Phenolic Profiles and Antioxidant Activity of Extracts from Peanut Plant Parts

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Abstract: Edible peanut seed represents approximately forty percent of the total mass of the peanut plant at harvest. This plant material is a potential source of nutraceutical compounds. Aqueous acetone was used to extract polar compounds from the leaves and roots of peanut plants, and from the shells of peanut seeds. The antioxidant activities of the extracts using ORAC were determined and compared. The identities of some of the compounds present were determined using LC-TOF-MS. This initial study indicates the potential of the under utilized parts of the peanut plant as a source of compounds of nutraceutical interest.

Keywords: Peanut plants, phenols, extraction, HPLC-MS, ORAC.

INTRODUCTION

Peanuts (*Arachis hypogaea*) are one of the most important legume crops in the semiarid and tropical regions of the world [1]. In 2006, over a million and a half acres of peanuts were planted in the United States [2]. From this, over four billion pounds of seed were harvested. The edible seed represents approximately forty percent of the total plant with the remaining plant material represented by the leaves, stems, and roots being left behind as soil conditioner or baled for animal feed. After shelling, peanut processing plants are left with shell material and testa or skins to dispose of as waste, or in some cases as very low value animal feed or plant mulch.

There has been increasing interest in the presence and availability of compounds in plant materials that may possess bioactive properties, in particular, antioxidant activity. Phenolic compounds are especially of interest due to their capacity to donate hydrogen ions and stop free radical oxidation. They are known to be present in every type of plant due to their production as secondary metabolites from terpenoid origins and the polyketide and shikimate pathways [3]. In plants, they function as defenses mechanisms and provide certain sensory characteristics [4].

This study was undertaken to determine if some greater value could be added to the nonseed portion of the peanut plant by identifying compounds with potential bioactvity. Our search of the literature has not revealed information of this type on peanut plant parts other than the skins [5, 6]. The extraction was done with a solvent system to optimize the recovery of phenolic compounds. The activity of the extracts from the leaves and roots of peanut plants and shells of peanut seeds were then determined using the Oxygen Radical Absorbance Capacity (ORAC) assay. Compounds present in the extracts were tentatively identified by their unique molecular masses obtained using High Pressure Liquid Chromatography interfaced directly with Time of Flight Mass Spectroscopy (LC-TOF-MS). This work is considered an initial study to try to elucidate the identities of the compounds present in the extracts and no attempts at quantification were performed.

MATERIALS AND METHODS

Extraction of Phenolic Compounds

Leaves, roots and shells from runner type peanuts grown in North Carolina, USA were separated from freshly dug plants, washed with water, freeze dried and ground to a fine powder. The extracts studied were prepared by using a published procedure [7], which described that the maximum recovery of phenolics is achieved using a 1:1 mixture of acetone (Thermo-Fisher Chemicals, Fairlawn, NJ, USA) and water (v/v). In brief, 0.2 g of lypholized plant part powder was extracted three times using 4 mL of the solvent mixture. The pooled extracts were used as prepared to perform the antioxidant assays. For the TOF-MS analysis, an aliquot of the extract was evaporated in a stream of nitrogen to remove the acetone and then an equal volume of methanol (Sigma Chemical Corp., St. Louis, MO, USA) was added to the remaining water.

Total Extractable Mass

To determine the total mass of the components soluble in the chosen solvent system, a 0.5 mL aliquot of each extract was added to a tared vial. The vials were subsequently dried under vacuum and the difference in mass after drying was used to calculate the total extractable mass.

Total Phenolics

Total phenolic content was determined colorimetrically using Folin-Ciocalteau (FC) reagent (Sigma Chemical Corp.) as described in the literature [8]. In brief, 20-500 µL of extract was mixed with 1.5 mL of FC reagent that had been previously diluted 1:10 with water. This mixture was then incubated for 5 min at 22°C and the visible absorbance at 725 nm was measured using a PharmaSpec spectrophoto-

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meter (Shimadzu, Japan). The resulting values were compared against a standard curve of Ferulic Acid (Sigma Chemical Corp.) prepared in the extraction solvent over a range of 1.5 to 50 mg mL^{-1} and reported in Table **1** in mg Ferulic Acid equivalents per 100 grams of dry extract (mg FA $100g^{-1}$).

Hydrophilic-Oxygen Radical Absorbance Capacity (H-ORAC) Assay

The H-ORAC procedure was adapted from the current literature [9]. Assays were prepared in black 96 microwell plates (Greiner Bio-one, Monroe, NC, USA). Fluorescein (Reidel-deHaen, Seelze, Germany) (1.17 mM) was prepared in 0.075 M Potassium Phosphate buffer, as was a 10 mM solution of 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH)(Sigma Chemical Corp.). Plant extracts from the leaves and roots were diluted 100 fold in the same phosphate buffer and then loaded into the plate wells. Shell extracts were added to the well plates undiluted. Aliquots (20 µL) of the fluorescein solution were added to the samples and incubated at 37°C for 15 min in a SAFIRE^2 Plate Reader (Tecan US, Inc., Durham, NC, USA). The AAPH solution (60 µL) was then added to each well and the plate was shaken orbitally inside the reader for 5 sec. Data points of relative fluorescence (Excitation = 483, Emission = 525) were acquired over 80 cycles of 1 min each with 5 sec of shaking between cycles. A standard curve of Trolox (Sigma Chemical Corp.) was also prepared over a range of 3 to 50 µM and analyzed in the same way as the samples. Antioxidant activity was reported as µM Trolox equivalents pre gram of dry extract.

Analysis of Extracts by High Pressure Liquid Chromatography-Time of Flight Mass Spectrometry (HPLC-TOF-MS)

A 5 µL injection of the solution prepared as described in the extraction section was injected on to an Agilent® Series 1100 liquid chromatogram (HPLC) (Agilent Technologies, Inc., Santa Clara, CA, USA) and separated using a C18 column (Restex Ultra Aqueous, 100 mm x 2.1 mm, (Restex Corp., Bellefonte, PA, USA)). The HPLC was interfaced directly to a Leco Unique® Time of flight mass spectrophotometer (TOF-MS) (Leco Corp, St. Joseph, MO, USA). The flow rate was 0.4 mL min^{-1} using a gradient of 0.1 % formic acid in water (A) and 50/50 v/v acetonitrile/methanol (B) as the mobile phase. The gradient program was 10 % B to start, increased to 95 % B in 30 min, held for 5 min, and then decreased to 5 % B in 5 min for a total run time of 40 min. The column was heated to 30°C and the autosampler was held at 10°C. The MS used a high flow electrospray ionization (ESI) source in the negative mode. The ESI voltage was -3500 V with a desolvation temperature of 300°C. The nebulizer pressure was 375 kPa using nitrogen as a desolvation gas at 7 L min^{-1}. The interface temperature was 100°C. The nozzle was set to -160 V and the skimmer was set to -60 V. Data was acquired at 1.56 spectra sec^{-1} using ChromaTOF software (Version 4.0, Leco Corp.). Identification of the compounds listed in Tables **1** through **3** was based on matches in literature sources to the unique masses found [10-12]. Figs. **1** through **3** are the chromatograms of the extracts from the leaves, roots and shells to show the separation achieved by the instrument.

RESULTS AND DISCUSSION

The extraction procedure used removed polar compounds from the plant parts without hydrolyzing them from sugars or other compounds which may naturally bind them. Based on discussions in the literature [9], the extraction solvent of equal parts acetone and water was chosen to extract the maximum amount of phenolic material. This would not necessarily translate to the highest H-ORAC values for the extracts. The extraction was also indiscriminate in the types of compounds removed. The leaves had the highest values for extractable material, total phenolics and H-ORAC activity as seen in Table **1**. The roots had values of total extractable material that was nearly as high, but the shells contained less than half of the roots. The shells are composed of a high percentage of lignin which would not be dissolved under the conditions used [13]. Using the molecular masses found and suggested formula given by the MS software, tentative identifications are listed in Tables **2** through **4**. The compounds assigned to the peaks in the chromatograms (Figs. **1** through **3**) were chosen based on molecular mass and feasibility for the type of sample. The water soluble compounds capable of providing protection against free radical damage were measured by the H-ORAC assay. It would be expected that many of these compounds would possess hydroxyl groups. From the MS profile of the leaves (Fig. **1**), the sample was seen to be contaminated with both an insecticide (Cyhalothrin) and an herbicide (Blazer®) (Peaks 5 and 12). As this study was not conducted in such a way to optimize extractions of compounds of this type, this does not imply that these are the only agricultural chemicals present on the leaves. It does however show that these compounds are present in the extracts. Since peanuts often need protection from invasive insect pests and weeds, it was to be expected that the possibility for such compounds to be present in the extracts existed. This may pose problems in the future if peanut leaves are to be considered for human or animal consumption.

Kaempferol (Peak 2) is a flavonol and Formononetin (Peak 14) is a flavone (See Table **2**). Both compounds were

Table 1. Assay Results for Plant Parts

Plant Part	Total Extract (mg mL^{-1})	Total Phenolics (mg FA $100g^{-1}$)	H-ORAC (:M Trolox g^{-1})
Leaves	$5.53 \pm 0.49^{a*}$	4166 ± 141^{a}	510 ± 65^{a}
Roots	4.73 ± 0.62^{a}	877 ± 49^{b}	207 ± 16^{b}
Shells	1.71 ± 0.27^{b}	425 ± 33^{c}	86 ± 5^{c}

*Values are means followed by standard deviations for at least three replicates of each measurement. Values within columns followed by the same letter did not differ significantly ($p<0.05$).

Table 2. Compounds Identified in Leaf Extracts Using LC-TOF-MS in the Negative Mode

Peak Number*	Unique Mass	Retention Time (min:sec)	Compound Assigned
2	282.0644	03:49	Kaempferol
4	577.2501	06:18	Apigen
5	447.1802	06:42	Cyhalothrin (insecticide)
7	595.1273	09:41	Astaxanthin
9	477.0635	10:42	Lantabetulic Acid
10	459.2525	13:45	Cassaine
11	285.0368	15:29	tetra hydroxyflavone
12	383.1686	16:30	Blazer® (herbicide)
13	467.2240	17:54	2-methyl-2(3-oxo-5-trityloxy-pentyl-) cyclohexane-1,3 dione
14	267.0689	18:24	Formononetin
15	323.1286	20:46	Otobain
16	383.1122	22:29	2-methyl-7-acetoxy-2',4',5'-trimethoxy-isoflavone
17	369.0983	22:38	Sesamolin
18	353.1050	22:54	Asarinin or Sesamin
19	353.4043	23:05	Asarinin or Sesamin
21	559.3148	26:20	Isogalpinone
22	277.2189	29:40	Linolenic acid
23	279.2350	30:50	Linoleic acid
24	255.2350	32:03	Palmitic acid
25	281.2494	32:08	Oleic acid

*Missing peak numbers were not identified.

Table 3. Compounds Identified in Root Extracts Using LC-TOF-MS in the Negative Mode

Peak Number*	Unique Mass	Retention Time (min:sec)	Compound Assigned
3	295.0473	05:12	Coutaric acid
4	431.1190	05:18	Inositol hexa acetate
5	250.0745	05:53	5,6-dimethoxy-3-methyl-7-nitro-1-indanone
6	474.2367	07:28	Tubulosine
7	417.0971	07:53	Saverogenin
12	227.0707	12:43	Resveratrol
13	285.0755	14:24	Sakuranetin
14	285.0436	15:01	Scutellarein
15	401.1215	18:01	Hexamethoxyflavone
16	299.0934	18:25	5-hydroxy-4',7'-dimethoxyflavone
17	315.0890	19:59	3,7,4'5-methoxy-6-methylflavanone
19	397.1271	24:24	Anthricin
20	295.2281	26:10	Verdolic acid
21	277.2181	29:37	Linolenic acid
22	279.2338	30:47	Linoleic acid
23	255.2336	32:00	Palmitic acid
24	281.2492	32:03	Oleic acid

*Missing peak numbers were not identified.

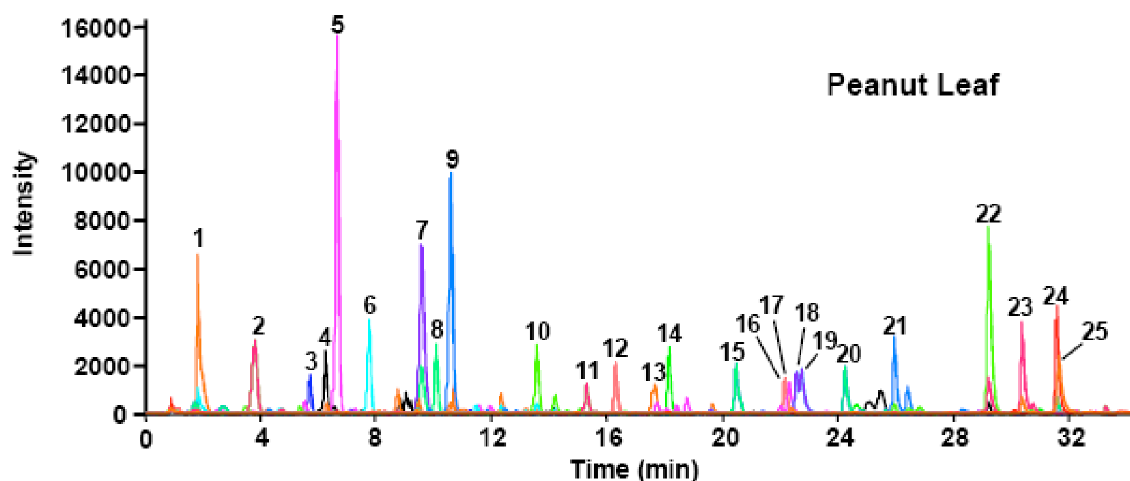

Fig. (1). Chromatogram of leaf extract using LC-TOF-MS in the negative mode.

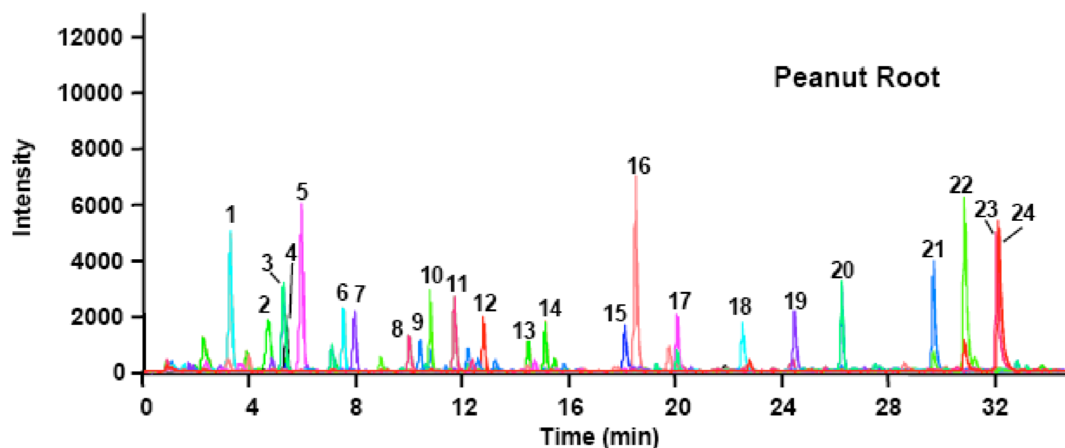

Fig. (2). Chromatogram of root extract using LC-TOF-MS in the negative mod.

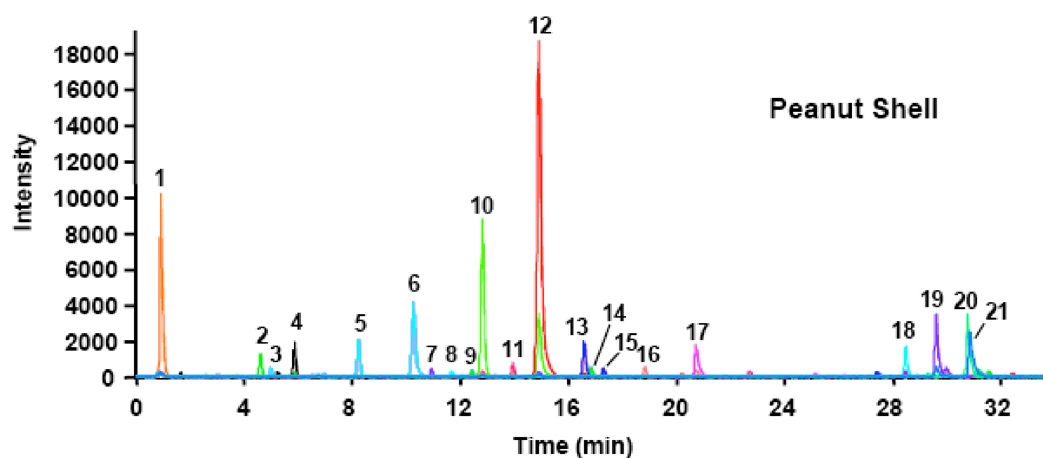

Fig. (3). Chromatogram of shell extract using LC-TOF-MS in the negative mode.

previously found in soybean, a legume like peanut [10]. Apigenin-7-rutinoside (Peak 4) is a flavone with a yellow color that is often seen bound with sugars in plants [10]. Another colored compound identified was the plant pigment, Astaxanthin (Peak 7). Peak 9 appeared to be some type of anthraquinone and was assigned to the triterpene, Lantabetulic Acid [10]. Cassaine is a phenolic acid found in bark and was assigned to Peak 10 [10]. Peaks 13 and 16 were assigned based on molecular weight only to be isoflavones [12]. Otobain (Peak 15) and Sesamolin (Peak 17) are compounds found in plant lipids [10]. Peaks 18 and 19 were very close in unique masses and were assigned to the isomers Asarinin

Table 4. Compounds Identified in Shell Extracts Using LC-TOF-MS in the Negative Mode

Peak Number*	Unique Mass	Retention Time (min:sec)	Compound Assigned
2	417.1100	04:48	Aloin
3	177.0198	05:12	Dihydroxycoumarin
4	461.1670	06:06	Peonidin-3-O-glucoside
5	177.0230	08:34	Dihydroxycoumarin
6	177.0209	10:41	Dihydroxycoumarin
7	371.0763	11:22	Syringin
8	227.0722	12:42	Resveratrol
9	269.0478	12:57	Trihydroxyflavone
10	287.0567	13:20	Fustin
11	337.1128	14:31	3-O-p-coumaroylquinic acid
12	285.0436	15:31	Luteolin
14	269.0448	17:30	Trihydroxyflavone
15	269.0563	18:00	Trihydroxyflavone
17	353.1031	21:34	Chlorogenic acid
18	277.2185	29:37	Linolenic acid
19	279.2354	30:48	Linoleic acid
20	255.2321	32:01	Palmitic acid
21	281.2504	32:06	Oleic acid

*Missing peak numbers were not identified.

and Sesamin, furans that have been found in bark [10]. Peak 21 was assigned to Isogalpinone, a compound that is usually an extraction artifact when working with natural products [14]. The remaining peaks were assigned to the main fatty acids found in peanuts.

The extracts from the roots produced a chromatogram (Fig. **2**) nearly as complicated as the leaves, but with peaks of less intensity. The extracts were pale yellow in color. Table **3** is the peak list. Peak 3 was identified as Coutaric Acid, a phenol found in wine which is known to be a secondary metabolite of pathogenic fungi [10]. Peak 5 was indentified as an indanone based on its fragmentation pattern [12]. Tubulosine (Peak 6) is an alkaloid previously isolated from sap [10] and Sarverogenin is a hydroxylated enolide found in seeds [10]. Compounds of this type have also been isolated from peanuts challenged by invasion by fungal invasion [15]. Other flavones and flavanones were identified in the roots (Peaks 13 through 17), including Sakuranetin previously found in bark (Peak 13) and Scutellarein (Peak 14) known to occur in leaves [10]. Peak 19 was identified as the ligan, Anthricin which has been isolated from plant material [10]. Vernolic acid (Peak 19) is an epoxylated fatty acid found in seed oils [10]. As with the leaves, some common fatty acids (Peaks 21-24) were also removed by the solvent mix.

The chromatogram of the shell extract was dominated by two peaks (Fig. **3**). Table **4** is the peak list. The one of greatest intensity was identified as Luetolin (Peak 12). This compound is a common flavone in plants and has been reported in peanut shells [10]. The next peak in order of intensity was identified as Fustin (Peak 10), another flavone that has previously been isolated from wood [10].

Although the roots had nearly as much extractable solids as the leaves, the ORAC activity was only about half as much compared to the leaves, and the total phenolics were much lower. The solvent mixture extracted the main fatty acids from the plant parts along with a number of flavone type molecules. The leaf extracts were more highly colored than those of the roots or the shells. As expected, some of the compounds were extracted as their glucosides [16]. The well known bioactive stilbene, Resveratrol was found in both the roots (Peak 12) and the shells (Peak 8) as has been previously reported [17,18].

Many compounds remained unidentified in all the samples. The TOF (time of flight) instrument allows for identification of unique masses even when the peaks in the chromatograms appear to be co-eluting as seen in the figures. In the case of the fatty acids, palmitic and oleic, it is expected with the column used that oleic would elute ahead of the palmitic, but in fact they were found at nearly the same retention time and identified by their characteristic masses. The presence of the fatty acids is listed here to show that they are present in the extracts, but their contribution to H-ORAC activity is probably minimal. This still needs to be determined. Hy-

drolysis and subsequent additional solvent extraction of these plant parts would be expected to yield smaller phenolic acid type molecules and such work is presently in progress. The numerous unidentified compounds need additional study.

CONCLUSIONS

The leaves and roots of the peanut plant and the protective shell of the peanut seed are not normally considered to be edible. This study has shown that these materials contain extractable compounds with antioxidant properties. This indicates that they have additional value beyond that of their current uses as low grade animal feed and soil conditioner. The compounds identified in the extracts are mainly phenolics that could be exploited for their nutraceutical properties.

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