (Ou *et al*., 2001). A 36ng/ml fluorescein and a 2.5% AAPH radical solution were prepared freshly in 75mM phosphate buffer (pH 7.4). The reaction was initiated by incubating 12µl of the extract in a black 96-well flat bottom plate with 138µl fluoresce in solution and 50µl AAPH solution. The decrease in fluorescence was then measured every 5min for 2h on a plate fluorometer (Fluoroskan, Thermo Electron Corporation). The ORAC values were determined by comparing the sample curve to the standard curve obtain for trolox. Results were expressed as µmol trolox equivalents per gram extract. The ferric reducing antioxidant power (FRAP) and the trolox equivalent antioxidant capacity (TEAC) was determined according to the spectrophotometric methods described by (Benzie, 1996) and (Pellegrini *et al*., 1999) respectively. For the FRAP assay, the extracts were incubated in a clear 96-well flat bottom plate in 300µl FRAP solution (250mM sodium acetate in acetic acid, pH 3.6; 0.83mM hydrochloric acid; 1.67mM iron (III) chloride hexahydrate) at 37ºC for 30Min. the reaction was measured at 593nm on a plate spectrometer (Multiskan, Thermo Electron Corporation) and the FRAP value determined by comparing to the standard ascorbic acid equivalents per gram extract.

Statistical Analysis: All data from assays of antioxidant parameters (FRAP, ORAC and TEAC) were analysed using their respective templates. Results were expressed as mean ± standard deviation of triplicate values per sample.

RESULTS AND DISCUSSION

Antimicrobial assay of the seed, pulp and peel: The antimicrobial activities of the methanolic extracts of the seed, pulp and peel of Allanblackia floribunda fruit was tested against Ten (10) strains of bacteria including six (6) Gram-negative isolates; *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella* sp. and *Pseudomonas aeruginosa*, and four (4) strains of Gram-positive; *Staphylococcus aureus*, *Bacilluscereus*, *Micrococcus luteus* and *Streptococcus pneumonia.* Different concentrations of the methanol extracts of each part of the fruit exhibited different antibacterial actions in a dose dependent manner against most of the tested organism. The inhibition zones as shown in Table 1 ranged from 25-10mm with most of the inhibition zone above 15mm. at 1000µg/mL the zone of inhibition for all the tested organism was above 15mm while at lesser concentration (250-62.5 µg/mL) the inhibition zone was low (with most zones below 15mm) and there was no inhibition against *Staphylococcus aureus*, *Streptococcus pneumonia* and *Salmonella typhi* at low concentration.

The methanol extract of the peel had the highest activity against all tested organisms at 1000 µg/mL, the seed had moderate activity, the inhibition zones ranged from 19-10mm with most zones below 15mm. It was also discovered that the seed extract was only active against *Shigella*sp at 500 µg/ml. Compared to the pulp and seed extracts, methanol extract of the peel exhibited low inhibitory effect against some of the tested organism but show showed no inhibitory effect against *Klebsiella pneumonia, Escherichia coli* and against *Bacilluscereus*, *Micrococcus luteus*&*Salmonella typhi* only at low concentration. From the result obtained in this work, the methanol extract of the pulp of *Allanblackia floribunda* fruit demonstrated better inhibitory efficiency against most of the test organisms.

Total polyphenol and flavonoid content of the seed, pulp, and peel extracts: Polyphenols shows numerous biological activities in human such as antimicrobial, anti-inflammatory and immunomodulatory activities. It also functions in plants as productive UV filters, plant development and insect's interaction. In order to investigate the phenolic content of the pulp, peel and seed of *Allanblackia floribunda*, the total polyphenol content of the methanol extracts of each part was determined. The total polyphenol content of the methanol extract of the peel (251.3 mg/g) was significantly ($p<0.05$) higher than the pulp (78.92mg/g) and both are significantly

		Zone of Inhibition (mm) by Concentrationof Extracts (µg/ml)									
Test Organism		*Staphylococcus aureus*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*	*Escherichia coli*	*Streptococcus pneumonia*	*Shigella sp*	*Bacillus cereus*	*Micrococcus luteus*	*Salmonella typhi*
Seed	**1000**	12	14	13	0	19	14	0	13	15	11
	500	11	13	11	13	14	14	11	12	14	10
	250	10	12	10	11	12	12	0	12	11	9
	125	9	11	0	10	0	0	0	10	10	0
	62.5	0	10	0	0	0	0	0	0	10	0
Pulp	**1000**	16	17	16	25	18	19	20	20	22	16
	500	14	13	12	25	16	11	20	20	13	10
	250	0	10	11	23	15	0	15	15	12	0
	125	0	0	11	21	12	0	10	10	10	0
	62.5	0	0	0	19	0	0	10	10	0	0
Peel	**1000**	15	15	19	0	0	24	0	0	0	0
	500	14	14	15	0	0	17	0	17	0	0
	250	12	14	13	0	0	15	12	13	16	20
	125	11	12	13	0	0	12	11	13	16	19
	62.5	0	12	10	0	0	0	0	12	14	12

Table 1. Antibacterial activities of the methanol extract of the seed, pulp and peel of Allanblackia floribunda fruit

Table 2. Total polyphenol and flavonoid content of the seed, pulp, and peel extracts.

Sample	Total Polyphenol (mgGAE/g)	Flavanol (mgCE/g)	Flavonol (mgQE/g)
Seed extract	5.56±1.79	ND	ND
Peel	251.3±1.01	158.37±12.70	ND
Pulp	78.92±3.62	200.83±2.54	ND

Note: r^2 shows direct correlation between the phenolics and FRAP assay, because the phenolics are directly responsible for the FRAP. Values in columns are means ± standard deviation of three determination (n=3). Abbreviations: GAE- Gallic acid equivalent, CE- Catechin equivalent.

Table 3. Total antioxidant capacity of the peel, pulp and seed of *Allanblackia floribunda* fruit

	Total antioxidant capacity of the peel, pulp and seed of *Allanblackia floribunda* fruit		
	Automated Oxygen Radical Capacity		
Sample	ORAC	FRAP µmol Vit C/gSample	TEAC µmol TE /gSample
Seed	13.33±7.21	130.58±9.88	49.80±1.55
Peel	1891±1.03	72041.35±2.04	15250±7.50
Pulp	704±4.62	2117.61±1.28	2087.7±0.18

higher than the methanol extract of the seed (5.56mg/g) as shown in Table 2. The polyphenol content of *Allanblackia floribunda* fruits reduces as we go from the peel to the seed. That is, peel>pulp>seed. This results correlated well with the FRAP, TEAC and ORAC assay, indicating that the antioxidant capacity is related to an additive effect of various polyphenols in the extract.

The flavanol and flavonol content of the extracts were analyzed to determine if other groups of the polyphenols from the flavonoid subgroup were present. It was discovered that the pulp had higher flavonol content followed by the peel, but was not determined in the seed. The flavanol content of the pulp is 200.83mg/g and the peel (158.37mg/g). Flavonol was not present in the three extracts, this implies that flavonol/proanthocyanidin contents are present in the pulp and peel which is responsible for their high activity. Though, flavonol which had been previously isolated from other parts of *Allanblackia floribunda* tree (leaves, root bark, stem bark and root) was not detected in all parts of *Allanblackia floribunda* fruit.

Total antioxidant capacity: Three different assays were used to determine the antioxidant capacity of the extracts. The results from the FRAP and TEAC correlated well with each other (r = 0.984 and 0.999). There was correlation between the FRAP assay and total phenolic (r=0.999) which indicates that the phenolics are likely responsible for the antioxidant activity of the extracts. In all the three assays, the seed showed a very low activity and that of the peel was significantly higher than the seed ($P<0.05$) while the pulp showed the highest activity as shown in Table 3.

Conclusion

The overall results of this study provide information on the antimicrobial and the antioxidant activity of the peel, pulp and seed of *Allanblackia floribunda* fruit. Based on the results in this study, the three parts showed very good activity against all tested organism but the pulp showed the best activity. The peel has high antioxidant activity compared to other parts of the fruits. This study also confirmed the ethnomedicinal use of these parts. However, further toxicological studies need to be done to confirm the results above.

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