



Extraction and Fractionation of Whole Black Seed Plantain (*Plantago rugelii* Decne) for *in-vitro* Antioxidant, Antibacterial and Phytochemical Screening

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ABSTRACT: This study was designed to explore new antioxidant and antimicrobial agents from the methanol whole plant crude extract and fractions of *Plantago rugelii*. The methanol extract and its fractions were prepared and screened for its phytochemical composition, *in-vitro* antioxidant potential and challenged with common pathogenic microorganisms for its antimicrobial activities using standard procedures. The phytochemical analysis revealed the presence of various pharmaceutically active secondary metabolites like alkaloids, phenolic, flavonoids, carbohydrates, glycosides, sterols, etc. In the DPPH assay, the aqueous methanol fraction was found to be the most effective among all the fractions in comparison to the ascorbic acid standard. Using the zone of inhibition as inhibitory parameter, the crude methanol extract exhibited the best antibacterial activity when challenged against all the clinical isolates except *Klebsiella pneumoniae*. The aqueous methanol extract exhibited the best percentage fungal inhibition when compared to other fractions. The standard drugs ciprofloxacin and fluconazole exhibited a near 100% activity except with *Proteus vulgaris* where the crude methanol extract has a higher value. This research holds promise for the exploration of various potentially active secondary metabolites which would help in developing pharmaceuticals, especially antioxidant and antimicrobial drugs. The isolation and characterization of the exact metabolites responsible for these activities is therefore recommended.

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There is accumulated evidence from researches to demonstrate the promising potentials of medicinal plants as presently used in various traditional, complementary and alternate systems of treatment of human diseases (Dahanukar *et al.*, 2000; Cowan, 1999). Medicinal plants are useful for healing and as well for curing human diseases because of the presence of phytochemical constituents (Fabricant and Farnsworth, 2001). Phytochemicals are naturally occurring compounds in medicinal plants which offer defense mechanism and protection from various diseases (Motaleb, 2011). Coupled with the significant increase in the incidence of side effects and the resistance that pathogenic microorganisms build against several antibiotics, research on medicinal plants has attracted a lot of attentions globally (Finch, 1998; Iwu *et al.*, 1999; Kunin, 1993). Most of the medicinal plants holding active secondary metabolites with high antioxidant property which are playing important role in the prevention of various diseases (Lobo *et al.*, 2010). Natural antioxidants from plant sources are potent and safe due to their harmless nature hence the upsurge in recent scientific search for

potential antioxidant with reduced side effects and economic viability (Auudy *et al.*, 2003).

Species of the genus *Plantago* (commonly known as plantain) have been used extensively for medicinal purposes (Blumenthal *et al.*, 2000). *Plantago rugelii* (Plantaginaceae) is found all over the world including Asia, Australia, New Zealand, Africa and Europe. The leaves of *P. rugelii* have been utilized as topical for wounds, bites, stings, bronchial infection, hepatitis, and jaundice among others. Its seeds have been established to be used in treating urinary infections (David, 2006). Hence the attempt is been made in this study to examine the various phytochemical constituents, antimicrobial and antioxidant efficacy of the various solvent extracts of *P. rugelii*.

MATERIALS AND METHODS

Plant materials collection, preparation and identification: Fresh *P. rugelii* whole plants comprising the root, stem and leaves were collected in June, 2012 from a forest in Owerre Olubor in Ika North East Local Government of Delta State Nigeria. The whole plants were washed with water to remove

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earthy impurities, identified and authenticated at the forest research institute of Nigeria (FRIN), Ibadan where a herbarium specimen was deposited and a voucher number 109775 issued. They were then air dried for three weeks, powdered with the aid of a clean mechanical grinder and stored in an air tight glass container until use

Chemicals: Methanol (Merck India), 1,1-Diphenyl-2-picrylhydrazyl (Merck India), acetone (Merck India), chloroform (Sigma Aldrich), ethyl acetate (Sigma Aldrich), petroleum ether (Sigma Aldrich) and other chemicals and reagents were all of analytical grade.

Extraction and Fractionation: The powdered whole plant comprising the root, stem and leaves was macerated with analytical grade methanol for 72 hours. The methanol was removed and residue immersed further in methanol for a further five days. Thereafter, the methanol was decanted and filtered with Whatman filter paper. The filtrate was concentrated under reduced pressure at 45°C in rotator evaporator (Stuart RE 300) and dried at room temperature to constant weight. This was crude methanol whole plant extract (CME). The CME was subjected to solvent-solvent extraction for four fractions, namely, pet ether, chloroform, ethyl acetate and aqueous fractions to obtain the PEF, CHF, EAF and AMF respectively. All the fractions were concentrated in the rotary evaporator at 45°C and stored at 4°C till use.

Phytochemical analysis: Simple chemical tests to detect the presence of carbohydrates, proteins and secondary metabolites in the CME, PEF, CF, EAF and AMF fractions were done in accordance with standard methods (Stahl, 1973; Sofowora, 1982; Trease and Evans, 1978).

Antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH): The DPPH assay has been largely used as a quick, reliable and reproducible parameters to search for the *in-vitro* antioxidant parameter of pure compounds as well as plant extracts. The scavenging effect of crude methanol extract and fractions of *P. rugelii* whole plant on DPPH radical was estimated with method described by Jain *et al.*, 2008. A solution of 0.1 mM DPPH in methanol was prepared, and 1.0 mL of this solution was mixed with 3.0 mL of extract in methanol containing 0.01 - 0.2 mg/mL of the extract. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference standard. The

ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH RSA}(\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

Where RSA = radical scavenging activity; A_0 was the absorbance of DPPH radical + methanol, A_1 was the absorbance of DPPH radical + sample extract /standard. Concentrations of 10, 25, 50, 100 and 200ug/ml of the crude extracts and its different fractions are used for the study (Jain *et al.*, 2008). The values are presented as the means of triplicate analysis.

Collection of the Microorganisms: The hospital strains were collected from different specimens (vaginal, urine, scars, etc.) of patients referred to the Medical Microbiology Department, University of Benin Teaching Hospital, Benin City Edo State Nigeria. These bacterial isolates were *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* while the fungal isolates are *Aspergillus niger* and *Candida albicans*

Confirmation of test organisms: Biochemical analysis methods as described by MacFaddin (MacFaddin, 2000), Fobres and Sham 2002 and Leboffe and Pierce, 2002 were carried out on each of the test bacteria isolates for confirmation. The Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986) was used for species authentication. The fungi isolates of *Aspergillus niger* and *Candida albicans* was identified via macroscopic and microscopic observations as well as germ tube test and chlamydo-spore production on cornmeal agar fortified with Tween 80 polysorbate.

Preparation of organisms: Preparation of standard bacterial suspensions: The average number of viable, bacterial organisms per ml of the stock suspensions was determined by means of the surface viable counting technique (Miles and Misra, 1938). About 10^8 - 10^9 colony forming units per ml were used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained (Sajid *et al.*, 2013). These bacterial suspensions were challenged with the dilutions of 25mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, and 125 mg/mL of the CME, PEF, CHF, EAF and AMF fractions respectively. Ciprofloxacin at 20mcg/mL was used as the positive control.

Preparation of standard fungal suspensions: The fungal cultures *Aspergillus niger* and *Candida albicans* were maintained on Sabouraud Dextrose Agar,

incubated at 25°C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100 ml) of sterile normal saline and the suspension was maintained for further use (Sajid *et al.*, 2013). These fungal suspensions were with the dilutions of 25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, and 125 mg/mL of the CME, PEF, CHF, EAF and AMF fractions respectively. Fluconazole at 100mcg/mL was used as the positive control.

Antimicrobial activity: Testing for antibacterial activity: The agar well diffusion method was used (Kavanagh, 1972) to assess the antibacterial activity of the prepared extracts. 0.6 ml of standardized bacterial stock suspensions of 10^8 - 10^9 colony forming units per ml was thoroughly mixed with 60 ml of sterile nutrient agar. 20 ml of the inoculated nutrient agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates, 4 cups, 10mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed. Alternate cups were filled with 0.1ml of each extracts prepared in dimethyl sulfoxide (DMSO) using micropipette and allowed to diffuse at room temperature for two hours.

The plates were then incubated in the upright position at 37°C for 18 hours. Three replicates were carried out for each extract against each of the test organism. The antibacterial activity of samples was compared with the standard drug ciprofloxacin (20 µg/mL) in DMSO. The antibacterial activity of samples compared with the standard drug ciprofloxacin was measured using the zone of inhibition as the inhibitory parameter (Sajid *et al.*, 2013).

Testing for anti-fungal activity: The same method as for bacteria was followed. Instead of nutrient agar media, yeast and mould extract agar was used. The inoculated medium was incubated at 25°C for two days for *Candida albicans* and three days for *Aspergillus niger*. This was carried out in triplicate for each fungus. Antibiotic susceptibility was carried out on DMSO and the test drug (fluconazole) to serve as negative and positive control respectively (Sajid *et al.*, 2013). The percent growth inhibition was calculated with reference to the negative control by applying the formula:

$$\% \text{ FI} = \frac{\text{LGn} - \text{LGs}}{\text{LGn}} \times 100 \quad - \quad - \quad (2)$$

Where FI = Fungal Inhibition, LGn and LGs are the linear growth of the negative control and sample respectively (Hameed, 2014).

Statistical Analysis: Obtained data were analyzed using Statistical Package for Social Sciences (SPSS 17.0, Chicago, IL) at 0.05 level of significance. Results were presented as mean and standard deviation (Mean \pm SD) for triplicate determination. The statistical significance between the control and each of the treated groups was determined by the Fischer's least significant difference post hoc test after one-way ANOVA. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Percentage yield of the crude methanol extract and fractions: The percentage yield of the methanol plant extract was 4.73%, while the percentage yield of the petroleum ether, chloroform, ethyl acetate and aqueous methanol fractions with respect to the crude methanol whole plant extract were 25.51%, 2.83%, 1.34% and 69.68% respectively.

Phytochemical Screening: The preliminary phytochemical screening of the fractions of *P. rugelii* showed the presence of important secondary metabolites which is presented in Table 1. The presence of these phytochemicals is responsible for the diverse traditional application of the plant. This finding is in agreement with the findings of Ekharo and Eboka, 2011 who reported the presence of these phytochemicals in the crude methanol whole plant of *P. rugelii*. The DPPH assay method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form, DPPH-H in the reaction (Singleton and Orthofer, 1999). DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants because it is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The crude methanol whole plant extract and its fractions showed a remarkable and concentration dependent increase in the radical scavenging. The free radical scavenging activity of the methanol whole plant extract and its fractions is in the order: AMF > EAF > CHF > PEF > CME indicating that the aqueous methanol fraction has the highest scavenging effect. Antioxidant activity of the plant is related to the presence of phenolic compounds such as flavonoids, phenolic acids and tannins. Polyphenols play an important role in the stabilization of lipid oxidation and may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis (Gursoy *et al.*, 2009). Flavonoids are widely distributed group of plant phenolic compounds responsible for the antioxidant activity of the plants.

Table 1. Preliminary phytochemical screening of the fractions of *P. rugelii*

Phytochemicals	Tests	Fractions of <i>P. rugelii</i>			
		PEF	CHF	EAF	AMF
Alkaloids	Mayer's	+	+	+	+
	Dragendroff's	+	+	+	+
	Wagner's	+	+	+	+
	Hagers	+	+	+	+
Carbohydrate/Reducing sugars	Molisch's	-	-	+	+
	Fehling's	-	-	+	+
	Benedict's	-	-	+	+
Cardiac glycoside	Keller	-	-	+	+
	Kilian's	-	-	+	+
Saponins	Frothing	+	+	-	-
Tannins	Ferric chloride	-	-	-	-
	Gelatin	-	-	-	-
Phytosterols	Salkowski	+	-	-	-
	Liebermann-Burchard	+	-	-	-
Triterpenes	Salkowski	+	-	-	-
	Liebermann-Burchard	+	-	-	-
Proteins	Biuret	-	-	-	-
	Ninhydrin	-	-	-	-
Phenolic compounds	Ferric chloride	+	+	+	+
	FolinCiocalteu's	+	+	+	+
Flavonoids	Shinado Test	+	-	+	+
	Ferric chloride	+	-	+	+
	Lead acetate	+	-	+	+
Anthraquinones	Borntrager's	-	-	-	-
	Ferric chloride	-	-	-	-

Key: (+) = presence of phytochemical; (-) = absence of phytochemical

DPPH radical scavenging assay: The radical scavenging property of the crude extract and fractions of *P. rugelii* is shown in Figure 1

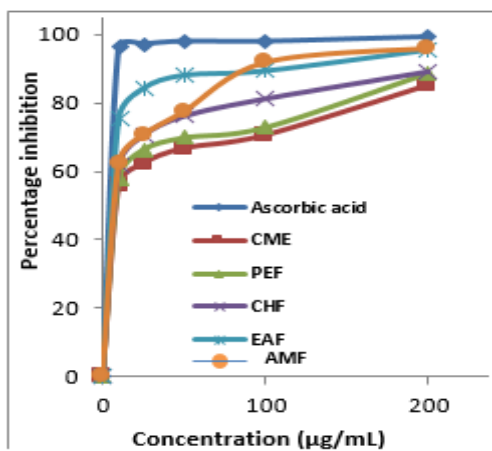


Figure 1. DPPH radical scavenging activity of crude *P. rugelii* extract, its fractions and the synthetic antioxidant (ascorbic acid) at

different concentrations. Each point is a mean from triplicate measurement.

It has been proven to display a wide range of pharmacological and biochemical activities including radical scavenging properties (Prasad *et al.*, 2009). These findings support earlier reports that plant metabolites like phenolic contents, tannins and flavonoids possess antioxidant activity (Rice-Evans *et al.*, 1997). These phytochemicals except tannins were reported to be present in the extract (Table 1). The result showed that the standard antioxidant (ascorbic acid) had stronger activity than the tested extracts, probably because the former contain more purified compounds than the latter.

Antibacterial and Antifungal activity

The result presented in Figure 2 and 3 reveals the *in vitro* susceptibility of the bacteria isolates and the percentage fungal inhibition (at 125 mg/mL) of the whole plant extract and fractions of *P. rugelii* in comparison to ciprofloxacin (20 µg/mL) and fluconazole standard (100 µg/mL) respectively.

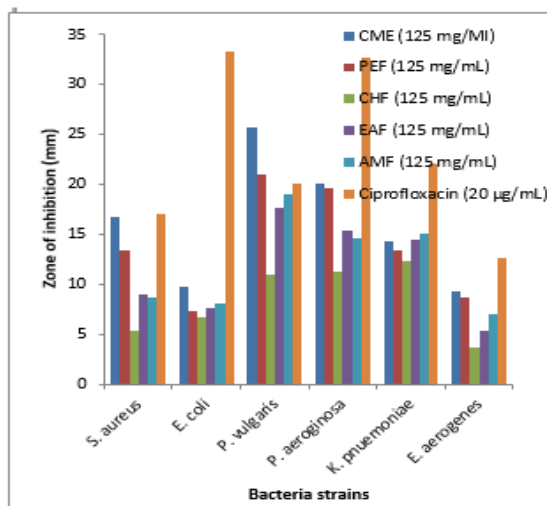


Figure 2: Antimicrobial activity (zone of inhibition) of *P. rugelii* crude extract and fractions against various human pathogens.

Natural antimicrobial compounds from plants act as therapeutics that can inhibit the growth of pathogens and have been used to overcome the side effects associated with the synthetic antimicrobial agents. The crude extract and its fraction showed varying activity against the bacteria isolates investigated. While the best activity was exhibited by the CMF on *P. vulgaris* with the inhibition zone (25.67±0.20), the least activity was exhibited by the CHF against *E. aerogenes* with the inhibition zone (3.67±0.09). The CHF generally showed the least activity amongst the fractions. The result of the anti-fungal assay indicated

that the CMF has the least activity against the challenged fungi with % inhibition of 13 and 24 while the best activity with % inhibition of 69 and 74 was obtained for the AMF for *Aspergillus niger* and *Candida albicans* respectively. Both ciprofloxacin and fluconazole) exhibited a 100% or near 100%.

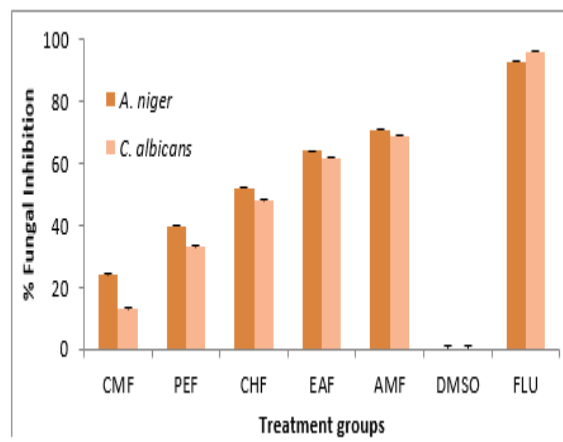


Figure 3: Comparison of the percentage fungal inhibition of *P. rugelii* crude extract, its fractions and fluconazole standard. DMSO = Dimethyl sulphoxide and FLU = Fluconazole. Data represent Mean \pm SD of three replicates

The antimicrobial activity recorded for the crude plant extract and its fraction is in agreement with the report of Cooper *et al.* (2006) was reported that the presence of more group of phytochemical diversity gives synergic effects in many biological applications. The disparity between the activities of the extracts and the standard antimicrobial drugs may be due to the mixtures of bioactive compounds present in the extracts compared to the pure compound contained in the standard antibiotics (Gatsing *et al.*, 2010).

Conclusion: The present study summarizes that *P. rugelii* is a good source of various metabolites like steroids, flavonoids, glycosides, alkaloids and carbohydrates. The crude methanol extract and the fractions of the plant showed promising broad spectrum antimicrobial activity and radical scavenging activity comparable to standard ciprofloxacin, fluconazole and the free radical scavenging activity of ascorbic acid. The finding of this study suggests that the leaves of this plant could be a potential source of natural antioxidant. These results encourage the researchers to do further *in vitro* and *in vivo* research that will explore the role of bioactive constituents responsible for these activities as well as carry out studies at molecular level.

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