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Studies on extraction and antioxidant potential of green coffee

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Abstract

Green coffee conserves were prepared from the species *Coffea arabica* and *Coffea robusta* by flaking, powdering and extraction with solvent mixtures of isopropanol and water in different ratios and their antioxidant properties were investigated. The yields of conserves were highest 27% for *C. arabica* and 29% for *C. robusta*, when isopropanol and water in ratio of 60:40 was employed. The total polyphenol content was determined and found to be higher (31.7–32.2%) in these conserves. At a concentration of 200 ppm, coffee conserves from Arabica and Robusta, exhibited 92% and 88% antioxidant activity respectively in comparison to 95% for BHA. The conserves were analyzed by HPLC and three phenolic compounds could be identified. The chlorogenic acid, the major compound in the purified extracts ($56 \pm 10\%$), was isolated and characterized by ^{1}H and ^{13}C NMR spectral data. While the caffeic acid part of the molecule was confirmed from the signals for aromatic protons and olefenic protons, the quinic acid group was evident from the signals for methine protons α to hydroxyl groups as well as for the methylene protons of the cyclohexane moiety. Similarly, ^{13}C spectra showed signals for two carbonyl carbons, apart from eight signals corresponding to six aromatic and two olefenic carbons and signals for the six carbons in the cycloalkane side chain.

Keywords: Coffee conserves; Antioxidant; Solvent mixtures; Chlorogenic acid; Polyphenols

1. Introduction

The coffee plant belongs to the genus of *Coffea* (Rubiaceae family). Coffee is a giant global industry and ranks second only to petroleum in terms of dollars traded worldwide. Presently, coffee production is about 6.3 million tons, with Brazil and Colombia contributing to nearly 44% of these figures. Nevertheless, the coffee industry, especially in India, is in a crisis as excess supply over demand has led to the lowest prices for the last three decades and development of value-added products from coffee are important to arrest this trend.

Chlorogenic acid, the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols for which green coffee beans, along with certain fruits and vegetables, are one of the richest dietary sources (Clifford, 1999). The antioxidant and antineoplastic properties of coffee extracts are reported (Kroyer, Kretschmer, & Washuettl, 1989; Rosenberg, 1990; Wattenberg & Lam, 1984). A study of Robusta and Arabica coffees from six different countries showed that Robusta samples contained significantly more reducing substances than Arabica samples and that protective activity measured *ex vivo* was significantly greater in roasted coffee (Daglia, Papetti, Gregotti, Berte, & Gazzani, 2000). Green coffee has a mild, green, bean-like aroma; the desirable characteristic aroma of coffee develops during the roasting process. A roasting time of 10 min is found to be ideal for producing coffee with optimal oxygen scavenging and chain breaking activities *in vitro* (Nicoli, Anese, Manzocco, & Lerici, 1997; DEL Castillo, Ames, & Gordon, 2002; Singh & Madhava Naidu, 2002). However, during the roasting process, the naturally occurring polyphenolic constituents are transformed to a complex mixture of Maillard reaction products. Using the ABTS.+ method, light roast or medium roast coffee and coffee melanoidins have been shown to contribute to a significantly higher antioxidant activity *in vitro* (Castellucio et al., 1995). Uric acid

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responsible for plasma antioxidant capacity, increases after tea drinking, whereas molecules other than uric acid (phenolic compounds) supposedly responsible for the higher plasma antioxidant capacity after coffee drinking (Natelle, 2002). Recently, antioxidant capacity of brewed coffees from different origins have been evaluated (Parras, Martinez-Tome, Jimenez, & Murcia, 2007).

Despite several reports on the antioxidant activity of coffee from roasted coffee, the antioxidant activity of coffee conserve from green coffee in particular is not well studied. A huge quantity of green coffee is available in India for value addition, because India is the fifth largest coffee producer in the world. Therefore, evaluation of its antioxidant properties is of interest primarily in order to find new promising sources for natural antioxidants for functional foods and nutraceuticals as well as utilization of green coffee other than for beverage use. The present paper describes the preparation of green coffee conserve from green coffee beans and optimization of extraction conditions to obtain an antioxidant rich conserve (Madhava Naidu, Sampathu, Raghavan, & Venkatesh Murthy, 2006). Here, green coffee was utilized to prepare the chlorogenic acid rich conserve, and its antioxidant activity was evaluated. The antioxidant activities of these conserves were evaluated by determination of radical scavenging activity, hydroxyl radical scavenging potential, and reducing power assay. Further, the chlorogenic acid of coffee conserves was purified and characterized by HPLC and NMR techniques.

2. Materials and methods

2.1. Materials

Coffee seeds viz., Arabica parchment (AP) and Robusta parchment (RP) were procured from the local market. Caffeic acid (CA), chlorogenic acid (CGA), ferulic acid (FE), β-carotene, 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, butylated hydroxyanisole (BHA) and Tween-80 were procured from Sigma–Aldrich, Chemical Co. (St. Louis, MO), USA. All solvents and chemicals used were of analytical grade and obtained from SD Fine Chemicals, Mumbai., India.

2.2. Extraction

Green coffee seeds were sterilized at 120 °C for 20 min, and subjected to flaking to obtain flakes of 0.5–1.0 mm thickness. Coffee flakes were extracted with hexane using Soxhlet apparatus. These flakes were dried using a cross flow drier at 55 ± 5 °C for about 2–3 h to bring down the moisture level to about 10%. The dry flakes were passed through a hammer mill fitted with 30 mesh (500 μm) to get a powder which was used as the raw material for preparation of conserve. These samples, 100 g each, were loaded in to separate glass columns and extracted with selected solvents, at a material to solvent ratio of 1:10. Solvent mixtures of isopropanol and water in ratio of 80:20; 70:30; and 60:40, respectively, were used for extraction. The solvent was added in 10 installments of 100 ml each allowing 30 min contact time over a period of 5 h. The extracts were drained and pooled. The pooled extracts were distilled on a rotavapor at 50 °C under reduced pressure (40 milli bar) and the product stored at 4 °C. Recovery of coffee constituents with reference to yield of conserves, polyphenol content and % CGA were computed.

2.3. Purification of chlorogenic acid

The procedure of Ky, Noirot, and Hamon (1997) was slightly modified and employed for purification of CGA. Coffee conserve was dispersed in water and ammonium sulfate was added to a final concentration of 20 g/l in order to precipitate proteins by an increase in ionic strength followed by addition of 4% phosphoric acid to render the CGA more soluble in ethyl acetate. Extracts were treated with petroleum ether (40–60 °C) to remove lipids and pigments. De-pigmented extracts were further treated with chloroform to remove caffeine and wax. The residue was washed with ethyl acetate, distilled and air dried to obtain a light creamy colored powder. The CGA in the powder was estimated by UV-Spectrophotometry (AOAC, 2005) and characterized by NMR (Bruker Avance 500 MHz).

2.4. Determination of total phenolic content

The total phenolic concentration was measured using the Folin-Ciocalteu method (Singleton & Rossi, 1965). In this procedure, 1 ml of appropriately diluted samples and a standard solution of gallic acid were added to a 25 ml volumetric flask containing 9 ml of double distilled water. A reagent blank using double distilled water was prepared. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of a 7% sodium carbonate solution was added with mixing. The solution was then immediately diluted to a volume of 25 ml with double distilled water and mixed thoroughly. After incubation for 90 min at room temperature, the absorbance at 765 nm was measured. The total phenolic contents of the samples are expressed in milligrams per serving of gallic acid equivalents (GAE).

2.5. DPPH radical scavenging activity

Free radical scavenging activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) by method of Shimada, Fujikawa, Yahara, and Nakamura (1992). Different concentrations (50, 100 and 200 ppm) of coffee conserves and BHA were taken in different test tubes. The volume was adjusted to 100 μl by adding MeOH. Five milliliter of a 0.1 mM methanol solution of DPPH was added to these test tubes and shaken vigorously. The tubes were then incu-

bated in the dark at room temperature for 20 min. A control sample was prepared as above without any conserve, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Control optical density} - \text{Sample optical density}}{\text{Control optical density}} \times 100$$

2.6. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Klein, Cohen, and Cederbaum (1991). Various concentrations (50, 100 and 200 ppm) of conserves in ethanol were placed in different test tubes and evaporated. One ml of iron–EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80–90 °C for 15 min. The reaction was terminated by the addition of ice-cold trichloroacetic acid (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 l with distilled water) was added to all the tubes and the tubes were allowed to stand at room temperature for 15 min for color development. Intensity of the yellow color formed was measured at 412 nm against reagent blank. Percentage hydroxyl radical scavenging was calculated by the following formula:

$$\text{Percentage hydroxyl radical scavenging activity} = 1 - (\text{difference in absorbance of sample} / \text{difference in absorbance of blank}) \times 100$$

2.7. Reducing power assay

The reducing power assay of coffee conserves was determined according to the method of Oyaizu (1986). Coffee conserves (50, 100 and 200 ppm) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was centrifuged at 3000 rpm (MSE Mistral 2000, UK) for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ solution (0.5 ml, 0.1%), and the absorbance was measured at 700 nm.

2.8. Identification of chlorogenic acids by HPLC analysis

The identification of individual phenolics was carried out using a Waters HPLC system, based on matching retention times of standards. The HPLC system consisted of, Waters 515 Binary HPLC pump, Waters automated gradient controller, and Waters 2487 Dual Wavelength Absorbance Detector. The column used was a hypersil C18 column (25 cm × 4.6 mm i.d. particle size 5 μm, pore size 100 Å). The compound were eluted with a gradient elution of mobile phase A [5% acetonitrile in 0.035%–trifluoroacetic acid (TFA)] and B (80% acetonitrile in 0.025% TFA) where B increased from 10% to 20% in 10 min, to 50% by 20 min, was maintained at 50% for 5 min and remained for 5 min before next injection. Elutes were detected by a Waters 2487 Dual Wavelength Detector at wavelength of 325 nm. Solutions of each standard at various concentration levels were injected into the HPLC system, and the peak areas were recorded. Thus, the calibration curves were prepared and response factors were calculated under the same conditions.

Experimental results were expressed as means ± SD of three parallel replicates. ANOVA, correlation analysis were performed using the SPSS 13.0 for Windows. P values <0.05 and 0.01 were regarded as significant and very significant, respectively. Analysis of variance was adopted to test the differences in the antioxidant activity by various assays between crude, purified and different standard compounds. 'F' indicates the relationship i.e. either difference or similarities between the analyzed parameters.

3. Results and discussion

3.1. Effect of solvent mixtures on yields of coffee conserves

Coffee seeds viz., Arabica parchment (AP) and Robusta parchment (RP) were procured and used in the present studies. Preparation of green coffee conserves from the species *Coffea arabica* and *Coffea robusta* by flaking, powdering and extraction with solvent mixtures of isopropanol and water in different ratios and their antioxidant properties were investigated. The yield of conserves obtained from arabica and robusta coffee using various solvent mixtures are shown in Table 1. In arabica coffee, solvent mixture ratio of 60:40 (isopropanol:water) gave the highest yield (27%) followed by solvent mixtures of 70:30 (26%) and 80:20 composition (21%). With increase in the water content in the solvent mixture, yield of conserves increased in both varieties. Also, in case of robusta coffee, the higher yield (29%) was obtained from 60:40 solvent mixture followed by solvent mixtures of 70:30 (24%) and 80:20 compositions (20%), respectively. The lowest yield (20%) was obtained from robusta coffee extracts using the solvent ratio of 80:20. The CGA content in the extracts was estimated spectrophotometrically and results are presented in Table 1.

Table 1
Yield, chlorogenic acid and polyphenol content in green coffee conserves

Sample	Solvent	Ratio of solvent	Yield of extracts (%)	Chlorogenic acid content (%)	Total polyphenols (%)
Robusta coffee	Isopropanol water	80:20	20.12 ± 0.75	20.89 ± 0.04	22.89 ± 0.06
		70:30	24.32 ± 0.62	25.19 ± 0.53	26.19 ± 0.63
		60:40	29.10 ± 0.25	29.71 ± 0.15	31.71 ± 0.25
Arabica coffee	Isopropanol water	80:20	21.12 ± 0.75	20.29 ± 0.04	23.29 ± 0.06
		70:30	26.10 ± 0.25	26.71 ± 0.15	28.71 ± 0.23
		60:40	27.32 ± 0.62	30.19 ± 0.53	32.19 ± 0.63

The total polyphenol content of purified conserve (both arabica and robusta) obtained from extraction with isopropanol plus water (60:40) was found to be 32.19 ± 0.63, 31.71 ± 0.25, respectively, as gallic acid equivalents. Here again isopropanol with lesser water content viz., 70:30 and 80:20 resulted in lower yields of polyphenols. The antioxidant activity appears to be directly correlated to the CGA and polyphenol contents of green coffee extracts; thus, the isopropanol:water (60:40) extract of coffee conserve (both Arabica and robusta) showed higher activity as compared to the other extracts (Fig. 1).

3.2. DPPH radical scavenging activity

The antioxidant activity of these conserves at 50, 100, 200 ppm concentrations (which were found to be optimal for these conserves in the preliminary study varying from 10 to 1000 ppm concentrations) was evaluated using the 1,1-diphenyl-2-picrylhydrazil radical scavenging activity. Free radical scavenging potential of coffee conserve extracted with aqueous isopropanol under different concentrations was tested by the DPPH method, and the results are shown in Fig. 1. Antioxidant reacts with DPPH, which is a stable free radical and converts it to α-α-diphenyl-β-picrylhydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant conserve. At 200 ppm concentration, arabica coffee conserves (60:40, 70:30 and 80:20) exhibited 92%, 87% and 76% antioxidant activity, respectively. At 200 ppm concentrations, conserves of robusta coffee extracted with isopropanol plus water (60:40, 70:30 and 80:20) exhibit 88%, 82% and 78% antioxidant activity, respectively. These results indicate that the both conserves have a noticeable effect on scavenging free radicals. The antioxidant activity of the natural plant extracts is generally attributed to their hydrogen donating ability (Klein et al., 1991). It is well known that free radicals cause auto-oxidation of unsaturated lipids in food (Kaur & Perkins, 1991). Antioxidants are believed to intercept the free radical chain of oxidation by donating hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate oxidation of the lipids (Sherwin, 1978). The data obtained reveal that the conserves function as free radical inhibitors and primary antioxidants that interact with free radicals. Differences in the antioxidant activity between the components of coffee conserves were found to be extremely significant ($F = 486.7726^{***}$). Further, these extracts were purified and subjected to evaluate antioxidant activity using hydroxyl radical scavenging activity and reducing power assay.

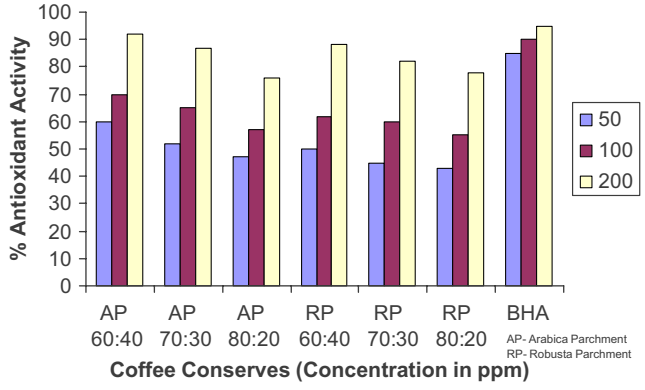

Fig. 1. Free radical scavenging activity of green coffee conserves.

3.3. Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Hochestein & Atallah, 1988). This radical has the capacity to conjugate with nucleotides in DNA and cause strand breakage, having far reaching implications leading ultimately to carcinogenesis, mutagenesis and cytotoxicity. In addition, these species are considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid–iron EDTA. The hydroxyl radicals were formed by the oxidation react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The hydroxyl radical scavenging activity of the isopropanol plus water conserves of coffee is shown in Fig. 2. Coffee conserves (both purified and crude) exhibit 80% and 92% hydroxyl radical scavenging activity at 200 ppm. The ability of the coffee conserves to quench hydroxyl radicals seems to be directly related to the prevention of propaga-

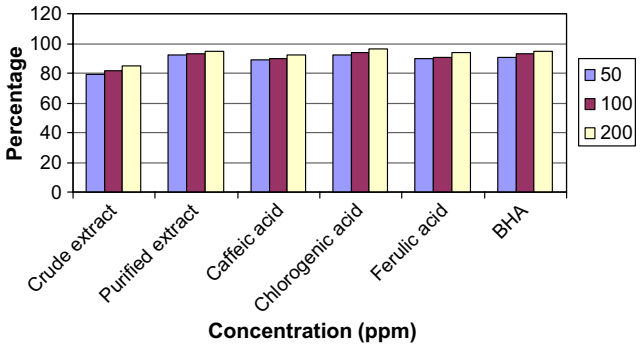

Fig. 2. Antioxidant activity by hydroxyl radical scavenging assay.

tion of the process of lipid peroxidation. They seem to be good scavengers of active oxygen species, thus reducing the rate of chain reaction. Differences in the antioxidant activity between the components of coffee conserves were found to be extremely significant ($F = 26.3825^{***}$).

3.4. Reducing power of coffee conserves

The antioxidant activities of natural components may have a reciprocal correlation with their reducing capacity (Duh, 1998). In this study, the Fe^{3+}–Fe^{2+} transformation was determined as reducing capacity. Results (Fig. 3) indicate that both the coffee conserves (crude and purified) exhibited effective reducing capacity at all concentrations. The reducing power of coffee conserves (as indicated by the absorbance at 700 nm) correlated well with increasing concentrations. Differences in the antioxidant activity between the components of coffee conserve were found to be extremely significant ($F = 3933.356^{***}$).

3.5. HPLC profiling of green coffee conserve, chemical composition, and quantification

The HPLC pattern of selected isopropanol:water extracts of green coffee conserve and purified conserve showed that CGA was the major component. Three compounds (Fig. 4) were identified and quantified. Identification was carried out using the external standard method. The CGA content in the coffee conserves based on the comparison of peak areas of each peak with that of authentic samples and from calibration curves was found to be in the range of 20–30%. Quantification of the phenolic compounds was done by peak normalization and application of the response factors obtained for individual pure standards of the compounds.

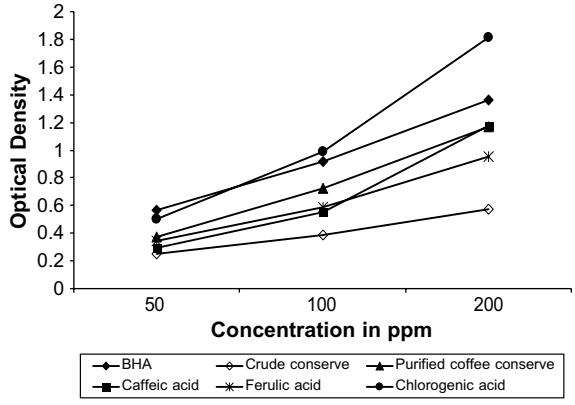

Fig. 3. Reducing power assay of coffee conserves compared with standard compounds.

After purification, the conserves was enriched with CGA and total CGA was found to be $56 \pm 11.0\%$ (Table 2). The polyphenols content as determined by spectrophotometric method was found to be $61 \pm 10\%$. Antioxidant activity assays were carried out with pure CGA, FE, and CA and a comparison of these with that of purified conserve. Antioxidant studies showed that the CGA made a greater contribution to total antioxidant activity than CA and FE.

3.6. Characterization of purified coffee conserve by NMR

Chlorogenic acid isolated from coffee was analyzed by ^{1}H and ^{13}C NMR (Figs. 5 and 6). While the caffeic acid part of the molecule was confirmed from the signals for aromatic protons [6.98 ppm (1H, d, $J = 2.00$ Hz), 6.92 ppm (1H, dd, $J = 2.00$ Hz and 8.14 Hz) and 6.76 ppm (1H, d, $J = 8.14$ Hz)] and olefenic protons [7.41 ppm (1H, d, $J = 15.96$ Hz) and 6.14 ppm (1H, d, $J = 15.96$ Hz)], the quinic acid group was evident from the signals for methine protons α to hydroxyl groups at 5.13 ppm (1H, m), 4.09 ppm (1H, m) and 3.72 ppm (1H, m) as well as for the methylene protons of the cyclohexane moiety at 1.91–2.11 ppm (4H, m). Similarly, ^{13}C spectra showed two carbonyl carbons at 176.99 and 168.39 ppm. Apart from these there were eight signals at 146.82, 145.91, 143.96, 126.66, 122.43, 115.93, 114.88 and 114.16 ppm corresponding to six aromatic and two olefenic carbons. The six carbons from the cyclohexane ring appeared at 74.69, 71.15, 70.40, 68.92, 36.31 and 36.19 ppm. In the ^{1}H NMR spectrum of the sample isolated from coffee, signals at 7.47 (1H, d, $J = 16$ Hz) ppm and 6.28 (1H, $d = 16$ Hz) corresponding to the olefenic protons and at 3.79 ppm (3H, S) from methoxy protons from ferulic acid, which is the minor component of the extract, were also observed. This is also substantiated by the HPLC profile of the sample which showed the presence of ferulic acid at 19%.

In conclusion, the green coffee contains polyphenols and CGA with high antioxidant properties. Isopropanol:water (60:40) afforded higher yield of conserves 29%, 27% in robusta and arabica coffee, respectively. At 200 ppm concentration, arabica coffee and robusta coffee conserves exhibited 92% and 88% antioxidant activity, respectively. The HPLC profiles of purified conserve were used to quantify the total CGA content and it was found to be in the range of $56 \pm 10\%$. Further, chlorogenic acid was the main

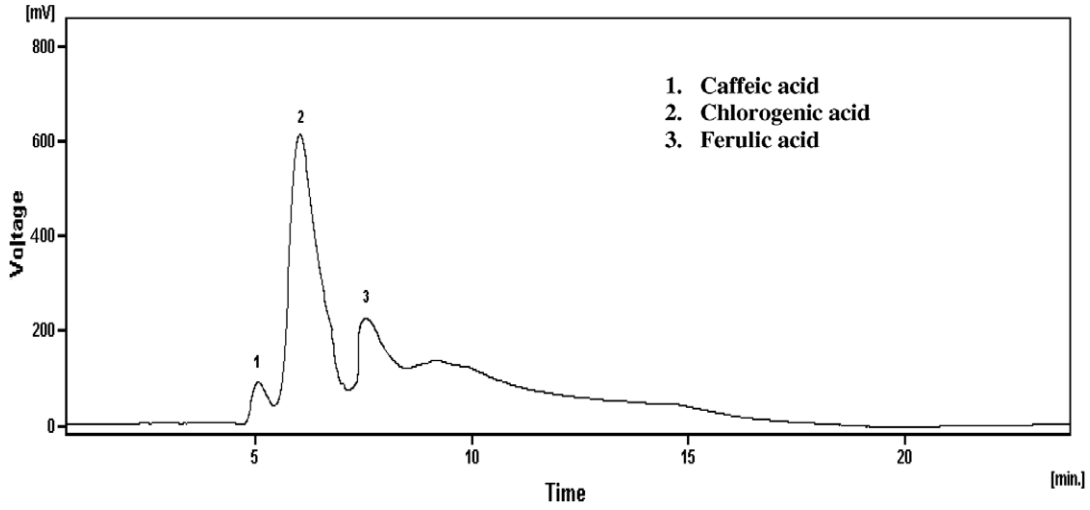

Fig. 4. HPLC profile of coffee conserve (purified conserve).

Table 2
Chemical profile of purified coffee conserve

Name of the compound	Retention time	Quantity (%)
Caffeic acid	5.05	6 ± 0.69
Chlorogenic acid	6.01	56 ± 11
Ferulic acid	7.54	4 ± 0.94

compound in the purified conserve as characterized by ^{1}H and ^{13}C NMR spectral studies. Results indicated that the conserves from green coffee possess potential antioxidant activity and could be used as nutraceuticals as well as preservatives in food formulations.

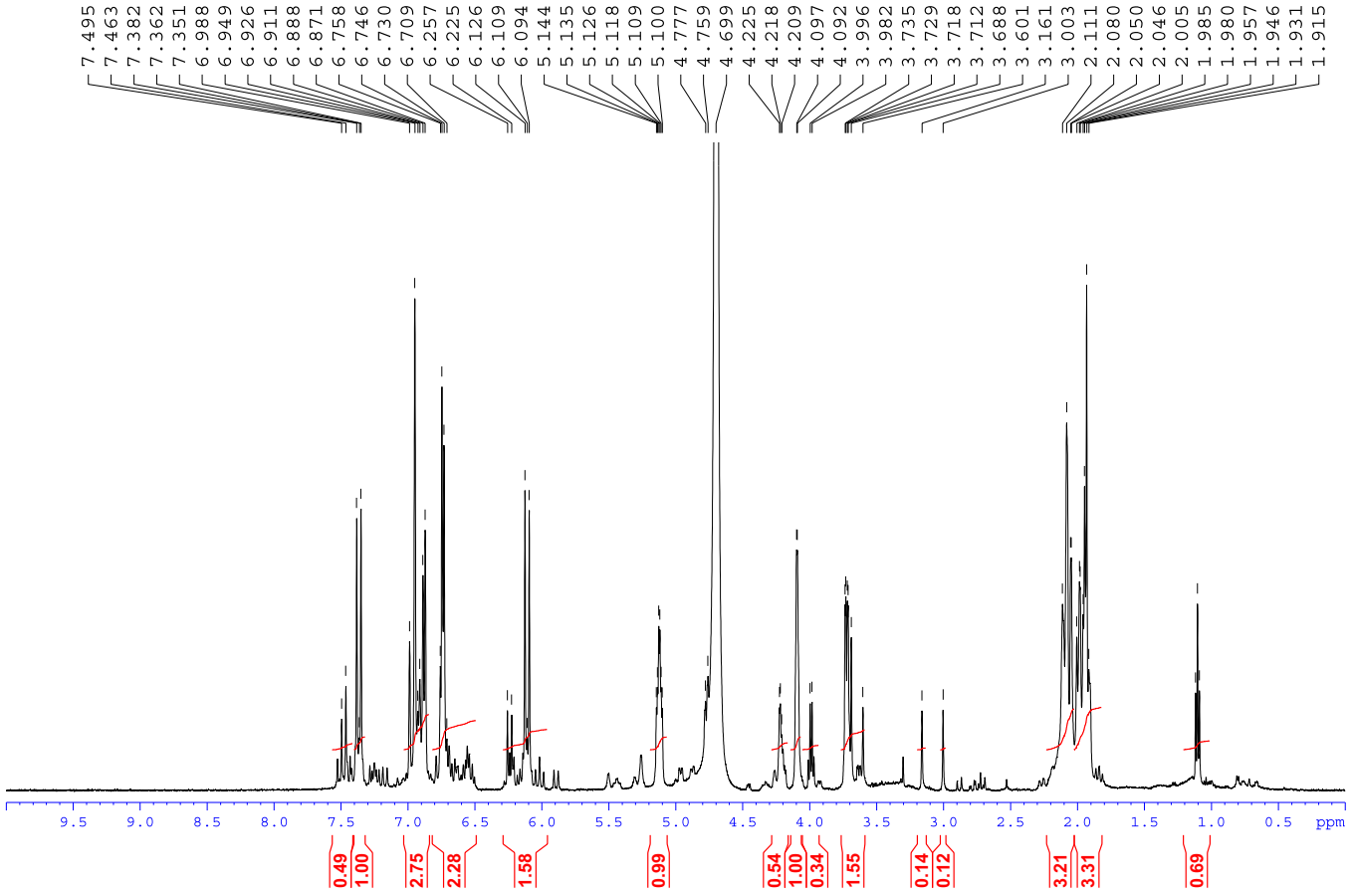

Fig. 5. ^{1}H NMR spectrum of chlorogenic acid isolated from coffee.

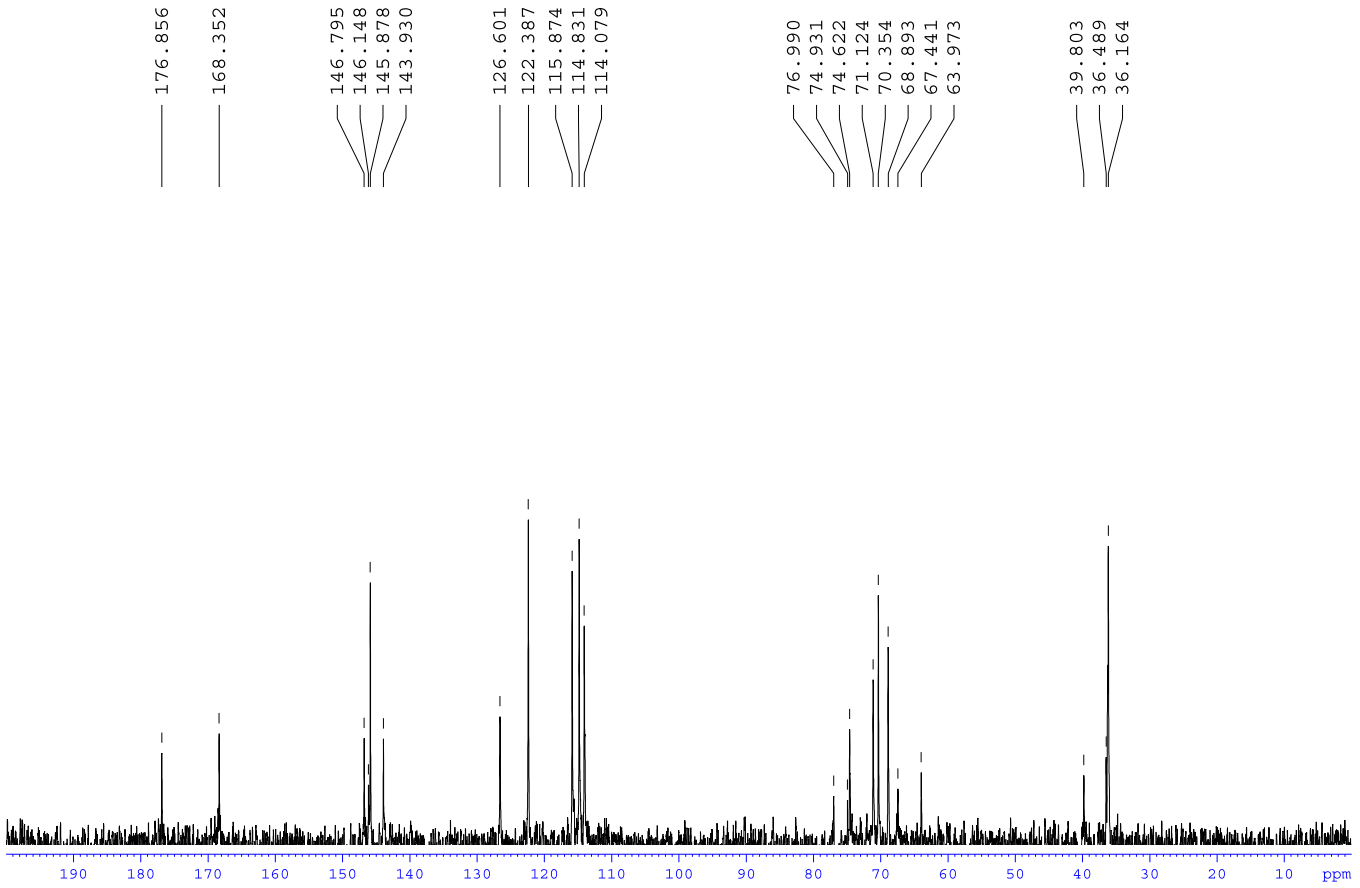

Fig. 6. ^{13}C NMR spectrum of chlorogenic acid isolated from coffee.

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