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Original article

Chemical composition, flavonoid - phenolic contents and radical scavenging activity of four major varieties of cardamom

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ABSTRACT

The relative levels of antioxidant activity, total flavonoid content, total phenolic content and reducing power of different organic and aqueous extracts with hexane sequentially extracted , dichloromethane, ethyl acetate, methanol and water of four different varieties of cardamom viz. Mysore, Malabar, Vazhukka and Guatemala have been studied. Ethyl acetate extract of all varieties showed greater activity. Based on the results Malabar variety was identified as the best source of antioxidant compounds. Chemical analysis of the samples was carried out to calculate the percentage amount of components present in the sample. The data given are in the normal range reported for the varieties. Chemical compositions of the essential oil of seed powder of these varieties were studied by GC and GC-MS. The main constituents identified were terpinyl acetate ranging between 61.65 % - 68.19 % followed by cineol (7.23 %-11.76 %).

1. Introduction

Cardamom (Elettaria cardamomum Maton), the Queen of all spices has a history as old as human race. It is one of the high priced and exotic spices in the world. It is the dried fruit of an herbaceous perennial plant belonging to the ginger family, Zingiberaceae. The plant is indigenous to southern India and Sri Lanka. It is also cultivated in Guatemala. There were initially three natural varieties of cardamom plant. They are Malabar, Mysore and Vazhukka, a naturally occurring hybrid between Malabar and Mysore varieties.

The major use of Cardamom on world wide is for domestic culinary purpose and in medicine. The seeds have a pleasant aroma and a characteristic warm, slightly pungent taste. The aroma and medicinal properties of cardamom are due to the volatile oil present in it and it is obtained from the seeds by steam distillation. The composition of cardamom oil has been studied by various workers [1-4] and the major compounds found were 1, 8 cineole (20-60 %) and α-terpinyl acetate (20-55 %). It has been established that the oils and extracts from spices usually used to flavour dishes are excellent source of natural antioxidant and they also find use as nutraceuticals, due to the presence of hydroxyl group in their phenolic compounds [5-13]. Cardamom has been used traditionally for a variety of conditions as a digestive, carminative, stimulant, breathe freshener and aphrodisiac. Current research has implicated cardamom's potential therapeutic value as an inhibitor of human platelet aggregation [14]. Cardamom has antioxidant properties and can increase levels of glutathione, a natural antioxidant in the body. Reports are available on the antioxidant activity of extract of cardamom from different countries [15-19]. In the present study four Cardamom samples Malabar, Mysore, Vazhukka and Guatemala obtained from Spices board, Cochin, Kerala, India were used. The objectives of the present study were an exhaustive study on these cardamom samples with respect to chemical analysis, essential oil analysis, total flavonoid content, total phenolic content and antioxidant properties of different extracts. Thus it was intended to investigate the distribution of active compounds in different extract so as to further characterize their specific antioxidant activities facilitating further isolation and purification of active compounds. No previous reports are available on the antioxidant activity of extracts of these varieties of cardamom.

2. Material and Methods

Four varieties of cardamom samples, Malabar, Mysore, Vazhukka and Guatemala were obtained from Spices board, Cochin, Kerala, India were used for the study. The seed and husk of all varieties were separated manually and seed husk ratio was determined. The seeds were powdered and divided in to three parts, and is used for triplicate analysis.

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2.1.Chemical analysis

Chemical analyses of cardamom seed powder was carried out by AOAC method. The analysis includes the estimation of moisture, volatile oil, starch, protein, ash and crude fibre.

2.2. Isolation of essential oil

The powdered cardamom subjected to hydro distillation using Clevenger type apparatus for 4-5 hours. The oil samples were separated from water and dried over anhydrous sodium sulphate and kept for further studies.

2.3.Volatile Oil Analysis
2.3.1.GC Analysis

Cardamom oils were analysed in a Hewlett Packard 5890 Gas chromatograph. The column used was fused silica capillary column (50 x 0.2 mm id, film thickness 0.25 µm), coated with cross linked methyl silicone. The oven temperature was 80-200^0 C at the rate of 2^0 C/ minute. Flame Ionization Detector was used as detector and temperature was 300^0 C and injection temperature was 250^0 C.

2.3.2.GC-MS Analysis

GC-MS analysis was carried out using Shimadzu, GC-MS, QP. 5050 A mass spectrometer with the same column and GC conditions. Helium was used as the carrier gas and ionization voltage used was 70 eV.

2.3.3.Compound identification

Identification of the components was achieved by comparing the retention time of authentic samples, confirmed by comparing the retention indices and the mass spectra of the samples with standard library (NIST) and library generated in the laboratory [20-23]. The retention indices were calculated for all constituents using a homologous series of n-alkanes, C_6 -C_{22}. The percentage composition of the identified compounds was computed from the GC peak area in methyl silicon column.

2.3.4.Preparation of extracts

Cardamom seed powder (300 g each) was sequentially extracted with 1 litre of hexane, dichloro methane, ethyl acetate, methanol and water at room temperature for 12 hour with constant stirring. The different extracts were examined for their phenolic content, total flavonoid content, total reducing power and radical scavenging activity by DPPH method.

2.4. Determination of total phenolic content

Total phenolic content of extracts was assessed by using the Folin-Ciocalteau reagent method [24]. The extracts were diluted with the same solvent used for extraction, to a suitable concentration for analysis. To 200 µl of the sample extracts 1.0 ml of FolinCiocalteau reagent and 0.8 ml of sodium carbonate (7.5 % w/v) were added and the contents were mixed well and allowed to stand for 30 min. Absorption at 765 nm was measured in a UVVIS spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milli grams per gram of sample, using a standard curve generated with gallic acid.

2.5. Estimation of total flavonoid content

The quantitative study of flavonoids was conducted by two complementary colorimetric methods, aluminum chloride method and 2, 4- dinitrophenylhydrazine (DNP) method. The sum of the flavonoid contents determined by the two methods represents total content of flavonoids.

2.5.1 Aluminium Chloride Colorimetric Method

The $AlCl_3$ colorimetric method was modified from the procedure reported by Woisky and Salatino [25]. Quercetin was used as the standard. 10 mg of the standard was dissolved in 20 ml ethanol. Different concentration of the standard and samples were taken and diluted to 1 ml and mixed with 0.1 ml 1 M potassium acetate solution, 0.1 ml 10 % $AlCl_3$ solution and 2.8 ml water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm with UV-VIS spectrophotometer. Blanks were prepared by replacing the amount of sample with methanol.

2.5. 2. 2, 4- dinitrophenyl hydrazine Colorimetric method.

This method was modified from the procedure described by Nagy and Grancai [26]. (±) Naringin was used as the reference standard. 10 mg of standard was made up to 25 ml using methanol. Different concentration of the standard was taken and made up to 1 ml. Then the solution was mixed with 2 ml of 1% 2, 4 DNP reagent and 2 ml of methanol and kept in a water bath at 50^{0}C for 50 minutes. After cooling to room temperature the reaction mixture was mixed with 5 ml of 1% KOH in 70% methanol and incubated at room temperature for 2 minutes. Then 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged for 10 minutes. The absorbance of the supernatant was measured at 495 nm. Experiment was repeated for all the extractives of different varieties.

2.6. Antioxidant activity studies
2.6.1 Determination of DPPH radical scavenging capacity

The antioxidant activity of different extractives were measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH method [27] modified by Sanchez-Moreno et al. [28]. A methanolic solution (0.1 ml) of the sample at various concentrations was added to 2.9 ml of DPPH (0.1 mM) solution in a glass cuvette. The content was well mixed immediately and the degree of reduction of absorbance was recorded at 30 th min using a UVVIS spectrophotometer at 517 nm. Optical densities at zero and at 30 th min were used for calculating percentage radical scavenging activity. Percentage DPPH radical scavenging activity was calculated by using the formula,

$$\% \text{ scavenging capacity} = [A_{Control} - A_{sample}] \times 100$$

where $A_{control}$ is the absorbance of control without tested sample and A_{sample} is the absorbance of sample and the experiments were carried out in triplicate. Percentage radical scavenging activity was plotted against the corresponding extract concentration to get the IC_{50} value.

2.7.Total Reducing Power

The reducing powers of the samples were determined by the method described by Oyaizu [29]. Different concentrations of the samples were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated for 30 min at 50°C. After incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation at 5000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% of ferric chloride and the absorbance of the resultant solution was measured at 700 nm against a blank. Increased absorbance indicates increased reducing power. Ascorbic acid was used as the positive control.

2.8.Statistical analysis

The experimental results were expressed as means ± SD of three

parallel measurements. The results were processed using Origin Pro 8 and the data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between sample means were calculated.

3. Results and Discussion

The seed and husk of all varieties were separated manually and ratio was tabulated in Table 1. The separated seeds were powdered and sieved in 0.5 mm mesh size. The powder was divided in to three parts, and all analytical data were collected from each part in duplicate and mean values were taken. The chemical analysis of the samples was carried out and results were shown in Table 2. The chemical analysis gave the general chemical composition of the sample and was characteristic of variety or grade. The data given are in the normal range reported for the varieties [4]. Volatile oil content of cardamom powder was estimated using Clevenger type apparatus by hydro distillation. Malabar cardamom has the maximum yield of volatile oil (8.79%) followed by Guatemala (8.60%), Mysore (7.99%), and Vazhukka (7.90%).

Table-1. Seed husk ratio of cardamom variety

Variety	Seed Husk ratio
Mysore	62:38
Malabar	76:24
Vazhukka	70:30
Guatemala	66:34

Table-2. Chemical analysis of cardamom seed powder (% on dry weight basis)

Constituents	Mysore	Malabar	Vazhukka	Guatemala
Moisture	10.33	9.0	11	10.33
Volatile oil	7.9	8.79	7.9	8.6
Starch	42.42	49.05	42.95	42.14
Protein	13.53	12.72	11.40	13.06
Ash	8.07	6.97	8.16	8.52
Crude fibre	13.16	8.50	11.70	12.09

3.1. Analysis of volatile oil

GC and GC-MS analysis of volatile oil from the cardamom seed powder samples were carried out. Percent compositions of major constituents identified in the oil are given in Table 3. Odour profile showed that the oil contains more than 55 constituents, out of which 29 were identified comprising 91.75% - 98.12% of total oil. The constituents were identified by comparing the retention time of authentic samples and comparing mass spectra with standard library (NIST) and comparing kovats index. The oils had little mono or sesquiterpene hydrocarbon and was dominantly made up of oxygenated compounds. Terpinyl acetate was the major constituent in the oil ranging between 61.65% - 68.19% and was highest in Vazhukka variety. 1, 8-cineol was the second major constituent (7.23% - 11.76%) and highest in Mysore variety. Linalool and geraniol contents are greater in Malabar (4.53% and 2.5 %) and Guatemala variety (5.96% and 2.47%). Neryl acetate (2.11%), d-limonene (2.23%), α-terpineol (5.51%) contents are somewhat higher in Mysore variety. Nerolidol content was in higher side in Malabar and Guatemala compared to other samples. Other compound, which recorded more than 1%, was terpine-4-ol. The characteristic flavour of cardamom was contributed by the esters, alcohols and 1, 8 cineol. The total content of monoterpene hydrocarbon was less than 5% in all variety except Mysore variety where it was 5.42%. The lower value found in the concentration of monoterpene hydrocarbons and monoterpene oxygenated particularly in the content of 1,8-cineol was due to the evaporation losses of these low boiling point components during deseeding and grinding of the seeds. The characteristic odour and flavour of cardamom is determined by the composition of its aromatic steam volatile oil.

Table 3. Chemical composition of essential oil of cardamom seed powder

Constituents	Mysore	Malabar	Vazhukka	Guatemala
α-Pinene	0.36	0.07	0.14	0.11
Sabinene	1.32	0.31	0.41	0.55
β-Myrcene	0.85	0.37	0.25	0.53
Δ3-Carene	0.12	0.05	0.08	0.07
D-Limonene	2.23	1.82	1.57	1.92
1,8 Cineole	11.76	7.55	7.23	9.29
γ-Terpinene	0.23	0.03	tr	0.11
Sabinene hydrate	0.32	0.22	0.23	0.26
Terpinolene	0.31	0.09	Tr	0.18
Linalool	0.94	4.53	0.86	5.96
β-Terpineol	0.64	0.2	0.21	0.22
Terpene-4-ol	1.51	1.18	1.34	1.53
Octyl acetate	0.06	0.07	0.11	0.11
α -Terpineol	5.51	3.69	2.91	4.92
Linalyl acetate	0.33	1.92	0.42	1.63
Verbeneol	0.36	0.3	0.22	0.43
Geraniol	1.66	2.5	1.52	2.47
Geranial	0.49	0.51	0.54	0.65
Ocimenol	0.26	0.41	0.62	0.32
Terpenyl acetate	64.45	63.32	68.19	61.65
Neryl acetate	2.11	1.61	1.75	1.58
3-Decen 2-ol	0.18	0.19	0.28	0.14
cis- Dodecen 5 enol	0.05	-	-	-
Cedrene	0.07	0.37	0.14	0.26
Eudesmene	0.32	0.62	0.39	0.38
β-Caryophyllene	0.12	0.23	0.4	0.14
Nerolidol	0.88	3.57	1.71	2.17
cis,trans Farnesol	0.16	0.84	0.14	0.38
cis,cis Farnesol	0.17	0.36	0.19	0.21

3.2. Successive extraction of cardamom seed powder

Cardamom seed powder was sequentially extracted with different solvents in the order of increasing polarity viz. hexane, dichloromethane, ethyl acetate, methanol and water. Each fraction collected was separately concentrated and used for the analysis. The yield of different extracts was given on dry weight basis (dwb) was shown in Table 4. Sequential extraction leads to an initial fractionation of constituents based mainly on their nature of polarity.

Table 4. Yield of extractives of cardamom seed powder (% on dwb)

Solvents	Mysore	Malabar	Vazhukka	Guatemala
Hexane	1.344	1.316	1.259	1.473
DCM	0.231	0.394	0.346	0.279
Ethyl Acetate	0.246	0.248	0.366	0.281
Methanol	2.225	2.717	2.809	2.739
Water	4.967	5.307	5.523	5.715

3.3.Total Phenolic content

The total phenolic content of the extracts was determined by Foiln-Ciocalteau method. Gallic acid was used as the standard.

Table 5 shows the amount of phenolic content in the different extracts of cardamom samples. The total phenolic content was expressed as gallic acid equivalents (GAE) per gram of dry material. Of all the extract analyzed ethyl acetate extract had highest phenolic content where as in the plant material it was in methanol, which is dependent on the content of the extract. The total phenolic content recorded highest in Malabar variety (2.13 g GAE) followed by Vazhukka (2.10 g GAE), Guatemala (1.76 g GAE) and Mysore (0.164 %).

Table 5. Total phenolic contents (% on dwb)

Extract	Mysore		Malabar		Vazhukka		Guatemala	
	Powder	Extract	Powder	Extract	Powder	Extract	Powder	Extract
Hexane	0.36	26.7	0.16	12.2	0.16	13.0	0.10	6.5
DCM	0.18	76.5	0.121	52.3	0.14	40.4	0.12	43.7
Ethyl acetate	0.37	149.3	0.46	185.6	0.45	123.8	0.36	128.9
Methanol	0.59	26.5	0.75	27.5	0.77	27.2	0.71	25.9
Water	0.14	2.89	0.64	12.1	0.58	10.9	0.47	8.2
Total	1.64	281.9	2.13	289.7	2.10	215.3	1.76	213.2

3.4.Total Flavonoid content

Flavonoid contents were determined by two complementary colorimetric methods, $AlCl_3$ and 2, 4-DNP methods. The content obtained by the two methods was added to evaluate the total content of the flavonoids. The results show that 2, 4-DNP methods gave higher values compared to $AlCl_3$ method. Literature reveals that flavones and flavonols react better and form more stable complexes with $AlCl_3$ while flavanones and dihydroflavonols reacted better with 2, 4-dinitro-phenyl hydrazine [26, 30, 31]. Total flavonoid content was higher in ethyl acetate extract of Malabar and Vazhukka, but in the case of Mysore and Guatemala, it was in dichloromethane extract. While considering in plant material flavonoid content was highest in methanol extract. As regards the total flavonoids, Malabar variety gave the maximum content (0.159%) followed by Vazhukka (0.137%), Guatemala (0.132%) and Mysore (0.090%). The amount of flavonoid was considered as the important index for evaluating the biological activity of any material. Numerous physiological activities had been attributed to flavonoids. The flavonoids are well known for their antioxidant, anti-inflammatory properties. Flavonoids protect cells against oxidative and free radical damage that has been linked to cancer, aging and cardiovascular diseases. The flavonoid content in the seed powder and the extracts were shown

Table 6. Flavonoid contents of cardamom seed powder (% on dwb)

Extract	Mysore		Malabar		Vazhukka		Guatemala	
	Powder	Extract	Powder	Extract	Powder	Extract	Powder	Extract
Hexane	0.017	1.33	0.022	16.8	0.013	1.01	0.023	1.53
DCM	0.022	10.23	0.025	63.9	0.021	5.91	0.028	10.10
Ethyl acetate	0.015	6.19	0.027	109.6	0.023	6.25	0.021	7.45
Methanol	0.025	1.15	0.071	26.1	0.070	2.48	0.036	1.32
Water	0.011	0.23	0.014	2.6	0.010	0.17	0.024	0.42
Total	0.090	19.13	0.159	219	0.14	15.82	0.132	20.82

3.5. DPPH radical scavenging activity

DPPH is a stable free radical with a characteristic absorption at 517 nm [purple color]. The reduction in DPPH radical was determined by the decrease of purple colour. It was found that DPPH radical scavenging activity of extracts increase with increase in concentration (Fig. 1). From the IC50 values (the concentration of the sample resulting in a 50 % inhibition of the free radical), it was revealed that ethyl acetate extract of Malabar cardamom shows highest DPPH radical scavenging activity (88.7 µg/ml) followed by Vazhukka (186 µg/ml), Guatemala (223 µg/ml) and Mysore (293 µg/ml). By comparing the IC50 values of samples with that of standard trolox (25.6 µg/ml), Malabar shows 4 times reduction from standard trolox, Vazhukka shows 7 times, Mysore 11 times and Guatemala 9 times reduction in DPPH activity of standard. Lower IC50 values of Malabar indicate the greater radical scavenging activity. The increase in activity could be due to the enrichment of phenolic antioxidants. Table 7 shows IC50 value for different cardamom samples in their extract in different solvents. There was no scavenging activity against hexane, dichloromethane and water extracts.

Table 7. DPPH activities of cardamom seed powder extract (IC50 µg/3mL 0.1mm DPPH)

Sample	IC_{50} (µg/3mL)			
	Mysore	Malabar	Vazhukka	Guatemala
Ethyl acetate extract	293	88.7	186	223
Methanol extract	1056	533.7	1108.9	1513.7

3.6.Reducing power activity

The presence of anti oxidants in the sample causes the reduction of Fe^{3+} cyanide complex to its ferrous (Fe^{2+}) form. Amount of $Fe^{+}2$ complex formed was measured by the optical density at 700 nm; increased absorbance indicates an increase in reducing power. Table 8 shows the total reducing power (OD at 700 nm) of the cardamom seed extracts at a concentration of 10 µg/ml. Among the fractions tested, ethyl acetate fractions showed the highest reducing power activity but less than that of ascorbic acid (0.52). The reducing power observed was highest in Malabar followed by Vazhukka, Guatemala and Mysore variety. The results showed that a good correlation exist between reducing power, DPPH radical scavenging activity and total phenolic content of the extract.

Table 8. Total reducing power of samples (odp700 of 10 µg/mL)

Extract	Mysore	Malabar	Vazhukka	Guatemala
Hexane	0.0305	0.0512	0.0236	0.0092
DCM	0.0983	0.0671	0.0332	0.0426
Ethyl acetate	0.1737	0.2184	0.2087	0.1887
Methanol	0.0193	0.1637	0.0864	0.0452
Water	0.0293	0.0331	0.0178	0.1005
Total	0.3511	0.5335	0.3697	0.3562

4. Conclusion

The present study showed that cardamom extracts contain phenolic and flavonoid compounds. In all the samples, ethyl acetate extracts of the samples had significant antioxidant activity, higher phenolic and flavonoid contents. Ethyl acetate extract with high phenolic content was highly effective in scavenging DPPH and the IC_{50} values were compared to that of standard antioxidant compound trolox. Lower IC_{50} value indicates higher DPPH radical scavenging property. Of the four-cardamom variety Malabar cardamom has got higher antioxidant activity, higher phenolic-flavonoid contents and reducing power. The scavenging effect of extracts was of great importance in biological system. Conclusively, this spice is a valuable source of a number of natural phytochemicals, which are essential for good health.

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