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Profiling of Phenolic Compounds in Sprouted Common Beans and Bambara Groundnuts

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Abstract

Consumption of sprouted legumes is a popular trend at household level in Africa at the moment. This study investigated the effect of sprouting on the phenolic phytochemicals of the red bambara groundnuts and red beans. Plant-derived phenolic compounds are important as antioxidants in our diet. Phenolic phytochemical profiling was performed using HPLC-PDA-ESI-MS and Folin Ciocalteu assay. There were noticeable changes in the phenolic profiles due to sprouting of the two legumes. The total polyphenol content increased by 1.3-fold and 3-fold after sprouting in red bambara groundnuts and red beans respectively. The HPLC-PDA-ESI-MS profiling of the methanolic extracts of the sprouts revealed new emerging compounds. In red bambara groundnuts, eleven new compounds emerged. The new compounds identified include caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside. In red beans, eight new compounds emerged. Catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and p-coumaric acid hexoside were new compounds identified. Sprouting therefore enhances the polyphenolic profiles of the two legumes.

Keywords: sprouting, polyphenols, bambara groundnuts, common beans

1. Introduction

Legumes are one of the important crops that are grown in many parts of the world to meet the nutritional needs of the majority households especially in developing countries. Germinating of legume seeds into sprouts for consumption is becoming popular at household level in Africa at the moment. Sprouting is the way of germinating seeds to be eaten either raw as salad vegetables or cooked. This preparation method is convenient as it guarantees the availability of fresh vegetables at any time of the year.

Processing of legumes has several effects on their chemical composition. Previous reports have documented changes that occur to some components of the seed legumes after sprouting. According to Marton et al. (2010), polysaccharides degrade into oligo and monosaccharides; fats into fatty acids and protein into free amino acids during germination. A study by Devi et al., (2015) showed a significant improvement in nutritional quality after sprouting of cowpeas at 25 ℃ for 24 h; protein increased by 9-12%, vitamin C increased by 4-38 times, phytic acid decreased by 4-16 times, trypsin inhibitor activity decreased by 28-55% and in-vitro protein digestibility increased by 8-20%. According to Jom et. al. (2011), germination alters the level of fatty acids, methyl esters, free fatty acids, monosaccharides and disaccharides detected by Gas Chromatography-Mass Spectrometry.

While studies focusing on changes that occur to macro and micronutrients due to sprouting of various legume seeds have been done previously, there are few reports on the effect of sprouting on phenolic phytochemical profiles. Understanding the fate of phenolic phytochemicals as a result of sprouting is important given the significant role they play as health promoting constituents in human nutrition. These compounds have antioxidant properties and may protect against major clinical conditions such as heart disease and cancer in which reactive oxygen species (i.e., superoxide anion, hydroxyl radicals and peroxy radicals) are involved (Rhodes and Price, 1997; Duthie and Crozier 2000). According to Croft (2016), dietary polyphenols or their metabolites act as signaling molecules that can increase nitrite oxide bioavailability and induce protective

enzymes.

The present study investigated the effect of sprouting on the phenolic phytochemical profiles of the red Zambian market classes of common beans and bambara groundnuts. The two legumes are landraces that have not been investigated in many aspects and rarely attract interest for commercialisation.

2. Materials and Methods

2.1 Sample Collection

The two legume samples were procured from the farmers in the Eastern region of Zambia immediately after harvest. The seeds were cleaned by winnowing removing dust, grit, chaff and any extraneous materials and stored in airtight containers at room temperature.

2.2 Sprouting of Common Beans and Bambara Groundnuts

100 seeds of each legume were soaked in tap water for 12 hours until fully imbibed. The seeds were then spread on a moist muslin cloth and allowed to germinate at room temperature (25 $\pm$ 1^{o}C) in the dark. The seeds were kept moist by sprinkling the water on a muslin cloth at regular intervals. Germination capacity was expressed as the percentage of the seeds that germinated (Jimenez Martınez et al., 2012). The sprouts were harvested on the 8th day of germination when 98 and 100% of the seeds had germinated in bambara groundnuts and common beans respectively. A seed was considered to have sprouted when the root growth of 2 mm had occurred.

2.3 Preparation of Extracts of Sprouted Beans and Bambara Groundnuts

Germination was stopped by putting the sprouted seeds into a-80^{o} C freezer for 5 hours following which the seeds were freeze dried for 72 hours. The dried seeds were ground into powder using a coffee grinder (Braun, Mexico). Approximately 15 g of seed powder in 150 ml of 70% methanol was sonicated for 30 minutes at 25^{o}C using the Eumax UD500SH 40 kHz ultrasonic bath. After extraction, the mixture was centrifuged at a speed of 10,000 rpm for 15 minute using Beckman Coulter JE centrifuge. The resulting supernatant was first concentrated to 30 ml by evaporation under reduced pressure in a rotary evaporator (Buchi R-210 model, Switzerland) to remove methanol. The extract was then frozen at -80 ^{o}C and freeze dried to obtain a powdered methanolic extract using the Telstar LyoQuest -85 freeze dryer. The freeze dried extracts were stored at -4^{o}C until further analysis

2.4 Determination of Total Polyphenols

Total polyphenols were determined by the Folin Ciocalteu assay according to the method of Makkar et al., (2000). To 100 µl of sample extract, 400 µl of distilled water was added followed by the addition of 250 µl Folin Ciocalteu reagent. 20 % Sodium carbonate (1.25 ml) was then added and the mixture was incubated for 40 min. Absorbancies were read at 725 nm after 40 minutes using a spectrophotometer (Ultrospec 1000 model, England) against the blank (70% methanol or water) depending on whether it was the water or 70 % methanol extract. The amount of total polyphenols was calculated as gallic acid equilvalents from the calibration curve of gallic acid standard solution and expressed as mg gallic acid equivalents/ 100 g DW.

HPLC -DAD-ESI-MS Instrumentation and chromatographic conditions

The freeze dried 70% methanolic extract powder of common beans and bambara groundnuts were analysed using a Waters ZMD 4000 system that was equipped with a Waters 2690 HPLC, Waters 996 photodiode array, ZMD mass spectrophotometer, 717 Plus autosampler, and a quaternary pump (Waters Corp, Milford, MA, USA). Separations were carried out on a 300 x 3.9 mm, 4 µm reversed phase Nova-Pak C18 (Waters) column that was maintained at 40^{o}C. The photodiode array detector (PDA) was linked directly to a sprayer needle where ions were generated by electrospray ionisation (ESI) in a negative mode. The mobile phase A consisted of 5% (v/v) acetonitrile/water, containing 0.1% (v/v) formic acid and mobile phase B consisted of 100% acetonitrile containing 0.1% (v/v) formic acid. The sample was injected at a volume of 25 µl. The elution profile consisted of a stepwise linear gradient from 0% to 28% solvent B for 22 minutes with a flow rate of 0.3 ml/min. The PDA detector was set to a scanning range of 200 to 700 nm and the UV-Vis absorption spectra were recorded online during the HPLC analysis. Phenolic acids and flavonols were detected at 280 and 360 nm, respectively. Continuous mass spectra data were recorded on a full scan negative ionisation mode for a mass range of m/z 85 to 1000. The capillary voltage was set at 2.5 kV, the cone at 20 V and the extractor at 5 V. Nitrogen gas was used for nebulising and drying at different fragmentation voltages. Data acquisition was controlled using MassLynx 4.1 (Micromass, Waters Corp., Beverly, MA, USA). The gradient solvent system used for the analysis of phenolic compounds is summarised in Table 1.

Table 1. Gradient solvent system for analysis of phenolic compounds by HPLC -PDA -ESI-MS

Time (minutes)	Composition of the mobile phase (%)	
	*Mobile phase (A)	*Mobile phase (B)
1	100	0
22	72	28
22.50	60	40
23	0	100
24.50	0	100
25	100	0
26	100	0

*Mobile phase A consisted of 5% (v/v) acetonitrile/water, containing 0.1% (v/v) formic; mobile phase B consisted of 100% acetonitrile containing 0.1% (v/v) formic acid.

2.5 Preparation of the Samples for HPLC -PDA-ESI-MS Analysis

Preparation of the test solution for HPLC-PDA-ESI-MS was done according to the procedure by Gülçin et. al., (2010) with slight modifications. One hundred mg of the freeze dried 70% methanolic extract was dissolved in 5 ml of ethanol-water (50:50 v/v). One hundred µl of the prepared extract was transferred into a 5 ml volumetric flask and diluted to the volume with ethanol-water (50:50). From the final solution, an aliquot of 1.5 ml was transferred into a capped autosampler vial and 25 µl of the sample was injected into the HPLC-PDA-ESI-MS system. Identification of phenolic compounds was accomplished using UV spectra and ESI-MS spectral data and by comparison with published data reported in the literature.

3. Results

3.1 Sprouts Characteristics

In Figure 1, unsprouted and sprouted red beans and red bambara groundnuts are compared. For this experiments, seeds were harvested on the 8th day of germination when 100% and 98% germination capacities were recorded in red beans and red bambara groundnuts respectively. Red bambara groundnuts showed a slightly lower germination capacity than red beans.

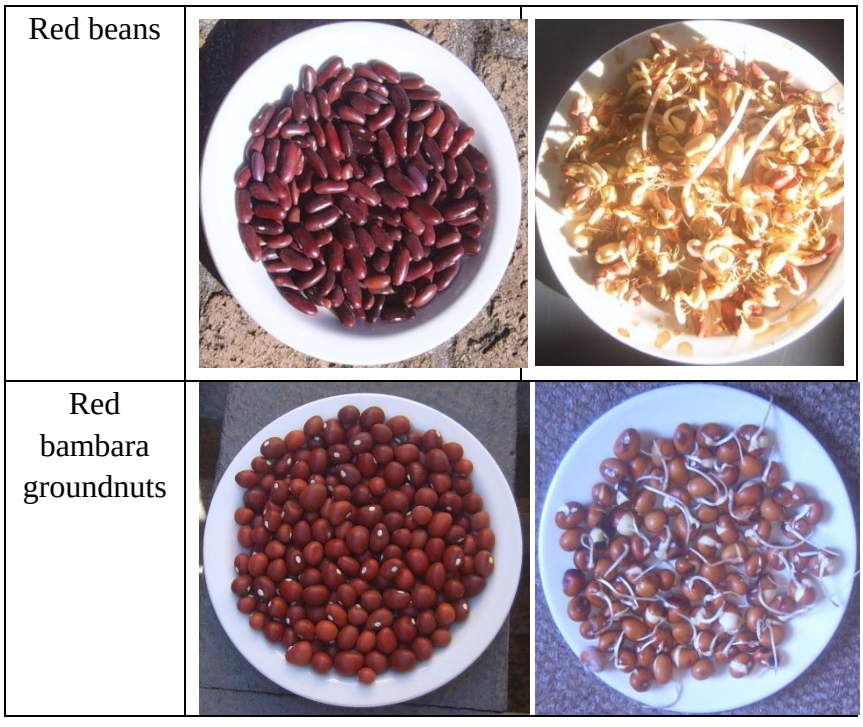

Figure 1. Sprouted red beans and red bambara groundnuts

3.2 Total Polyphenol Levels in Sprouted Red Bambara Groundnuts and Red Beans

The total polyphenol concentrations of the sprouts of red bambara groundnuts and red beans are presented in Table 2. Sprouting displayed a positive effect on the total polyphenol concentration of both the red bambara groundnuts and red beans. In red bambara groundnuts, the total polyphenol content increased by 1.3-fold after sprouting. The difference in the total polyphenol concentration of the sprouted and unsprouted seeds was significant.

In red beans, total polyphenol concentration increased by 3-fold after sprouting. As observed above with respect to red bambara groundnuts, the difference in the total polyphenol concentration of the sprouted and unsprouted seeds was significant. Increase in the concentration of total polyphenols after sprouting has been reported in other leguminous seeds. Oloyo (2004) reported a 5-fold increase in total polyphenol content of *Cajanus* seeds after 5 days of germination.

Table 2. Polyphenol concentration of different day sprouts of red bambara groundnuts and red beans

Market classes of bambara groundnuts and common beans		Total polyphenols (mg GAE / 100 g DW)
Red bambara groundnuts	Control (unsprouted)	135.9 ± 2.0^{a}
	Sprouted	174.2 ± 4.8^{b}
Red beans	Control (unsprouted)	84.5 ± 4.8^{a}
	Sprouted	253.1 ± 5.7^{b}

Means in the same column for each legume type with different superscripts were significantly $p<0.05$) different.

3.3 HPLC-PDA-ESI-MS Profiles of the Sprouted Red Bambara Groundnuts and Red Beans

Figures 2 and 3 present the HPLC-PDA-ESI-MS chromatograms of methanolic extract from the unsprouted and sprouted red bambara groundnuts. Compound with negatively charged $[M-H]^-$ ions at m/z 273, 191, 467, 205, 387, 609 and 245 were observed in both the unsprouted and sprouted red bambara groundnuts. Identification of some compounds was done based on mass spectra obtained in the negative mode by using their fragmentation pattern and data from published literature (Wu and Prior 2005; Seeram et al., 2006). A deprotonated molecule $[M-H]^-$ at m/z 191 yielded MS/MS spectrum with an ion at m/z 127 upon fragmentation, characteristic of quinic acid (Gouveia and Castilho 2011). A compound with a negatively charged ion $[M-H]^-$ at m/z 205 yielded fragments at m/z (179, 143, 129) upon MS/MS fragmentation, indicating the presence of caffeic acid. The fragmentation pattern is characterised by the loss of a hydrocarbon moiety (amu 26). This molecule was identified as caffeic acid derivative. A negatively charged $[M-H]^-$ ion at m/z 387 with MS/MS fragmentation at m/z 207 was identified as medioresinol, a phenolic lignin. Hassain et al., (2010) reported a compound in lamiaceae with similar fragmentation pattern that was identified as medioresinol. a deprotonated molecule [M-H]- at m/z 609, upon MS/MS fragmentation yielded a fragment at m/z 301 (indicative of the presence of quercetin fragment). This compound was identified as quercetin-3-O-rutinoside. Lin et al., (2008) reported a compound in common beans with similar fragmentation pattern that was identified as quercetin-3-O-rutinoside by confirmation with a standard. However, there were eleven emergent deprotonated molecules [M-H-] of m/z 341, 391, 205, 405, 189, 389, 463, 271, 543, 529 and 447 that emerged in the HPLC-PDA-ESI-MS profile of sprouted red bambara groundnuts (Figure 3).

The HPLC-PDA-ESI-MS chromatograms of the methanolic extract from the unsprouted and sprouted red beans are presented in Figures 4 and 5. Deprotonated molecules $[M-H^-]$ with m/z of 191, 385, 259, 387, 273, and 263 were observed in both unsprouted and sprouted red beans. A compound with a negatively charged $[M-H]^-$ ion at m/z 191 fragmented to yield an ion at m/z 127 and was identified as quinic acid. There were four compounds that gave a negatively charged $[M-H]^-$ ion at 385 and eluted at different retention times but fragmented to yield MS/MS spectra with ions at m/z 193, indicative of ferulic acid fragment. Four isomers of ferulic acid derivatives have been reported previously in common beans by Lin et al., (2008). A deprotonated molecule $[M-H]^-$ at m/z 259 yielded MS/MS fragment ions at m/z (241, 223, 197), indicating the presence of a syringic acid fragment. This compound was identified as a syringic acid derivative. A negatively charged $[M-H]^-$ ion at m/z 387 with MS/MS fragmentation at m/z 207 was identified as medioresinol, a phenolic lignin. Hassain et al., (2010) reported a compound in lamiaceae with similar fragmentation pattern that was identified as medioresinol. However, there were eight new deprotonated molecules $[M-H^-]$ of m/z 297, 587, 451, 429, 609, 463, 505 and 489 that emerged in the HPLC-PDA-ESI-MS profile of the sprouted red beans only (Figure 5). The emergence of the new compounds could be attributed to sprouting.

For both red bambara groundnuts and red beans, there were variations in the peak heights (concentrations) for

the compounds that were detected in the unsprouted and sprouted seeds. Peak heights were higher in the extracts of the sprouted than the unsprouted seeds. It has to be noted however that a slight drift in the retention times of peaks was observed in these runs (compare Figures 2 & 3 for red bambara groundnuts and 4 & 5 for red beans). This may be attributed to minor temperature changes or the increase in the back pressure in the column. Shifts in laboratory temperature and a slight increase of the back pressure in the column may cause drifting retention times in long automated operations (Waters Corporation, 2002).

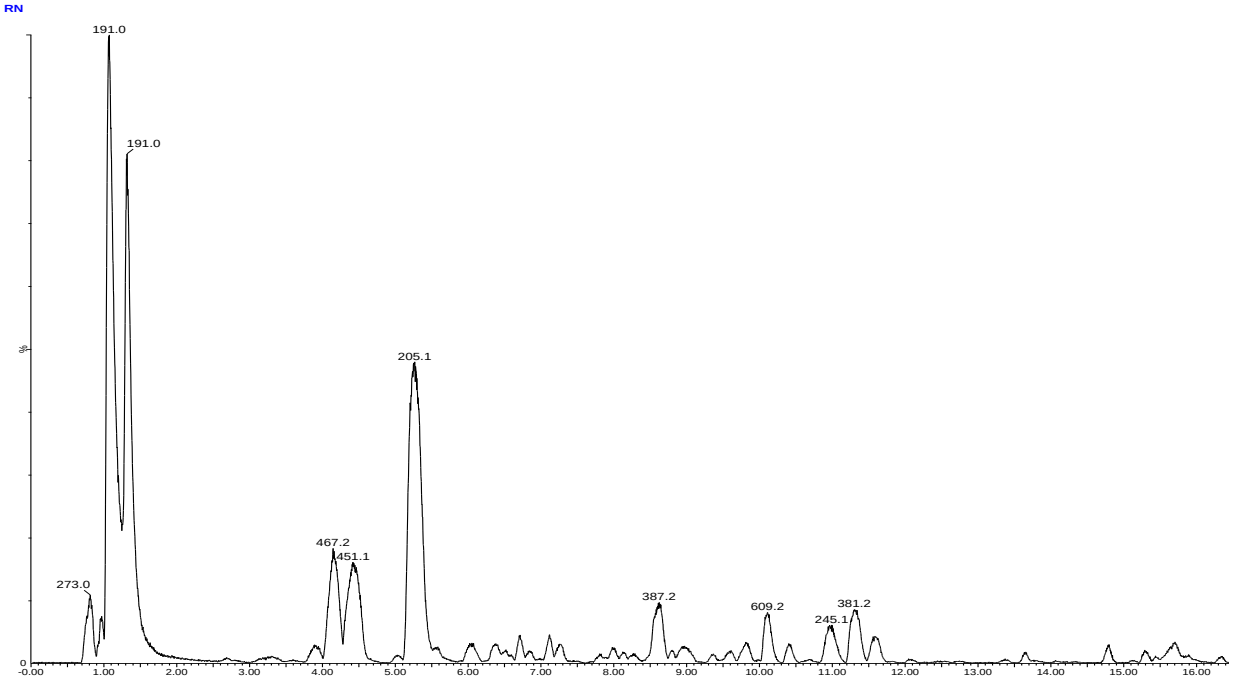

Figure 2. HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red bambara groundnuts

Number on top of each peak is the m/z of the deprotonated molecule [M-H]-of each compound.

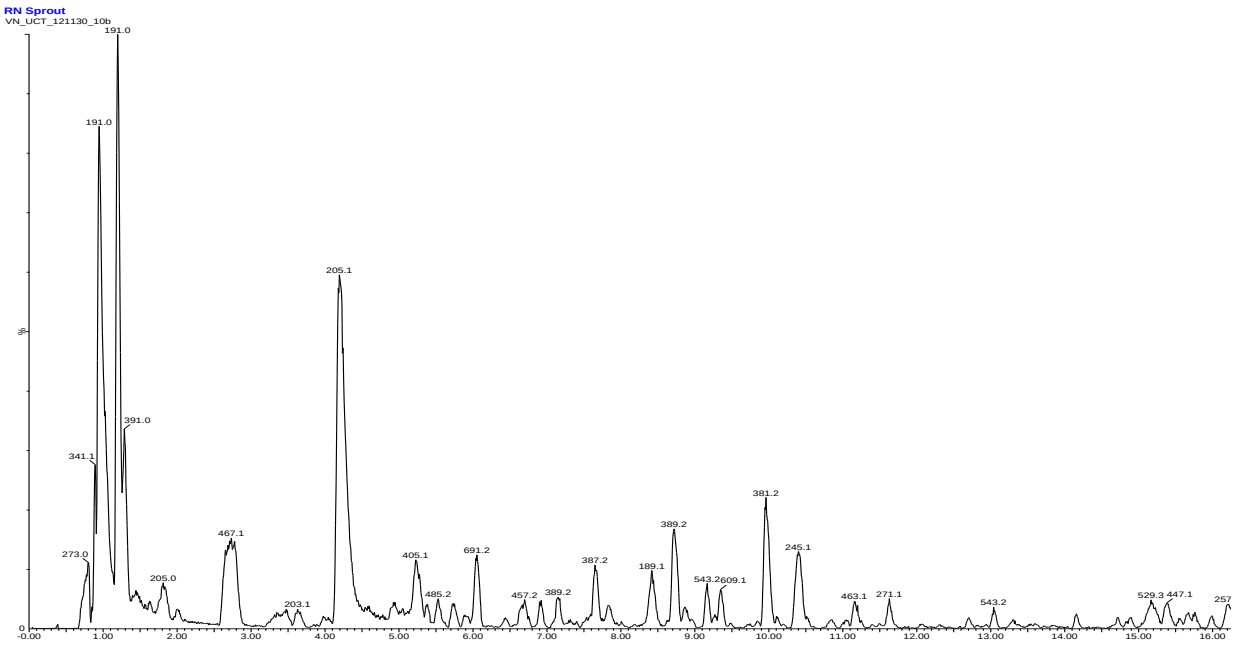

Figure 3. HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red bambara groundnuts

Number on top of each peak is the m/z of the deprotonated molecule [M-H]-of each compound.

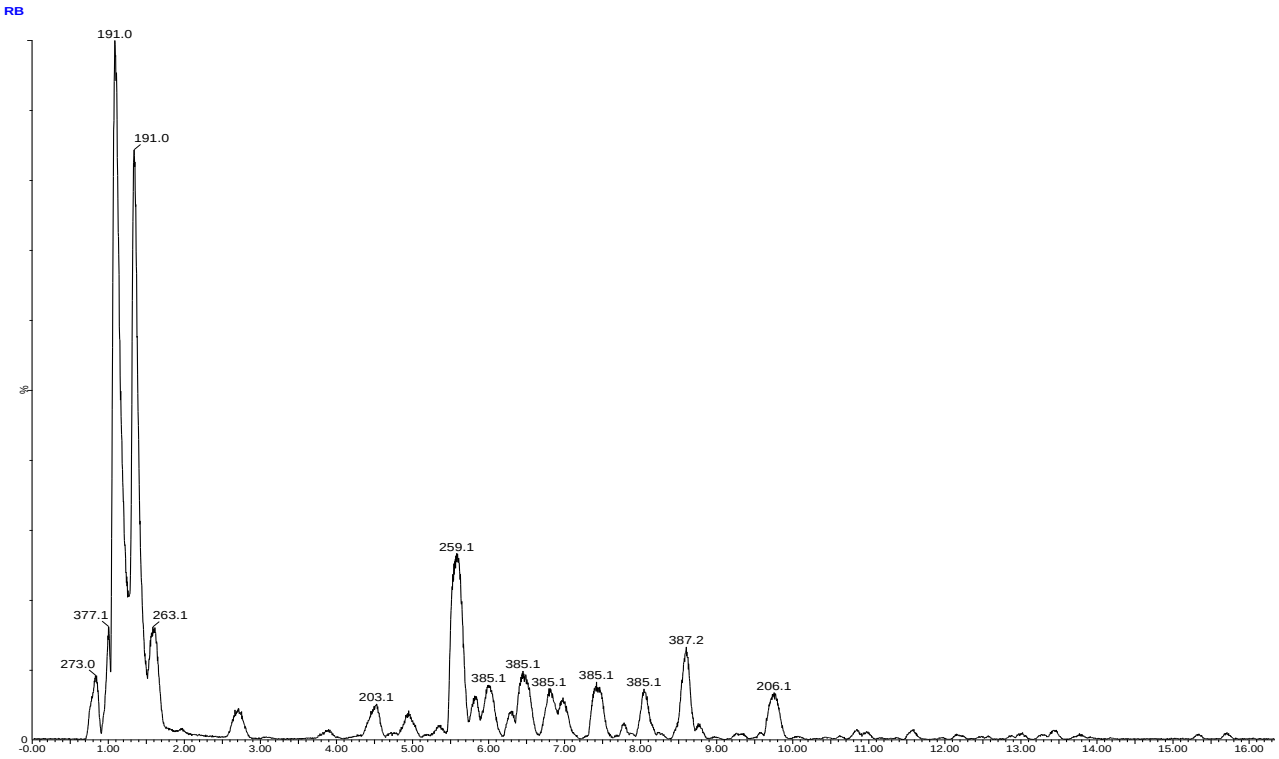

Figure 4. HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of the unsprouted red beans

Number on top of each peak is the m/z of the deprotonated molecule [M-H]-of each compound.

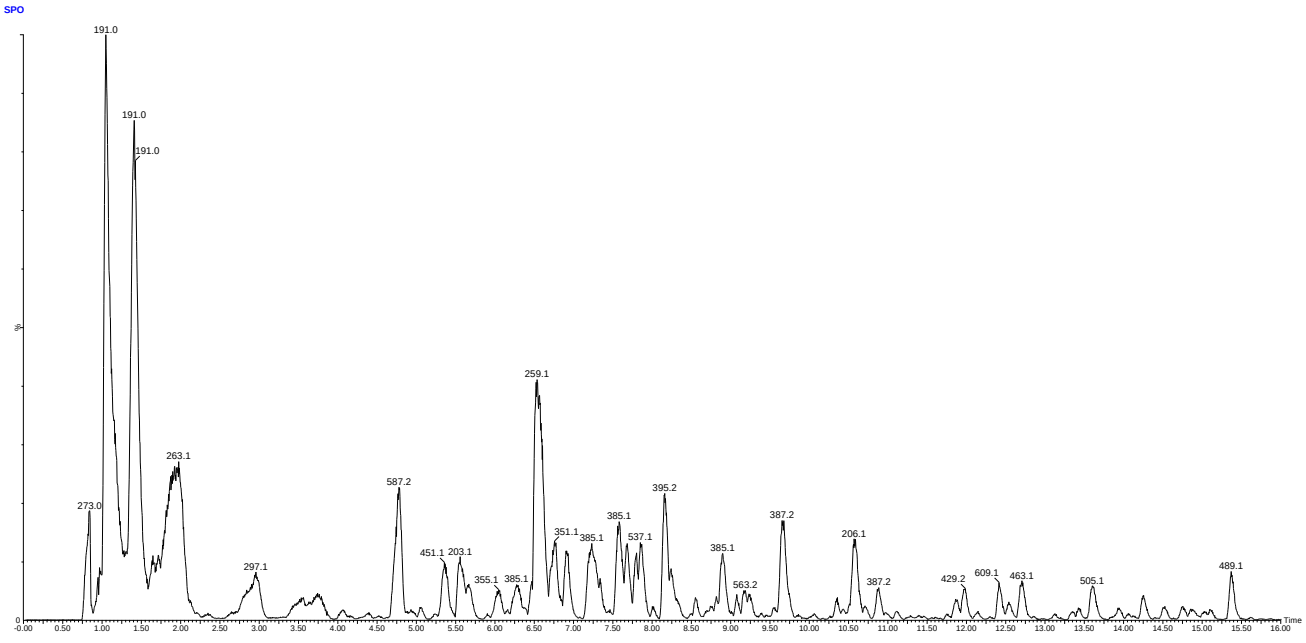

Figure 5. HPLC-PDA-ESI-MS chromatogram of 70% methanol extract of sprouted red beans. Number on top of each eak is the m/z of the deprotonated molecule [M-H]-of each compound

Based on the literature data and fragmentation pattern, attempts were made to identify the new emerging compounds in both the spouted red beans and red bambara groundnuts. Some emergent compounds that were identified in red bambara groundnuts and red beans are presented in Tables 3 and 4 respectively.

Table 3. Emerging phenolic compound tentatively identified in sprouted red bambara groundnuts

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
341	179, 161	Caffeic acid hexoside [v]
389	185,157,143	resveratrol glucoside [w]
271	151,119	Naringenin [x]
529	368,367, 179	caffeic acid derivative [y]
447	285	kaemferol glucoside [z]

[v] Hassain et al., 2010, [w] Amandeep et al., 2010, [x] Rabaneda et al., 2003, [y] Gouveia and Castilho 2011, [z] Lin et al., 2008

Table 4. Emerging phenolic compound tentatively identified in sprouted red beans

Parent ion [m/z]	Fragment ions [m/z]	Tentative identification
451	289	Catechin glucoside [a]
429	217, 285	Luteolin hexoside [b]
609	301	Quercetin -3-rutinoside [c]
463	301	Quercetin -3-O-lucoside [c]
505	301	Quercetin glucoside acylated [a]
489	155, 163, 327	*p*- coumaric acid hexoside [b]

[a]Estrella et al., 2011, [b]Palafox-Carlos et al., 2012, [c]Lin et al., 2008

4. Discussion

The red beans and red bambara groundnuts used in the sprouting experiment were suitable for sprouting, though the germination capacity in red beans was higher compared to red bambara groundnuts. Changes that occurred as a result of sprouting were positive and beneficial in both common beans and bambara groundnuts. According to Brajdes and Vizireanu (2012), germination is the only process of agro-food processing which provides significant increase of the nutritional value by increasing the bioavailability of vitamins, bioelements and other biologically active compounds.

Sprouting displayed a positive effect on the total polyphenol levels of the red bambara groundnuts and red beans. After 8 days of sprouting in red bambara groundnuts, total polyphenol levels increased 1.3-fold, whereas in red beans, there was a 3-fold increase. The HPLC-PDA-ESI-MS chromatograms of the methanolic extracts of the sprouted red bambara groundnuts and red beans revealed new emerging compounds. In red bambara groundnuts, eleven new compounds emerged. The new compounds tentatively identified include caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside. In red beans, eight new compounds emerged. Catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and *p*-coumaric acid hexoside were new compounds tentatively identified.

The increase in the total polyphenol levels as a result of sprouting has been reported previously in other seeds. Nwanguma et al., (1996) found an increase of several fold in total phenolic content in all the four sorghum varieties after germination. According to the findings of Jimenez Martınez et al., (2012), total polyphenol concentration increases by 2-fold in *Campestris* L. seeds after 8 days of germination. Oloyo (2004) reported a 5-fold increase in total polyphenol content of *Cajanus* seeds after 5 days of germination. Methanolic extract of mungbeans that was sprouted for 7 days had total phenolics ranging from 166.5 to 191.7 mg ferulic acid equivalents (FAE) kg /DW whereas that for dry seeds ranged from 97.8 to 101 (FAE) kg/DW (Kim et al., 2012). A study by Brajdes and Vizireanu (2012) reported a number of important changes in the amount of biologically active compounds when buckwheat was germinated for 7 days. The amount of polyphenols increased from 50.36 to 298.3 mg GA / 100g DW, the amount of rutin increased from 13.66 to 283.42 mg / 100g DW, the amount of quercetin increased from 4.77 to 223.76 mg / 100g DW, whereas the amount of ascorbic acid increased from 0 to 1.09 mg / 100g DW

Changes in the total polyphenol concentration during sprouting may be attributed to the enzymatic activies in the seed during germination process. Polyphenols in the cell are bound to the cellular components (carbohydrates, pectin, lignin and proteins). During the enzymatic degradation of the said cellular components, bound phenolics may be released and made available for quantification. According to Brajdes and Vizireanu (2012), the increase of phenolic compounds in the germinated seeds can be explained by an increase in the amount of free forms occurring as a consequence of hydrolytic enzyme activity, due to the breakdown of the cell wall during germination. It can be assumed that conjugated phenolic acids are released from the breakdown of cell walls,

maybe to protect the inner parts of the caryopsis which is still needed to support the developing germ (Engert et al., 2011).

Studies on the enzymatic release of bound phenolics from celluar components have been reported previously. Sinapic acid and *p*-coumaric acid from wheat bran were released by human colonic cinnamoyl esterase (Andreasen et al., 2001). The release of the ferrulic acid of soluble feruloylated oligosaccharides by microbial esterase in the human colony was reported (Kroon et al., 1997). Arnous (2009) reported the release of phenolic acid (both hydroxycinnamic and hydroxybenzoic), anthocyanins and quercetin during enzymatic (pectinolytic and cellulolytic) degradation of the cell wall polysaccharides of grape skin. Condensed tannins or proanthocyanidins that exist as oligomer and polymers may disintegrate into simple phenolics due to the activities of the hydrolytic enzymes. A study by Cheng et al., (2006) suggests that polyphenolic compounds such as tannins are broken and simple phenolics are released.

5. Conclusion

The study has revealed that sprouting enhances the polyphenolic profiles of common beans and bambara groundnuts. After 8 days of sprouting of the red bambara groundnuts (at 98% germination capacity), and red beans (at 100% germination capacity), the concentration of total polyphenols were higher compared to the unsprouted seeds. HPLC-PDA-ESI-MS advanced analytical technique revealed eleven new compounds in red bambara groundnuts and eight in red beans. Caffeic acid hexoside, resveratrol glucoside, a caffeic acid derivative, naringenin and kaempferol glucoside were new compounds tentatively identified in sprouted red bambara groundnuts. In red beans, new compounds tentatively identified include catechin glucoside, quercetin-3-O-glucoside, quercetin-3-rutinoside, luteolin hexoside, quercetin glucoside acylated and *p*-coumaric acid hexoside.

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