

ANTIOXIDANT PROPERTIES OF AFRICAN OIL BEAN (*Pentaclethra macrophylla* Benth) SEED PHENOLICS AS INFLUENCED BY EXTRACTION SOLVENTS AND HEAT TREATMENTS

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ABSTRACT

Three commonly used extraction solvents (70% ethanol, 80% acetone and acidic 70% acetone) were evaluated for yields of phenolic substances and subsequent antioxidant capacity of the extracts from three samples (raw, dried and autoclaved) of African oil bean (*Pentaclethra Macrophylla* Benth) seed in a 3x3x4 factorial design. The contents of selected phytochemicals, namely, total phenolic content (TPC), total flavonoid content (TFC) and condensed tannin content (CTC) were investigated. In addition, antioxidant activities were tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results show that for extraction, 70% ethanol gave more yields of extracts from raw and dried samples, while 80% acetone gave more yield of extract from the autoclaved sample. Moreover, the acidic 70% acetone gave the highest yield among the three solvents for extracting TPC from the three samples while for the TFC 80% acetone gave the highest yield. The best extraction solvent for DPPH antioxidant assay was 70% ethanol.

Key words: *Pentaclethra macrophylla*, seed extract, heat treatment, extraction solvent, DPPH

INTRODUCTION

The African oil bean tree, *Pentaclethra macrophylla* Benth (*Leguminosae*, subfamily *mimosoidae*), is a large leguminous, nodule-forming multipurpose tree species occurring naturally in the humid lowlands and some parts of the sub-humid zones of West and Central Africa (Enujiugha, 2008). The edible part of the plant is the seed which can be consumed as roasted snack, or as fermented condiment in soups and porridges (Enujiugha and Olagundoye, 2001; Enujiugha *et al.*, 2008), or preserved via thermal processing in lacquered cans (Enujiugha and Akanbi, 2010). *Pentaclethra macrophylla* seeds are known to be highly nutritious (Enujiugha and Ayodele-Oni, 2003) and contain appreciable amounts of thermostable amylases and lipases (Enujiugha *et al.*, 2002, 2004). The seeds also have high phytase activity to take care of the high phytate content that decreases divalent minerals' absorption (Enujiugha, 2005). The medicinal and therapeutic properties of the oil bean seed are well-documented as including snake bite antidote, anti-diarrheal, and anti-hypertensive, among others (Enujiugha, 2008). However, the seed antioxidant properties are not currently established. Current research efforts are geared towards exploitation of natural sources of antioxidants in light of the carcinogenic tendencies of synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Generally, antioxidants are

compounds that detoxify reactive oxygen species and prevent their damage to cellular macromolecules and organelles through different mechanisms (Shahidi, 2000). It has been widely reported that a group of anti-oxidant compounds of different chemical structures present in plant foods are involved in the prevention of certain diseases, such as cardiovascular diseases, age-related diseases, inflammatory processes and certain kinds of cancer (Halliwell, 1997). Phenolic compounds including flavonoids and condensed tannins present naturally in vegetables, fruits, grains, legumes and pulses possess the ability to reduce oxidative damage believed to cause many diseases. On the other hand, anti-oxidants are commonly used as food additives that prevent lipid peroxidation in food products, especially fatty foods, thereby extending their shelf-life.

Phenolic compounds being natural antioxidants are secondary metabolites commonly found in both edible and non-edible parts of plants. Evaluation of antioxidant activity of phenolic compounds from lesser-known underutilized leguminous seeds has been of interest in recent years as a way to increase their utilization potentials. Antioxidant activity has been reported for extracts from unconventional legumes such as African locust bean (Enujiugha, 2010), African yam bean (Enujiugha *et al.*, 2012), and Bambara groundnut. The objectives of this research work were to extract the phenolic compounds from African oil bean (*Pentaclethra macrophylla* Benth) seed using different

solvents, determine the effect of dry and wet heat treatments on the extracted seed phenolics and evaluate the free radical scavenging properties of the phenolic extracts using 2,2-diphenyl-1-picrylhydrazyl (DPPH).

MATERIALS AND METHODS

Chemicals and Reagents

All solvents used were of analytical grade and include methanol, ethanol, acetone, vanillin, Folin-Ciocalteu's reagent, acetic acid, tannic acid, NaNO_2 , AlCl_3 , H_2SO_4 , HCl , DPPH (2,2 - diphenyl -1- picrylhydrazyl), BHT, Gallic acid and catechin.

Preparation of the African oil bean seed samples

African oil bean (*Pentaclethra macrophylla* Benth) seeds were purchased from a local market in Nri, south-eastern Nigeria. The beans were sorted and cleaned, and dehulled using mechanical method (cracking in a mortar and separating cotyledons with kitchen knife). The seeds were divided into three parts. The first portion (raw sample) served as control while the second portion was dried in an air oven for 6 h at 60°C and 1 h at 100°C. The third portion was heated using autoclave for 10 min at 121°C, and then dried at 40°C for 6 h. The three samples were then milled separately into flour prior to extraction.

Extraction of the samples

Raw and processed (dry and autoclaved) seed flour samples were first defatted with petroleum ether (40°–60°C b.p.) using Soxhlet apparatus in the laboratory then extracted by simultaneously stirring with 100 ml of acidic 70% acetone, 80% acetone and 70% ethanol at 25°C for 24 h (Siddhuraju and Becker, 2003), and then filtered using Whatman no.4 filter paper. The residues were each re-extracted with 150 ml of acidic 70% acetone, 80% acetone and 70% ethanol as described above for 3 h. The combined solvent extract was evaporated under reduced pressure using rotary vacuum-evaporator at 45°C. The remaining water was removed by lyophilisation. The freeze-dried extract thus obtained was used directly for total phenolics, total flavonoid and condensed tannins estimation and also for the assessment of antioxidant capacity by the use of DPPH assay. All the extracts were combined and stored at 4°C in the dark until further analysis. Extraction was performed in triplicates for the individual samples.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) was determined by a Folin-Ciocalteu assay (Singleton and Lamuela-Raventos, 1999) using gallic acid (GA) as the standard (Enujiugha *et al.*, 2012). The mixture of sample (0.5 ml

in 50 mg/ml methanolic solution), distilled water (0.5 ml), 3 ml Folin-Ciocalteu reagent solution and 0.5 ml NaCO_3 were vortexed and incubated for 8 min at room temperature. Then a further dose (0.5 ml) of distilled water was added. The mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm against distilled water as blank. The total phenolic content was expressed as gallic acid equivalent [mg of GAE/g sample] through the calibration curve of gallic acid. Linearity range of the calibration curve was 50-1000 $\mu\text{g/ml}$ ($r = 0.99$). The determination was conducted in triplicate.

Determination of total flavonoid content (TFC)

Total flavonoid content was determined using a colorimetric method described previously (Enujiugha, 2010). Briefly, a dose of 0.25 ml of the seed extract or (+) – catechin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by adding 75 μL of a 50% NaNO_2 solution. After 6 min, 150 μL of a 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution was added and was allowed to stand for another 5 min before adding 0.5 ml of 1 M NaOH . The mixture was made up to 2.5 ml with distilled water and mixed thoroughly. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm using a UV-Visible spectrophotometer (UV 160A, Shimadzu, Kyoto, Japan). The results were calculated and expressed as micrograms of (+) – catechin equivalent (mg CAE/g sample) using the calibration curve of (+) – catechin. Linearity range of the calibration curve was 10 to 1000 $\mu\text{g/ml}$ ($r = 0.99$). The determination was carried out in triplicate.

Determination of condensed tannin content (CTC)

The analysis of condensed tannin content (CTC) was carried out according to the method of Broadhurst and Jones (1978). To the suitably diluted sample (50 mg/ml), 3 ml of 4% methanolic vanillin solution and 1.5 ml concentrated hydrochloric acid were added. The mixture was allowed to stand for 5 min and then absorption was measured at 500 nm against methanol as blank. The amount of condensed tannin was calculated and expressed as mg catechin equivalent per gram of sample (mg CAE/g sample) using calibration curve of (+) – catechin. Linearity of the Calibration curve ranged from 200 to 1000 $\mu\text{g/ml}$. The determination was conducted in triplicate.

Free radical scavenging activity on 2, 2–diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant activity of the African oil bean seed extracts was measured in term of hydrogen donating or radical scavenging ability, using the stable radical

DPPH according to the method of Brand-Williams *et al.* (1995), with slight modifications. A methanol solution (0.1 ml) of the sample extracts at various concentrations (50 mg/ml, 10 mg/ml, 5 mg/ml) was added to 3.9 ml (0.25 mM) of DPPH solution. The solution was allowed to stand in the dark for 30 min and absorbance of sample (A_{sample}) at 517 nm was determined with a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) against methanol as blank. A negative control of DPPH was taken ($A_{reference}$). The percentage concentration of DPPH in the reaction medium was calculated using the following formula:

$$DPPH \% = 1 - \frac{Abs_{sample}}{Abs_{reference}} \times \frac{100}{1}$$

Where $Abs_{reference} = 1.41$.

The free radical scavenging capacity of the seed extracts was expressed as an equivalent of that of Trolox. Every sample was extracted in triplicate and the results were calculated and expressed as micromoles curve of Trolox equivalents (TE) per gram of seed using the calibration curve of Trolox. Linearity range of the calibration curve was 20 to 1000 μ M ($r = 0.99$).

Table 1: Weights of the extracts from raw, dried and autoclaved samples of African Oil Bean (*pentaclethra macrophylla benth*) seed after freeze drying.

SOLVENTS / SAMPLES	70% ETHANOL	80% ACETONE	ACIDIC 70% ACETONE
RAW (g)	4.83	2.36	0.92
DRIED (g)	3.91	3.25	1.76
AUTOCLAVED (g)	4.23	4.45	2.83

Total Phenolic Contents (TPC)

Total phenolic contents (TPC) of the extracts from African oil bean seed are presented in table 2. Natural phenolics exert their beneficial health effects mainly through their antioxidant activity (Fang *et al.*, 2002). These compounds are capable of decreasing oxygen concentration, intercepting singlet oxygen, preventing 1st-chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalysts, decomposing primary products of oxidation to non-radical species and breaking chains to prevent continued hydrogen reaction from substances (Shahidi and Naczk 2004). Phenolic compounds contribute to the overall antioxidant activity of plant foods.

Statistical analysis

Analyses were performed in triplicate and the data were analyzed by analysis of variance (ANOVA) using SPSS 15.0. Duncan's multiple range test was used to detect any significant differences between solvents used and heat treatment methods employed.

RESULTS AND DISCUSSION

Extraction

The results in Table 1 show the weights of the extracts from the raw, dry and autoclaved samples of African oil bean seed after freeze drying. The raw sample ranged from 0.92g to 4.83g while the dry sample ranged from 1.76g to 3.91g and the autoclaved sample ranged from 3.83g to 4.45g. The extract from raw and dried sample were in the following order from lowest to highest: acidic 70% acetone < 80% acetone < 70% ethanol while autoclaved sample was acidic 70% acetone < 70% ethanol < 80% acetone. In raw and dried sample 70% ethanol is the best extracting solvent which has the best extract but in autoclaved sample 80% acetone was the best extraction solvent. According to Xu and Chang (2007), 70% ethanol was the best extraction solvents for antioxidants assay.

The extracts from different extraction solvents differed significantly ($p < 0.05$) in their TPC. The TPC of raw African oil bean seed extracted by different solvent ranged from 2.083 to 3.020mgGAE/g; dried sample ranges from 1.890 to 3.660mgGAE/g and autoclaved sample ranged from 2.250 to 3.010mgGAE/g.

Overall, the TPC of dried and autoclaved samples of African oil bean seed were less affected by the different solvent used as evidenced by their smaller variation in the range of TPC yield compared to larger variation of raw sample. The TPC extracted by the solvents in raw sample were in the following order from lowest to highest: 80%acetone< 70%ethanol < acidic 70% acetone while in dried sample the TPC extracted by these solvents were in the following order: 70%ethanol

< 80% acetone < acidic 70% acetone and in autoclaved sample it was 80% acetone < 70% ethanol < acidic 70% acetone. The results show that acidic 70% acetone had the highest yield among the three solvents for extracting

phenolics from raw, dried and autoclaved sample of African oil bean seed. According to Xu and Chang (2007) acidic 70% acetone was the best for lentil, black soybean and the two colored common bean (*Phaseolus Vulgaris*).

Table 2: Effects of Different Extraction Solvents and Treatments on Phenolic Profiles of Raw, Dried and Autoclaved Samples of African Oil Bean (*Pentaclethra Macrophylla* Benth) Seeds.

Samples	Solvents	Total Phenolic Content (mg GAE equivalents / g)	Total Flavonoid Content (mg CAE equivalents / g)	Condensed Tannin Content (mg CAE equivalents / g)
70% ETHANOL	RAW	2.687±0.01 ²	1.483±0.00 ⁶	0.237±0.01 ²
	DRIED	1.890±0.01 ⁰	1.157±0.01 ²	0.270±0.01 ⁰
	AUTOCLAVED	2.507±0.01 ²	0.863±0.06 ⁰	0.227±0.01 ²
80% ACETONE	RAW	2.083±0.01 ²	2.870±0.01 ⁰	0.337±0.01 ²
	DRIED	2.147±0.01 ²	1.210±0.01 ⁰	0.307±0.00 ⁶
	AUTOCLAVED	2.250±0.01 ⁷	2.470±0.01 ⁰	0.227±0.01 ²
ACIDIC 70% ACETONE	RAW	3.020±0.01 ⁷	1.870±0.01 ⁷	0.240±0.02 ⁰
	DRIED	3.660±0.01 ⁰	1.950±0.01 ⁰	0.24±0.01 ²
	AUTOCLAVED	3.010±0.01 ⁷	1.813±0.01 ⁵	0.450±0.01 ⁰

Data are expressed as mean ± standard deviation (n=30);

Values with the same superscript on the same column are not significantly different (P< 0.05)

Total Flavonoid Contents (TFC)

In order to estimate the potential role of flavonoid on antioxidant activity of the African oil bean seeds, Total Flavonoid Contents (TFC) of the extracts were determined and the results are presented in Table 2. Flavonoids are widespread plant secondary metabolites, including flavonols and condensed tannins. Epidemiological studies suggest that the consumption of flavonoid-rich foods protect against human diseases associated with oxidative stress. In vitro, flavonoids from several plant sources have shown free-radical scavenging activity and protection against oxidative stress. As components of vegetables and fruits they are regularly present in human food. However, there are only a few reports on identification and qualification of flavonoids in food legumes. The TFC of raw sample of African oil bean seed extract ranged from 1.483 to 2.870 while that of dried sample of ranged from 1.157 to 1.950 and the autoclaved sample ranged from 0.863 to 2.470. The TFC yield by the extraction solvent was in the following order in raw and autoclaved sample: 70% ethanol < acidic 70% acetone < 80% acetone while in dried sample, 70% ethanol < 80% acetone < acidic 70% acetone.

The results show that 80% acetone had the highest yield among the three solvents for extracting flavonoid content from all samples. This is in agreement with the results reported by Xu and Chang (2007) where 80% acetone was the best among the six selected solvents for

extracting flavonoids from yellow pea, green pea and chickpea.

Condensed Tannin Content

The effects of various solvent extraction systems on the recovery of condensed tannins from African oil bean seed are represented in Table 2. Tannins are produced via condensation of simple phenolics and have a variety of molecular structures. They are generally divided into hydrolysable and condensed proanthocyanidins (polymers of flavan-3-ols) (Haslam 1989). Tannins are biological active compounds and may have beneficial or adverse nutritional effects. Early works suggest that phenolic substances occur primarily in the seeds of certain pigmented cultivars of sorghum, millets and legumes (Salunkhe *et al* 1982). Condensed tannins, the predominant phenolic compound in legumes seeds, were widely found in lentil, pea, colored soybean and common beans (Takahata *et al.*, 2001; Troszynska and Ciska, 2002; Beninger and Hosfield, 2003; Amarowicz *et al.*, 2004). Tannins are located mainly in the testa and play an important role in the defense system of seeds that are exposed to oxidative damage by many environmental factors (Troszynska and Ciska, 2002).

Sample extract from different extraction solvents differed significantly (P<0.05) in their Condensed Tannin Content (CTC). The CTC of raw sample of African oil bean seed ranged from 0.240 to 0.337 meanwhile dried sample ranged from 0.247 to 0.307 and autoclaved sample ranged from 0.227 to 0.450.

The CTC yield by extraction solvent were in the following order for raw sample: 70% ethanol > acidic 70% acetone < 80% acetone while in dried sample: acidic 70% acetone < 70% ethanol < 80% acetone and in autoclaved sample, it was 70% ethanol < 80% acetone < acidic 70% acetone.

The result shows that 80% acetone had the highest yield among the three solvents for extracting condense tannin from raw and dried sample of the African oil bean seed while acidic 70% acetone was the best for autoclaved sample. Zlotek et al. (2015) reported similar results in the extraction of Basil leaves.

Radical DPPH Scavenging Activity

It is generally recognized that free radical produced in the body are partly associated with the etiology of cancer and other chronic disease. Dietary antioxidants, capable of scavenging free radicals are able to reduce the risk of the disease. Therefore, it is important to determine the radical scavenging effect of antioxidants in legumes. DPPH is a free radical which is stable at room temperature and produces a violet solution in ethanol. Reductions of DPPH by antioxidants result in loss of absorbance. Thus, the degree of discoloration of the solution indicates the scavenging efficiency of the added substances. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. The DPPH values of the antioxidant extracts are presented in Table 3. Sample extracts from the different extraction solvents differed significantly ($P < 0.05$) in their DPPH

values. The DPPH value was determined using three concentrations: 50mg/g, 10mg/g and 5mg/g.

The DPPH value of 50mg/g in raw sample of African oil bean seed ranged from 0.04 to 0.08 while dried sample ranged from 0.07 to 0.08 and autoclaved sample ranged from 0.06 to 0.08. The DPPH value of 50mg/g concentration was affected by the extracting solvent in the following order from low to high: 80% acetone < acidic 70% acetone < 70% ethanol in raw sample, while in dried sample it was acidic 70% acetone < 70% ethanol < 80% acetone and in autoclaved sample it was 80% acetone < acidic 70% acetone < 70% ethanol. The results show that 70% ethanol was the best for extracting for raw sample of African oil bean seed while 80% acetone was the best for extraction of dried samples of the seed and 70% ethanol and acidic 70% acetone was the best for extraction of autoclaved sample of African oil bean seed.

In 10mg/g concentration, the DPPH value ranged from 0.06 to 0.08 in raw while in dried it ranged from 0.03 to 0.08 and in autoclaved sample from 0.07 to 0.09. The DPPH value of this concentration were also affected by the extraction solvent in the following order: in raw sample, 80% acetone < 70% ethanol > acidic 70% acetone while in dried sample it was 80% acetone < acidic 70% acetone < 70% ethanol and in autoclaved sample it was 80% acetone > acidic 70% acetone < 70% ethanol. 70% ethanol was the best extraction solvent for DPPH antioxidant assay for the three samples.

Table 3: Effects of Different Extraction Solvents And Treatments On Antioxidant Activities Of Raw, Dried And Autoclaved Sample Of African Oil Beans (*Pentaclethra Macrophylla* Benth) Seed.

Samples	Solvents	CONCENTRATIONS (mg/ml)		
		50% DPPH scavenging capacity	10% DPPH scavenging capacity	5% DPPH scavenging capacity
RAW	70%			
	ETHANOL	0.083±0.006 ^c	0.083±0.006 ^c	0.090±0.006 ^c
	80%			
	ACETONE	0.043±0.006	0.060±0.006 ^b	0.077±0.006 ^b
DRIED	ACIDIC 70%			
	ACETONE	0.070±0.006 ^b	0.077±0.006	0.080±0.006 ^b
	70%			
	ETHANOL	0.080±0.006 ^b	0.080±0.006 ^c	0.087±0.006
AUTOCLAVED	80%			
	ACETONE	0.090±0.006 ^c	0.033±0.006	0.047±0.006 ^b
	ACIDIC 70%			
	ACETONE	0.067±0.006	0.070±0.006 ^b	0.080±0.006 ^c
AUTOCLAVED	70%			
	ETHANOL	0.080±0.006 ^c	0.087±0.006 ^c	0.083±0.006 ^c
	80%			
	ACETONE	0.063±0.006 ^b	0.070±0.006 ^b	0.073±0.006 ^c
AUTOCLAVED	ACIDIC 70%			
	ACETONE	0.073±0.006 ^c	0.073±0.012 ^{2b}	0.080±0.006 ^{2b}

Data are expressed as mean ± standard deviation (n=30);

Values with the same superscript on the same column are not significantly different ($P < 0.05$)

In 5mg/g concentration of African oil bean seed, the DPPH value ranged from 0.08 to 0.09 in raw sample while in dried sample it ranged from 0.04 to 0.09 and from 0.07 to 0.08 in autoclaved sample. The DPPH values were in the following order: in raw sample it was 80% acetone > acidic 70% acetone < 70% ethanol, while in dried sample it was 80% acetone < acidic 70% acetone < 70% ethanol, and in autoclaved sample it was 80% acetone < 70% ethanol > acidic 70% acetone. The results show that 70% ethanol was the best extracting solvent for DPPH antioxidant assay for raw, dried and autoclaved samples. The results were consistent with a report on wheat antioxidant extraction (Zhou and Yu, 2004b) which recommended 70% ethanol as the best solvent for extracting antioxidants from wheat bran.

CONCLUSION

This study demonstrated that the extraction solvents play an important role in the extraction of important bioactive groups from African oil bean seeds. The optimal conditions for polyphenol extraction from African oil bean seeds were significantly different depending on the types of treatment (raw, dried or autoclaved) used. The most effective solvent for total phenolics content was acidic 70% acetone while the most effective solvent for condensed tannin and flavonoid extraction was 80% acetone. However, the 70% ethanol extracted sample showed the greatest antioxidant activity in the DPPH free radical assay. This would imply that 70% ethanol is best for antioxidant extraction from African oil bean seeds.

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