Isolation and Characterization of Phytochemicals from Caraway Seeds
2.1. Introduction

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There are more than one thousand known phytochemicals present in herbs and spices. Some of the bioactive phytochemicals are phenolic compounds including phenolic acids and flavonoids. Phenolic compounds are the most ubiquitous group of plant secondary metabolites distributed in various dietary and medicinal plants. Phenolic compounds have many diverse functions in plants viz., antimicrobial and antifungal, insect feeding deterrence, protection from damage by solar UV radiation, chelation of toxic heavy metals and as an antioxidant against free radicals generated during the photosynthetic process (Gould and Lister, 2006). Epidemiological studies have shown that consumption of foods, rich in phenolic compounds can be correlated with reduced incidence of cardiovascular diseases (Criqui and Ringel, 1994). The phytochemicals retard the progression of arteriosclerosis by acting as antioxidants towards low density lipoproteins (LDL) and neutralize the free radicals mediated oxidative stress (Frankel et al., 1993; Frankel et al., 1995; Meyer et al., 1998).

2.1.1. Phenolics

Phenolic compounds embrace a wide range of secondary metabolites that are synthesised from carbohydrates via the shikimate pathway. Phenolics encompass approximately 8,000 naturally occurring compounds, all these possess the common structural feature, a phenol.

2.1.1.1. Classification of phenolic compounds

Current classification divides the broad category of phenolics into polyphenols and simple phenols based solely on the number of phenol sub units present (Clifford,
Phenolic acids are phenols that possess one carboxylic acid functional group. Polyphenols possessing at least two phenol subunits include the flavonoids and those compounds possessing three or more phenol subunits are known as tannins.

### 2.1.1.1. Phenolic acids

Naturally occurring phenolic acids contain two distinguishing constitutive carbon frame works: hydroxy benzoic and the hydroxy cinnamic structures. Although the basic skeleton remains the same, the number and positions of the hydroxyl groups on the aromatic ring create the variety.

#### 2.1.1.1.1. Benzoic acid derivatives

![Benzoic acid derivatives](image)

Gallic acid $R_1=R_2=R_3=OH$, Protocatechuic acid $R_1=H$, $R_2=R_3=OH$
Vanillic acid $R_1=H$, $R_2=OH$, $R_3=OCH_3$, Syringic acid $R_2=OH$, $R_1=R_3=OCH_3$

#### 2.1.1.1.2. Cinnamic acid derivatives

![Cinnamic acid derivatives](image)

Ferulic acid $R_1=R_2=H$, $R_3=OH$, $R_4=OCH_3$, p-Coumaric acid $R_1=R_2=R_4=H$, $R_3=OH$
O-Coumaric acid $R_2=R_3=H$, $R_1=OH$, Caffeic acid $R_1=R_2=H$, $R_3=R_4=OH$
Sinapic acid $R_1=H$, $R_3=OH$, $R_2=R_4=OCH_3$
2.1.1.2. Flavonoids

Flavonoids are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain (C$_6$-C$_3$-C$_6$ system). Many flavonoids are easily recognized as flower pigments in most flowering plant families.

![Chemical structure of flavonoids](image)

The chemical structure of flavonoids are based on a C-15 skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3 or 4. Sometimes, the six-member heterocyclic ring C occurs in an isomeric open form or is replaced by a five membered ring. Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification. There are 6 major subgroups of flavonoids, they are:

2.1.1.2.1. Chalcones

![Chemical structure of chalcones](image)

Butein 2’=4’=3=4=OH, Okanin 2’=3’=4’=3=4=OH, Licochalcone 4’=3=4=OH, 2=OCH$_3$, Chalconarigenin 2’=4’=6’=4=OH
2.1.1.2.2. Flavone

![Flavone Structure]

- Apigenin $5=7=4'=$OH,
- Diosmetin $5=7=3'=$OH, $4'=$OCH$_3$,
- Sinenstein $5=6=7=3'=$OCH$_3$,
- Isovitexin $5=7=4'=$OH, $6=$Glucose

- Luteolin $5=7=3'=$OCH$_3$
- Tricin $5=7=4'=$OH, $3'=$OCH$_3$
- Nobelitin $5=6=7=8=3'=$OCH$_3$

2.1.1.2.3. Flavonol

![Flavonol Structure]

- Fisetin $7=3'=$OH,
- Morin $5=7=2'=4'=$OH,
- Quercetin $5=7=3'=$OH,
- Isorhamnetin $5=7=4'=$OH, $3'=$OCH$_3$,
- Gossypetin $5=7=8=3'=$OH

- Kaempferol $5=7=4'=$OH
- Herbacetin $5=7=8=4'=$OH
- Robenetin $7=3'=$OH
- Myricetin $5=7=3'=$OH

2.1.1.2.4. Flavonol glycosides

- Rutin – Quercetin 3-O- rhamnosylglucoside,
- Hyperin quercetin 3-O-β-D-galactopyranoside
2.1.1.2.5. Flavanone

\[ \text{Naringenin } 5=7=4'=\text{OH,} \]
\[ \text{Friedictylol } 5=7=3'=4'=\text{OH,} \]

2.1.1.2.6. Flavanone glycosides

\[ \text{Prunin – Narigenin } 7\text{-glucoside,} \]
\[ \text{Naringin – Narigenin } 7\text{-neohesperidoside} \]
\[ \text{Narirutin - Narigenin } 7\text{-rutinoside,} \]
\[ \text{Hesperidin – Hesperitin } 7\text{-rutinoside} \]

2.1.1.2. Identification and characterization of phenolic compounds

Although, the number of identified phenolic compounds is increasing exponentially, the phenolic content of most plants constitutes a complex mixture, the chemical nature of which has not yet been completely elucidated for many plant species. Isolation and structural elucidation of these phytochemicals are the initial steps to understand their physiological and biological action.

Most of the phenolic acid derivatives present in the plant matrix are stored in vacuoles. The commonly used solvents for extraction are water, methanol, ethanol, acetone and ethyl acetate. Extraction periods vary from 1 to 6 h using Soxhlet’s apparatus (Sun et al., 2001). Mechanical means are employed to enhance molecular interaction viz., vortexing (Montedoro et al., 1992), sonication, mechanical stirring and continuous rotary extraction (Guillen et al., 1996).
Hydrolysis of the ester bond to carboxylic acid has been one of the strategies employed to gain a more specific picture of the phenolic acid profile in plants. There are two main procedures to cleave the ester bond, namely acid hydrolysis and alkali hydrolysis or saponification. A third, less prevalent technique is cleavage through the use of enzymes (esterases). Although reaction time and temperature for the acid hydrolysis conditions vary a great deal, the general method involves extraction of plant material in aqueous or alcoholic solvents at reflux or above reflux temperatures for 30 min to over night at room temperature. Saponification involves extraction of the sample with 1-4M NaOH. Enzymatic reactions have been reported to release phenolic acids (mainly ferulic and p-coumaric acids). Pectinases, cellulases and amylases are usually used for cleaving the carbohydrate linkages. Acid hydrolysis results in the cleavage of acetal or hemiacetal bonds between carbohydrate moieties and the hydroxyl groups of the aromatic ring and not by ester cleavage reactions.

Historically, the most common colorimetric method employed for the quantification of phenolic content was the Folin-Ciocalteu reagent (FCR). FCR involves the reduction of a phosphomolybdic-phosphotungstic acid to a blue coloured complex by phenols in alkaline solution. The blue coloured phosphomolybdic-phosphotungstic phenol complex generated in solution gives detectable absorbance at 760 nm (Singleton and Rossi, 1965). Gallic acid is used as a standard and the total phenol content is expressed as gallic acid equivalents (GAE). Identification of individual phenolic compound is not possible with this method.

For the last two decades, the analytical technique that has dominated the separation and characterization of phenolic compounds is HPLC with reverse phase (RP)
column technology coupled with mass spectroscopy (MS). Many reviews have been published on separation of the flavonoids and phenolic acids (Robards and Antolovich, 1997; Robards et al., 1999; Escarpa and Gonzalez, 2001). Considering the diversity of stationary phases available, columns chosen for the determination of phenolic acids are exclusively composed of a C$_{18}$ stationary phase with an internal diameter (i.d) ranging from 2.1 to 5mm, particle size of 3 or 5µm and column length of 100 to 300mm. Investigations using mass spectrometry coupled to liquid chromatography report using columns with smaller i.d of 1.1mm to 2.1mm (Cartoni et al., 2000).

Although there is a large variation in percent composition of solvent systems, the types of solvents used for separation of phenolic acids remain fairly consistent. Gradient elution systems are more frequently employed than their isocratic counterparts. Linear gradients involving an aqueous phase and an organic phase are commonly used in the separation of phenolic compounds. Typically, an acid is added to the aqueous and alcoholic phase (Delage et al., 1991). Acetic acid is the most commonly used acidic additive, but, sulphuric acid, perchloric acid, phosphoric acid (Spanos and Wrolstad, 1990), trifluoroacetic acid (Beveridge et al., 2000), hydrochloric acid (Delage et al., 1991) and formic acids are also being used (Salagoity-Auguste and Bertrand, 1984; Benassi and Cecchi, 1998). Methanol and acetonitrile are the predominantly used organic solvents with the acidic aqueous medium, but propanol, butanol, tetrahydrofuran and ethyl acetate are also employed in separation of phenolic acids. In certain investigations, the mobile phases (A and B) consist of mixtures of the organic and aqueous solvents. Borzillo et al., (2000) suggested solvent A as methanol-acetic acid-water (10:2:88, v/v/v) and solvent B as methanol-acetic acid-water (90:2:8, v/v/v). In some of other methods,
the mobile phases contain buffers such as \( \text{H}_3\text{PO}_4/\text{KH}_2\text{PO}_4 \) or acetic acid/ammonium acetate and sodium citrate buffer (pH 5.4). The run times for the methods vary a great deal, ranging from 30 to 150 min and the column is kept at constant temperature for reproducible separation of phenolic acids.

Detection techniques for HPLC separation of phenolic compounds are by UV-Visible with photodiode array (PDA) detector, with monitoring wavelengths from 190-380 nm. Phenolic acids with benzoic acid carbon framework have their \( \lambda_{\text{max}} \) in the range of 200-290 nm. The only exception is gentisic acid, which has an absorbance that extends to 355 nm. The cinnamate derivatives, due to the additional conjugation, show an additional broad absorbance band from 270 to 360 nm. The single most common wavelength routinely used for monitoring phenolic compounds is at 254 nm.

Reverse phase-high performance liquid chromatography (RP-HPLC) represents the most popular and reliable technique for the analysis of phenolic acids. Compound elution typical of RP-HPLC is that polar compounds elute first, followed by those of decreased polarity (Nogata et al., 1994). Detection is usually based on absorption of UV or less commonly, visible radiation at various wavelengths which are the characteristic of the class of phenolic compounds (Bengoechea et al., 1995). Mass spectrometric determination of molecular weight of the individual components in the sample enables more precise identification of compounds and provides specific information on both qualitative and quantitative detection of phenolic compounds in high resolution chromatography (Angerosa et al., 1995).
Development of analytical methods for phenolic acids using LC (Liquid chromatography) coupled to MS detection is an emerging field. Diverting a portion of the solvent allows for a small flow rate (11µL/min) into the electron spray interface (ESI) and MS for ion generation and detection, respectively. The mass detection portion was carried out in the negative ion mode (i.e., [M-H]). High sensitivity with detection limits ranging from 1-6ng can be detected with LC-MS-ESI technique. Retention time and mass [M-H]¯ confirm the identification of phenolic compounds. The interface between the liquid chromatography and the MS detector are carried out with atmospheric pressure ionization (API). After determining the precursor ion in full scan negative-ion mode, the product ions are determined using MS/MS.

In this chapter, the data on proximate composition of caraway seeds, total phenolic compounds, separation and quantification of individual phenolic compounds from caraway phenolic extract by HPLC-MS method are presented.

2.2. Materials and Methods

2.2.1. Chemicals

Standard fatty acid esters and phenolic compounds namely gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol were purchased from Sigma Chemical Co., MO, USA. Glucose, potassium persulfate, copper sulphate, boric acid and ammonium sulphate were purchased from Hi-media, Mumbai, India. Folin-Ciocalteu reagent was purchased from Sisco research laboratories, Mumbai, India. All other chemicals and solvents used were of analytical grade.
2.2.2. Caraway seeds

Caraway seeds were obtained from local market, identified and authenticated by Dr. H.B. Singh, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India.

2.2.3. Garbling

The dirt and extraneous matter from the seeds were removed by hand.

2.2.4. Grinding

Grinding of the seeds was done in a mixer grinder.

2.2.5. Determination of proximate composition

2.2.5.1. Moisture

The moisture content of caraway seeds was determined according to the standard method of AOAC. The sample (2g) was weighed in an aluminium dish and placed in a hot air oven maintained at 130±1°C for 4h and was then cooled to room temperature in a desiccator and the weight loss in percentage was reported as moisture content of the seeds.

2.2.5.2. Carbohydrate

The carbohydrate content of caraway seeds was estimated by phenol-sulphuric acid method (Dubois et al., 1956). The sample (0.5mL) was mixed with 0.3mL of 5% phenol to which 1.8mL of concentrated sulphuric acid was added and mixed thoroughly. After 20min of incubation at ambient temperature, the absorbance was measured at
480nm using a spectrophotometer. The carbohydrate content was determined by referring to the standard graph prepared using D-glucose (0-50µg/0.5mL).

2.2.5.3. Protein

The protein content of caraway seeds was estimated by micro-Kjeldahl method according to the method of AOAC. The sample (1g) was digested with concentrated \( \text{H}_2\text{SO}_4 \) (20mL) in the presence of catalytic mixture (98 parts of potassium sulphate and 2 parts of copper sulphate) till the solution became clear. The contents of the flask were cooled and the volume was made upto 100mL with water in a volumetric flask. The 5mL of the digested material was steam distilled in presence of 10mL of 40% NaOH. The liberated ammonia was absorbed into a container containing 10ml of 2% boric acid and a few drops of methyl red indicator was added. This solution was titrated with 0.01N HCl, till it became bluish green. Simultaneously, a running blank was processed as above, with water in place of the sample. Ammonium sulphate solution was used as the standard to estimate the amount of nitrogen content of the sample.

2.2.5.4. Lipid

Total lipid content of caraway seeds was determined according to the method of AOAC. 10g of the powdered caraway seeds was packed in a thimble and extracted with 200mL of hexane in a Soxhlet’s apparatus at 60°C for 16h. The extract was transferred to a previously weighed, dry flat bottom flask and the solvent was evaporated over hot water bath. The flask was dried, cooled and final weight was taken. The fat content was expressed as g/100g of sample (percentage).
2.2.5. Ash

The ash content of caraway seeds was determined by gravimetric method according to the procedure described by AOAC. 10g of caraway seeds was weighed in a clean silica crucible and heated in a muffle furnace for 5h at 550ºC and the crucible was cooled in a desiccator. The weight of the ash was determined and expressed as percentage of original sample.

2.2.6. Aqueous extract of caraway seeds

The sample (10g) of defatted caraway seed powder was extracted with 100mL of distilled water by magnetic stirring overnight and the process was repeated thrice. The pooled extract was lyophilized and stored under refrigeration until use.

2.2.7. Aqueous methanol extract of caraway seeds

100 g of defatted caraway seed powder was soaked in 1L mixture of methanol and water (1:1 v/v) and kept at ambient temperature for 48h with occasional shaking. The solvent was filtered with handmade filter paper and the procedure was repeated thrice. The pooled extract was concentrated and treated with 3 volumes of hexane and the aqueous phase was recovered, concentrated and dried. The dried extract was refrigerated until use.

2.2.8. Water-methanol-acetone extract of caraway seeds

10g of the defatted caraway seed powder was extracted with a mixture of 100mL of water, methanol and acetone (7:7:6 v/v/v) with constant stirring on a magnetic stirrer. The extraction was repeated thrice and filtered using filter paper. The pooled extract was centrifuged and concentrated to half volume in a rota vapour at ambient temperature.
Caraway seeds Powder

Defatted with hexane → Hexane extract

Defatted powder

Extraction with water, methanol and acetone (7:7:6) → Residue

Extract

The extract is filtered, centrifuged and concentrated under vacuum

Concentrated extract

Hydrolysis with 2N HCL

Hydrolyzed extract

Phase separation with hexane (1:3 v/v)

Defatted and hydrolyzed extract

Phase separation with Ethyl acetate (1:3 v/v)

Ethyl acetate phase

Concentrated and dried under vacuum

Dried extract

Figure 2. Flow chart showing the extraction and hydrolysis of phenolic compounds from caraway seeds.
The concentrated extract was hydrolyzed with 2N HCl and treated with three volume of hexane to remove traces of fatty matter. The hydrolyzed phenolics were extracted into equal volume of ethyl acetate and the process was repeated thrice. The ethyl acetate phase was concentrated and dried. The dried extracts were stored under refrigeration until use.

2.2.9. Estimation of total phenolic content

Total phenolic content of caraway extract was measured according to Folin-Dennis method as described by Singleton and Rossi (1965). 50µg of caraway extract was dissolved in 0.5mL of methanol and the sample was incubated with 2.5mL of 10% FC reagent for 2min at room temperature. To this, 2mL of 7.5% Na₂CO₃ was added and incubated for 1h at ambient temperature. The absorbance was measured at 765nm against a blank using UV-Visible spectrophotometer. Gallic acid was used as standard. Total phenolic content in the extract was expressed as gallic acid equivalents (GAE).

2.2.10. Separation and identification of phenolic compounds of caraway extract by HPLC and LC-MS methods

2.2.10.1. HPLC

Phenolic extract of caraway was dissolved in methanol and subjected to HPLC for qualitative and quantitative analysis. The HPLC system (Shimadzu) is equipped with dual pump LC-10AT binary system, UV detector SPD-10A, Phenomenex Luna reverse phase C_{18} column (i.d. 4.6mm×250mm) and the data was integrated by Shimadzu Class VP series software. The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile. The gradient programme for HPLC was 20% B at 0min, 30% at 15min and
finally to 60% at 40min. The amount of phenolic compounds was calculated by comparing the peak area (254nm) of the individual phenolic compounds with that of standards. Known quantities of phenolic compound standards such as caffeic acid, ellagic acid, ferulic acid, gallic acid, protocatechuic acid, quercetin and kaempferol were used for the identification and quantification of phenolic compounds present in the extract of caraway seeds.

### 2.2.10.2. HPLC-ESI-MS

An API 200 triple quadrupole mass spectrometer was used for determining the mass of the phenolic compounds. Analyses were performed on a Turbo ions spray source in negative mode by using nebuliser gas (N$_2$), focusing potential $-400V$, entrance potential 10, declustering potential (DP) 25–60 and collision energy (CE) 15–35. Full scan acquisition was performed by scanning from $m/z$ 150–700u at a cycle time of 2s. MS product ions were produced by collision-associated dissociation (CAD) of the selected precursor ions in collision cell. In all experiments, both the quadrupoles (Q$_1$ and Q$_2$) were operated at unit resolution. Product ion scan of selected molecules were carried out in order to confirm the structure of compounds.

### 2.2.11. Statistical analysis

Statistical analysis was done using the software SPSS. The differences in mean values were tested using one-way analysis of variance (ANOVA) and Duncan’s multiple range test (DMRT) was used to determine the significant differences amongst the test materials. Differences were considered to be significant at $P\leq 0.05$. 

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2.3. Results

Caraway seed powder was used for determining the proximate composition and defatted seed powder for profile of phenolic compounds. The phenolic compounds in the phenolic extract were separated and quantified by reverse phase HPLC coupled with mass spectrometry.

2.3.1. Proximate composition of caraway seeds

The proximate composition of caraway seeds is presented in Table 1. The data indicates that the caraway seeds are good source of carbohydrate and fiber. The carbohydrate content was 50.1% and the fiber content was 25.3% whereas the amount of protein, lipid and ash were found to be 7.6%, 8.7% and 4.1%, respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>4.2 ± 0.10</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>50.1 ± 0.96</td>
</tr>
<tr>
<td>Protein</td>
<td>7.6 ± 0.06</td>
</tr>
<tr>
<td>Lipid</td>
<td>8.7 ± 1.50</td>
</tr>
<tr>
<td>Ash</td>
<td>4.1 ± 0.47</td>
</tr>
<tr>
<td>Fiber</td>
<td>25.3 ± 1.56</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of three estimations

2.3.2. Total phenol content

Caraway seeds were extracted with water, 50% aqueous methanol and a mixture of water-methanol-acetone, separately. The total phenolic content in these extracts was determined by FC method and the values were expressed as GAE (Figure 3). Significant
variation in the phenolic content was observed in different extracts of caraway. The water-methanol-acetone extract showed highest total phenolic content of 50.20mg/g. The water extract showed lowest total phenolic content of 8.76mg/g. However, 50% aqueous methanol extracted total phenols to an extent of 12.63mg/g. Thus, water-methanol-acetone extracted 5.73 fold higher total phenols as compared to aqueous extract.

![Figure 3. Total phenolic content of different extracts of caraway. Values are mean ± SEM of three estimations.](image)

The total phenolic content was lowest in aqueous extract. Hence water-methanol-acetone extract with highest total phenol content was used for further studies such as characterization of phenolic compounds, antioxidant, anti-inflammatory and antimicrobial properties of caraway.
2.3.3. Isolation and identification of phenolic compounds by HPLC and LC-MS

Most phenolic compounds are present normally as glycosides in plants. Hence caraway phenolic extract using water-methanol-acetone was subjected to acid hydrolysis (with 2N HCl) to break the glycoside linkages and the free phenolic compounds in hydrolysate were separated, identified and quantified by LC-MS. HPLC profile of phenolic compounds is shown in Figure 4.

![HPLC chromatogram of phenolic extract of caraway.](image)

The peaks identified are 1) Gallic acid, 2) Protocatechuic acid, 3) Caffeic acid, 4) Ellagic acid, 5) Ferulic acid, 6) Quercetin and 7) Kaempferol.

The identification and quantification of individual phenolic compound was achieved by comparing retention time and the peak area of phenolic compounds present in the extract with that of standards. Interestingly, caraway contained a mixture of phenolic acids including gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic...
acid and flavonols such as quercetin and kaempferol (Table 2). Quantitatively, different phenolic acids in caraway extract were found to be 0.475mg of caffeic acid/g, 0.350mg of ferulic acid/g, 0.148 mg of gallic acid/g, 0.125mg of ellagic acid/g and 0.105mg of protocatechuic acid/g. Further, two flavonoid compounds were estimated to be 0.129mg of quercetin/g and 0.69mg of kaempferol/g. Caffeic and ferulic acids were the major phenolic acids and quercetin was the major flavonoid among phenolic compounds present in caraway. The concentration of various phenolic compounds identified from phenolic extract of caraway was in the decreasing order of caffeic acid> ferulic acid> gallic acid > quercetin > ellagic acid > protocatechuic acid > kaempferol. The structure of phenolic compounds was further confirmed by LC-MS. LC-MS characteristics of identified phenolic compounds are given in Table 6 and Figure 5 and 6.

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Concentration (µg/g of seed powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>475 ± 18</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>350 ± 14</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>148 ± 11</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>125 ± 13</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>105 ± 16</td>
</tr>
<tr>
<td>Quercetin</td>
<td>129 ± 15</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>69 ± 12</td>
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Table 6. HPLC retention time and fragments of phenolic compounds identified from phenolic extract of caraway through LC-MS

<table>
<thead>
<tr>
<th>Retention time</th>
<th>[M-H]⁻</th>
<th>Fragmented ion</th>
<th>Corresponding fragment</th>
<th>Compound</th>
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<tr>
<td>6.15</td>
<td>169</td>
<td>125</td>
<td>M-COO⁻</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>8.19</td>
<td>153</td>
<td>109</td>
<td>M-COO⁻</td>
<td>Protocatechuic acid</td>
</tr>
<tr>
<td>13.25</td>
<td>179</td>
<td>135</td>
<td>M-COO⁻</td>
<td>Caffeic acid</td>
</tr>
<tr>
<td>17.44</td>
<td>300.8</td>
<td>170</td>
<td>M-125</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>Trihydroxy benzene fragment</td>
<td></td>
</tr>
<tr>
<td>21.02</td>
<td>193</td>
<td>178</td>
<td>M-O⁻</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>149</td>
<td>M-COO⁻</td>
<td></td>
</tr>
<tr>
<td>29.00</td>
<td>301.1</td>
<td>151</td>
<td>M- Free phenol at 2 position and a portion of the benzopyranone ring moiety</td>
<td>Quercetin</td>
</tr>
<tr>
<td>37.09</td>
<td>285</td>
<td>133</td>
<td>M-151</td>
<td>Kaempferol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>151</td>
<td>Free phenol at position 2 and a portion of the benzopyranone part</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. Mass spectra of phenolic compounds present in caraway phenolic extract.
2.4. Discussion

The proximate composition, total phenols and individual phenolic compounds were estimated to understand phytochemical profile of caraway seeds. Caraway seeds contained 4.2% moisture, 50.1% carbohydrate, 7.6% protein, 8.7% fat, 4.1% ash and 25.3% of fibre. A comparative account of proximate composition of black cumin and cumin seeds are presented in (Table 7). Caraway seeds are rich in carbohydrate and fibre content. In comparison, black cumin seeds (Nigella sativa) as well as cumin seeds (Cuminum cyminum) are rich in carbohydrate, protein and fat content (Takruri and Dameh, 1998).
Table 7. A comparative analysis of proximate composition of different cumin varieties (g/100g)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Black cumin (Nigella sativa)</th>
<th>Cumin (Cuminum cyminum)</th>
<th>Caraway (Carum carvi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>11.9</td>
<td>3.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>36.6</td>
<td>24.1</td>
<td>50.1</td>
</tr>
<tr>
<td>Protein</td>
<td>18.7</td>
<td>23.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Lipid</td>
<td>15.0</td>
<td>36.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Ash</td>
<td>5.8</td>
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<td>4.1</td>
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<tr>
<td>Fiber</td>
<td>12.0</td>
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<td>25.3</td>
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</tbody>
</table>

The present study has focused on the isolation and characterization of phenolic compounds from caraway and their biological effects in terms of antioxidant, anti-inflammatory and antimicrobial activity. Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological properties. Epidemiological studies have shown that consumption of food, rich in phenolic content can reduce the risk of heart disease by slowing the progression of atherosclerosis by acting as antioxidants towards low-density lipoprotein (LDL) (Kinsella et al., 1993). Traditional methods for the determination of the phenolic content relied on colorimetric measurement of total phenols using one of a number of reagents of varying selectivity. Folin-Ciocalteu reagent is the classic reagent recommended for total phenols (Conde et al., 1997; Khokhar and Magnusdottir, 2002). Eventhough FC reagent is nonspecific to phenolic compounds, the total phenol estimation by FC reagent is convenient and has become a routine assay in studying phenolic antioxidants.
The total phenolic content of caraway showed wide variation with the solvents used for extraction. Often a combination of solvents will provide optimum recovery of all phenols or at least a limited range of phenols (Robards, 2003). With dried materials alcoholic solvents presumably rupture cell membranes and enhance the extraction of endocellular materials (Robards, 2003). Extraction method and solvent choice are generally critical and no single solvent will provide optimum recovery of all phenols or even a limited range of phenols. Aqueous mixture of methanol (Conde et al., 1997), ethanol (He et al., 1997) or acetone (Guyot et al., 1998) are often the solvents of choice for recovery of a wide range of phenols from diverse sample types including oats (Peterson et al., 2001), fruits and vegetables (Wang and Sporns, 2000; Lister et al., 1994) and spices (Areias et al., 2000). Hexane is generally employed for extracting non-polar components of a biological sample and it is widely employed in the extraction of lipids. Moreover preliminary cleanup of the sample by a non-polar solvent will give more exact recovery of polar compounds like phenolics. The present study employed water, methanol and acetone as solvents to extract the total phenolics from defatted powder of caraway seeds. Phenolic extract extracted with aqueous methanol and acetone of caraway seeds resulted in highest total phenol content (50.20±10mg/g) followed by 50% aqueous methanol 12.63±5mg/g, while aqueous medium extracted lowest total phenol content of 8.76±1mg/g indicating combination of aqueous and organic polar solvents extract more total phenols than aqueous medium alone.

Phenolics are a diverse class of secondary plant metabolites. All plant phenolic compounds share one common feature, namely an aromatic ring with at least one hydroxyl substitute, but may vary greatly in their complexity from simple phenols to the
highly polymerized tannins and lignins. They occur predominantly as conjugates with sugars (mono-, di-, or oligosaccharides), with glucuronic or galacturonic acids, or even with other phenols that are linked to hydroxyl groups or, less frequently, aromatic carbon atoms. In plants, phenolic compounds fulfil essential physiological purposes, such as protection from ultraviolet radiation, pathogens and predators, contribute to their colour and flavour and facilitate growth and reproduction (Bravo, 1998; Harborne and Williams, 2000; Heim et al., 2002).

The phenolic compounds from caraway seeds were extracted with aqueous 70% methanol and 70% acetone to facilitate extraction of both low and high molecular weight phenolic compounds. Thus, with the above solvent system the present investigation could extract a number of phenolic acids and flavonols from caraway (Table 5). The total phenol content of this extract was estimated to be 50.2±10mg/g of defatted powder.

Qualitative and quantitative analysis of individual phenolic compounds is difficult because most phenolic compounds are normally present as glycosides in plants. Hydrolysis of phenolic glycosides to their corresponding aglycones offers a practical method for the quantification of phenolic acids in foods. The rate of acid/base hydrolysis of glycosides depends on the acid/base strength, nature of the sugar moiety and its position in the phenolic acid nucleus. In this study a simple extraction protocol involving 70% methanol and 70% acetone was adopted for extraction of phenolic glycosides followed by a hydrolysis step with 2N HCl and phenolic acids were analyzed as aglycones. Aqueous methanol acetone extract of caraway was subjected to acid hydrolysis (2N HCl) for 30min to break the glycoside linkages. The protocol adopted to
extract phenolic compounds from caraway seeds is presented in Figure 2. Further, the phenolic compounds in hydrolysate were separated, identified and quantified by LC-MS.

HPLC-MS is a fast and reliable method for structural analysis of non-volatile phenolic compounds, since better techniques (interfacing systems) have been developed for the removal of the liquid mobile phase before ionization (Careri et al., 1998). Pietta et al., (1994) showed that thermospray LC-MS is an excellent technique for the analysis of flavonol glycosides from medicinal plants. Positive ion fast atom bombardment MS and tandem MS have been used to study the glycosidic linkages in diglycosyl flavonoids (Li and Claeys, 1994). HPLC electron spray ionisation (ESI)-MS offers advantages in terms of sensitivity and capacity for the analysis of large, thermally labile and highly polar compounds (Robards and Antolowich, 1997; Careri et al., 1998).

Figure 7. Pie Diagram showing different phenolic compounds present in caraway seeds and their relative concentration.
The phenolic compounds in caraway were separated and identified by reverse phase HPLC. The reverse phase C$_{18}$ column combined with a gradient elution system of increasing hydrophobicity was chosen because all compounds of interest were successfully separated. The two solvents were used for gradient elution were 2% acetic acid in water as solvent A and acetonitrile as solvent B. The identification of the individual phenolic compounds was achieved by comparing retention time and the peak area of extracted compounds with that of reference standards. Interestingly, caraway contained a number of phenolic acids including gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and also flavonols such as quercetin and kaempferol (Figure 7). Caffeic and ferulic acids were found to be most abundant among phenolic acids present in caraway seeds. Quercetin was found to be the main flavonol in caraway.

2.5. Conclusion

Caraway seeds contained a mixture of important phenolic compounds such as gallic acid, protocatechuic acid, caffeic acid, ellagic acid, ferulic acid and also flavonols such as quercetin and kaempferol. The biological properties of these phenolic compounds such as antioxidant, anti-inflammatory and antimicrobial activities were studied in detail in in vitro model systems and presented in chapter 3, 4 and 5.