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Development of Extraction Protocol for Phenolic Compounds in African Pear Seed (*Dacryodes edulis*)

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ABSTRACT

The efficiency of four solvents (methanol, ethanol, acetone and ethyl acetate) at 20, 40, 60, 80 and 100% aqueous solution and three extraction methods (vortex mixing, sonication and sonication/vortexing) in extracting the phenolics of African pear were evaluated. Extracts were analyzed for phenolic content by HPLC and Folin–Ciocalteu assays. Two major phenolic peaks were isolated in the HPLC identified as peak 1 and peak 2. The main component, peak 2 was found to be gallic acid. Acetone at 80% aqueous solution gave maximum yield in extracting the total phenolics, yielding as high as 1597.3mg GAE/g. Peak 1 component was more efficiently extracted by acetone at 40% aqueous solution giving a yield of 267229mAbs in peak area whereas peak 2 component was better extracted by acetone at 60% aqueous solution with a peak area of 5825970mAbs. Most effective method of extracting the total phenolics was found to be vortex/sonicator (1004.8ppm). Practical Application: The potential health benefits of phenolic compounds prompted our interest in developing extraction protocols and analytical methodologies for their detection and measurement from African pear. African pear with a long standing history of healing properties could serve as a major source of some important phenolic compounds. A well-articulated extraction protocol in terms of the proper solvent mixture and extraction method will help both pharmacists and food processors to extract these phenolics and use them in their drug and food formulations, thereby expanding the utility of this plant.

Keywords: HPLC, Phenolics, Solvent, Extraction, African pear.

INTRODUCTION

Phenolic acids are plant secondary metabolites widely spread throughout the plant kingdom (Bruneton, 1993; Herrmann, 1989). Recent interest in phenolic acids stems from their potential as protective factors against cancer and heart diseases in part because of their potent antioxidative properties (Breinholt, 1999). They are also known to exhibit antibacterial effects (Weston *et al*., 1999) antimutagenic and anti-inflammatory activities in bacteria and mammalian (Loarca-Pina *et al*., 1998; Ihantola-Vormisto *et al*., 1997; Kaur *et al*., 1997). Besides, some phenolic acids were reported to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase (Middleton *et al*., 2000). According to Nacoulma (1996) therapeutic uses of medicinal plants in folk medicine could be attributed to their free phenolic acids. The potential healthful of phenolic acids have prompted the interest in developing analytical methodologies for their detection and measurement from plant sources. A number of analytical techniques have been presented for the analysis of phenolic acids, including thin-layer chromatography (Schmidtlein and Herrmann, 1975), gas-liquid chromatography (Schulz and Herrmann, 1980), gas chromatography-mass spectrometry (Wu *et al*., 1999), or capillary electrophoretic methods (Fernandez *et al*., 1996). However, high-performance liquid chromatography is presently the most widely used method (Amakura *et al*., 2000, Escarpa and Gonzales, 2000, Hahn *et al*., 1983). Despite the numerous HPLC methods for phenolic acids determination described in the literature, an optimized method, convenient for all phenolic acids from complex mixture like plant extracts is not available to our knowledge. Hence, this study aimed to optimize and develop extraction protocol based

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on solvent mixture as well as extraction methodology for phenolics in African pear seed.

The focus of this research is on African pear *Dacryodes edulis*, named "African pear" or "Safou". It is a tropical tree producing a consumable fruit, which softens when heated and then would be eaten with cassava or *asa* dessert. The native area of Safou extends from Sierra Leone to Uganda to the east and to Angola to the south. It is an attractive tree, usually 8 - 12 m in height, but sometimes reaching 20 - 25 m in dense forest stands. Safou belongs to the family of Burseraceae. The wounded bark exudes a limpid resin that becomes opaque while solidifying. The burning resin releases a strong odour (Raponda and Sillans, 1961). D. *edulis* has a long history of use in folk medicine. Traditional healers in Nigeria and in the Democratic Republic of Congo use the plant to treat various infections. It is used in traditional medicine as a remedy for parasitic skin diseases, jigger, mouthwash, tonsillitis and drepanocytosis (Burkill, 1994, Mpianaa *et al*., 2007). Essential oils from different parts of D. *edulis* have been isolated and analyzed. Essential oil of untreated, boiled and roasted fruits contains many constituents among which a-pinene, b-pinene, myrcene, limonene and sabinene were found to be the main compounds (Jirovetz *et al*., 2004). The stem bark essential oil contains predominantly terpinen-4-ol, a-thujene and a-pinene, whilst aphellandrene is the major component of the root bark oil. b-caryophyllene is a dominant constituent of the leaf oil (Onocha *et al*., 1999). The resin has been reported to yield a peppery essential oil that is rich in sabinene, b-phellandrene and limonene (Burkill, 1994).

MATERIALS AND METHODS

The fruits of African pear were purchased from the local market in Owerri, Imo State, Nigeria. The fruits were cleaned and the pulp part neatly removed from the seed. The thin elastic membrane endocarp protecting the pulp from the seeds was removed to expose the small seeds inside. The seeds were then dried under the sun, milled into powder (passing 0.5mm screen) using a laboratory hammer mill. The powder was stored in cellophane and kept in a freezer.

The initial preliminary cutting, removing of the endocarp, drying and milling were carried out at the Federal University of Technology, Owerri, Imo State, Nigeria while the rest of the experiments were carried out at North Dakota State University, US.

Comparison of extraction procedures

The dried African pear seed powdered sample was extracted with MeOH – H_2O (20:80, v/v). Extractions were carried out using three different procedures namely, vortex mixing, sonication, and sonication- vortexing. Extractions were carried out using the same solid to solvent ratio and solvent mixture as described by Justesen (2000). For each, approximately 250 ± 1mg of the powdered sample was placed in a 16 x 125mm screw-capped vial and 10ml of the MeOH – H_2O (20:80, v/v) solvent mixture added. For sonication, the vials were placed in an ultrasonic sonicator bath at $40^{O}C$ for 30min. In combined sonication and vortexing, the vials were first vortex-mixed for about 1min before the soniction and vortex-mixed (1min) after sonication. Extraction with vortex mixing was performed by vortex mixing the vials for 2min (three times) on a Vortex – Genie 2 scientific industry (Bohemia, N.Y. USA). After extraction with the different procedures, the mixture was centrifuged at low speed (10000 x g) for 10min. The supernatant was transferred into a 25-ml volumetric flask. The residue was re suspended in an additional 5ml of MeOH – H_2O (20:80, v/v), gently mixed manually for 30s and centrifuged for 5min. The supernatant was combined with the first extract. The volume of combined supernatant was made up to 25ml with extraction solvent and 2ml aliquots of extracts were filtered through a 0.45 µl PVDF syringe filter for phenolic assay by the FC method and HPLC analysis. For each sample, extraction and analyses were carried out in duplicate.

Comparison of the extraction efficiency of solvent composition

A systematic variation of different portions of two solvent mixtures (20:80, 40:60, 60:40 and 80:20 v/v) of methanol, acetone, ethanol, and ethyl acetate to water was used to compare the extraction efficiency of the phenolic compounds from African pear (seed). Also the efficiency of 100% (100:0) of each solvent was evaluated. The volume of the combined extract was adjusted to 25ml with the corresponding extraction solvent and appropriate aliquots filtered through a 0.45-µm PVDF syringe filter prior to total phenolic and HPLC assay. Duplicate extractions, FC assays and HPLC analyses were carried out for each sample. All extractions were carried out with approximately 250 ± 1mg of dried powdered flour of the African pear seed (particle size < 0.5mm) by using ultrasonic bath (soncation) and vortexing. The extraction efficiency is computed using the maximum yield produced by any of the solvents (as 100% yield) and calculating other solvents' efficiency by dividing this maximum yield with the individual solvent's yield as enunciated by Luthria *et al*., (2006).

Determination of total phenolics (TP) by Folin-Ciocalteau (FC) assay

The TP content was determined using FC assay (Singleton 1974) with gallic acid as a standard on a Varian Bio 50 UV spectrophotometer. The assay was carried out by pipetting 60µl of the African pear extract into an 8ml vial. This was followed by addition of 4.74ml of water. This mixture was vortex mixed for 10-20s and 300 µl of FC reagent were added. The mixture was vortex mixed for an additional 10 – 20s, 900 µl of filtered 200g/L sodium carbonate solution were added after 1min and before 8min of addition of the FC reagent. This was recordrd as time zero; the mixture was vortexd 20 – 30s after the addition of sodium carbonate. After 2h ± 3min, at ambient temperature, the absorbance of the colored reaction product was measured at 765nm. A calibration curve was created using standard gallic acid solutions each time an analysis was run. The level of TP in the extract was calculated from the calibration curve. The

results were expressed in mg of gallic acid equivalent per gram ($mgGAEg^{-1}$) of the dried African pear seed.

Determination of the phenolic compounds by HPLC

The samples (10µl of the extract) were separated using an HPLC system (LCMS-QP8000, Shimadzu) coupled with a photodiode-array detector (DAD) and a reverse-phase C18 lunar column (phenomenex, Torrance, CA, USA). The column was themostatistically controlled at $40^{O}C$ and the flow rate was set to $0.5mlmin^{-1}$. The mobile phase consisted of only one solvent (Isocratic elution) made up of methanol-water (80:20). Dual wavelengths (270 and 350 recommended for Phenolics) were used to detect the eluent composition. The detection was carried out using a photodiode-array UV detector. Extraction efficiency was estimated by the peak area.

Identification of the phenolic compounds

The phenolic compounds were identified by comparing the retention times of the different HPLC peaks with the retention times of phenolic standards injected into the HPLC under the same conditions. The phenolic standards used were benzoic acid, caffenic acid, erusic acid, flavones, gallic acid, ferusic acid, chlorogenic acid, flavanone, ellagic acid, trans cinamic acid, 5, 7, dihydroxy flavones, sinapic acid, protocathchin, O-comouric acid, M- comouric acid and P- comouric acid. The standards were injected into the HPLC at 10µl as the sample extract using the same HPLC system (LCMS-QP8000, Shimadzu) coupled with a photodiode-array detector (DAD) and a reverse-phase C18 lunar column (phenomenex, Torrance, CA, USA) under the same conditions.

RESULTS AND DISCUSSIONS

When the solvent extract of the African pear seed was injected into the HPLC, two major peaks representing the two major components in African pear were found as shown in the chromatogram (Figure.1). The peak areas and retention times are clearly shown in Table.1. The peak 1 was found to contain about 26% of the total phenolics while peak 2 contained about 74%. When the retention times of the phenolic standards were compared with the retention times of the two components found in the African pear, it was only the retention time of gallic acid standard (2.265min) was found to compare with that of peak 2(2.238min), peak 1didn't have a match. The major component in African pear, seed peak 2 is therefore suspected to be gallic acid. The peak 1 component could not be identified from all the phenolic acid standards injected.

The extraction efficiencies of the solvent mixtures in terms of the total phenolics are shown in Figure.2. As may be noted the most efficient solvent mixture was found to be acetone, especially at 80% aqueous solution giving a total phenolic yield of 1597.3mg GAE/g (100% efficiency). This was followed by ethanol with a yield of 1278.2mgGAE/g (80% efficiency). The least performance was noted with ethyl acetate especially at 100% yielding as low as 13.1mgGAE/g (8% efficiency).

Figure.1 The Hplc Hcromatogram of Phenolics of African Pear Seed (*Dacryodes edulis*)

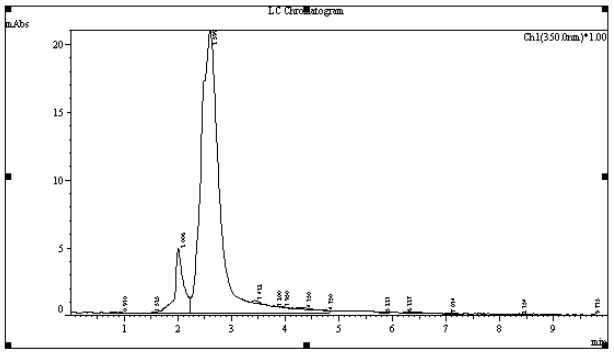

Figure.2 Extraction Efficiency of Solvent Mixtures on African Pear Seed Total Phenolics

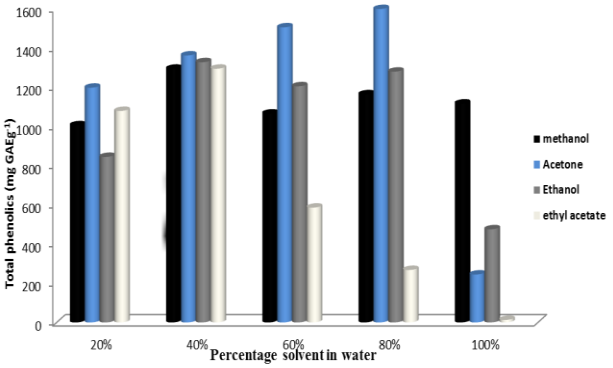

Figure.3 HPLC Extraction Efficiency of Solvent Mixtures on African Pear Seed Peak 1

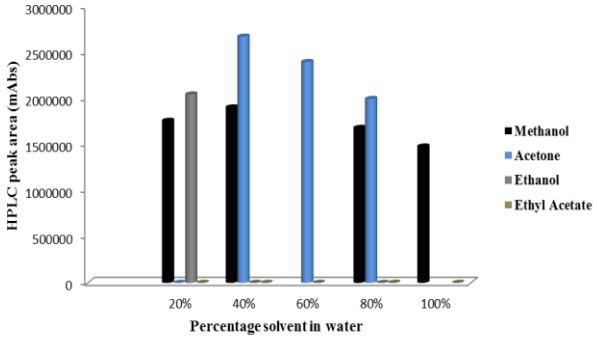

Figure.4 HPLC Extraction Efficiency of Solvent Mixture on African Pear Seed Peak 2

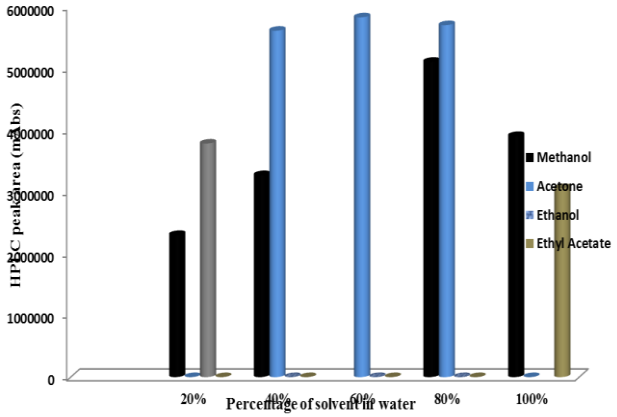

Figure.5 Effiency of the Extraction Methods Used on African Pear Seed Total Phenolics

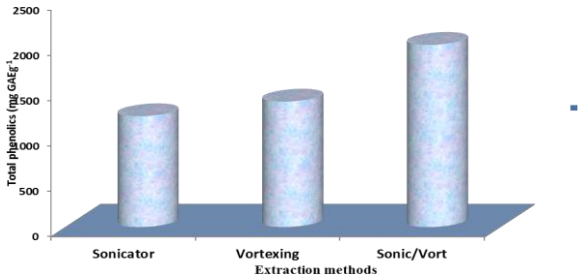

Table.1 The HPLC Profile Of The Components In African Pear Seed *(Dacryodes edulis)*

Peak#	R.Time	I.Time	F.Time	Area	Height	A/H
1	0.093	0.050	0.333	561	55	10.19
2	1.117	1.075	1.317	667	44	15.15
3	1.780	1.317	1.975	1987680	89111	22.30
4	2.238	1.975	7.125	5650258	246219	22.94
5	6.125	5.992	6.308	920	73	12.52
6	6.592	6.392	6.908	1755	139	12.59
7	7.008	6.958	7.108	221	39	5.69
				7642062	335681	-

In HPLC analysis, peak 1 component was more efficiently extracted by acetone at 40% aqueous solution yielding as high as 267229mAbs in peak area (100% efficiency) followed by ethanol at 20% aqueous solution with 2044650mAbs peak area (77% efficiency). Ethyl acetate performed very poorly (Figure.3). On the other hand peak 2 component was best extracted by acetone at 60% aqueous solution with a peak area of 5825970mAbs (100% efficiency), then at 80% aqueous solution (5699769mAbs) and at 40% aqueous solution (5609575mAbs) with extraction efficiencies of 98% and 96% respectively (Figure.4). The least performed extraction solvent for the peak 2 component (gallic acid) was ethyl acetate, especially at 20% aqueous solution with 0mAbs peak area.

This extraction efficiency trend of the various solvents can be explained by the nature of the phenolics and polarity of the solvents. According to Tsao and Deng (2004) polar phenolic compounds usually respond more to aqueous polar solvents in extraction whereas non-polar phenolics are better extracted with nonaqueous solvents. In this case African pear seed seems to contain more of a moderately polar phenolic compound. Acetone with moderate polarity (dielectric constant of 21) doing better as extractant than the rest whereas ethyl acetate, which is nonpolar (dielectric constant of 6) performed very poorly (Lowery and Richardson 1987).

Comparison of extraction procedures

The performance of the various extraction methods in terms of the total phenolics (TP) are given in Figure.5. The most effective method was vortex/sonicator (1004.83mg GAE/g), followed by vortexing alone (691.73mg GAE/g) and then sonicator alone (611.13mg GAE/g). That means that vortexing when combined with sonication gave the maximum extraction efficiency (100%), vortexing alone gave efficiency of 69% while sonication yielded extraction efficiency of 61%. Intensity of agitation as well as environmental conditions prevailing during the extraction influences the extraction efficiency. Ultrasonic cavitation creates shear forces that break cell walls mechanically and improve material transfer (Http/www.hielscher.com/ultrasonics, 2009).The relative high yield under the combined sonicator/vortexing method should be as a result of the level of agitation achieved in vortexing alongside the cavitation shear force in sonication. These effects would obviously accomplish more than when a single operation is used like vortexing or sonication alone.

Health benefits of African pear

As earlier highlighted from the result, African pear seed contains some phenolics, the major one being identified as gallic acid. The presence of these phenolics can help to explain some of the health claims established with this fruit (Marles and Farnsworth, 1995; Gray and Flatt, 1997).

Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenyl natural products from different plants, is known to have anti-oxidant, anti-inflammatory, anti-microbial, and radical scavenging activities. According to Kim *et al*. (2006) gallic acid decreased the phorbol 12-myristate 13-acetate plus calcium ionophore, stimulated pro-inflammatory cytokine gene expression and production such as TNF-α and IL-6 in human mast cells. The inhibitory effect of gallic acid on the pro-inflammatory cytokine was nuclear factor-κB and p38 mitogen-activated protein kinase dependent. In addition, gallic acid inhibited compound 48/80-induced systemic allergic reaction and IgE-mediated local allergic reaction (http://toxsci.oxfordjournal, 2009). Thus African pear may be said to have some phenolic compounds with health benefits. Further research may be conducted to identify the exact compound representing the peak one component in the HPLC.

CONCLUSION

African pear seed (*Dacryodes edulis*, G. Don Lam) contains two major phenolic compounds, designated as peak 1 and peak 2. Peak 2 which has 74% of the total phenolics was identified as gallic acid. Peak 1 component was more efficiently extracted by acetone at 40% aqueous solution giving a yield of 267229mAbs in peak area whereas peak 2 component was better extracted by acetone at 60% aqueous solution with a peak area of 5825970mAbs. Acetone at 80% aqueous solution gave the maximum yield in terms of total phenolic, producing as high as 1597.3mg GAE/g. The most effective extraction method for the total phenolics was found to be a combination of ultrasonic bath and vortexing.

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