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Electrofocusing of Methanolic Extracts for Identification of Individual Flavonol Biomolecules in *Camellia* Species

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An effort has been made to isolate individual catechin compounds from green tea leaves in their pure form by electrophoresis. In the present study total polyphenol extraction was carried out initially and estimated through spectrophotometric and HPLC methods. Extracted polyphenol was separated on 0.7% agarose gel and visualized at 360 nm. Fragmented individual compounds were gel eluted with methanol and confirmed as (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG) by HPLC. The method developed describes a suitable method for the isolation of valuable molecules in tea.

KEYWORDS: Polyphenol extract; total catechins; agarose gel electrophoresis; gel elution

INTRODUCTION

Tea is a widely cultivated plantation crop and heavily consumed as a beverage. India produces marketable tea to meet the demand of national and international markets. Besides being components of a popular beverage, tea polyphenols have gained more importance because of their antioxidant nature and antibacterial and antiallergic activities (*1, 2*).

Tea shoots contain as high as 35% polyphenols to total dry matter content, of which two-thirds is contributed by catechins, a major biochemical component (*3*). Crop shoots registered as high as 25% of catechins (*4*), but it varies with genotype. Total catechins comprised dihydroxylated [(−)-epicatechin (EC) and (−)-epicatechin gallate (ECG)] and trihydroxylated [(−)-epigallocatechin (EGC) and (−)-epigallocatechine gallate (EGCG)] catechin fractions. Both dihydroxylated and trihydroxylated fractions are known for their therapeutic effects (*5*−*7*). A special focus is given to the extraction of polyphenols, particularly individual compounds due to their abundance in tea plants. The extraction of polyphenols from tea plants in higher quantity can be an additional source of revenue to the tea industry. Even though many procedures are available to extract the polyphenols from plant materials, separation of individual components of interest deserves attention.

Separation of catechin using carbon dioxide extraction from green tea leaves has been reported by Chang et al. (*8*). According to Bronner and Beecher (*9*), high-performance liquid chromatography is the conventional means of analyzing catechins in tea and other biological constituents. However, owing to the high cost involved in separation, a simple, rapid, and easy to perform alternative method is warranted. Davis et al. (*10*) have reported a traditional extraction of caffeine from black tea using organic solvents, but, to our knowledge, no study has separated individual catechins using electrofocusing on agarose gel. Exploiting the potential nature of migration under an electric field of a biological component, electrofocusing of crude extract was attempted.

There are a multitude of procedures available for the extraction of total polyphenols from plants. Due to the complex nature of the individual molecules, previous attempts for their separation were rather unsuccessful. Electrophoresis, as a method, is generally used in the separation of proteins, amino acids, and nucleic acids. The inherent charge-to-mass ratio of biomolecules under applied voltage, which travels toward an opposite charge under electrofocusing, is attempted for separation. Even though agarose gel electrophoresis is routinely used in the molecular biology laboratory for the separation of nucleic acids, studies using this technique for polyphenol separation are limited (*11*). In light of the above discussion, this study presents an easy extraction technique coupled with identification of catechin fractions from crude extracts of green tea leaves.

MATERIALS AND METHODS

Chemicals. HPLC grade acetonitrile, methanol, and Folin−Ciocalteu reagents were purchased from Merck (Darmstadt, Germany). Agarose (low EEO) and Tris were obtained from Gnetix, USA. Authentic reference standards of EGC, EC, ECG, and EGCG were procured from Sigma Chemical Co. (St. Louis, MO). Other solvents and chemicals used were of analytical grade procured locally. All materials were used without further purification. Catechin standards were dissolved in methanol to a concentration of 1 g/mL and stored at −20 °C.

Plant Material. Crop shoots comprising two leaves and a bud were collected from field-grown, mature tea bushes of UPASI 17, representing the 'Cambod' cultivar, were used for sample preparation. These plants were grown at the United Planters' Association of Southern India (UPASI) Experimental Farm, which is located at an altitude of 1150

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m above MSL. All of the cultural operations in the field were carried out according to UPASI recommendations (*12*).

Extraction of Total Polyphenols. Total polyphenols were extracted from tea leaves following the procedure described by Antolovich et al. (*13*) with a slight deviation (*14*, *15*). In the present study, methanol alone was used as the solvent for extraction of polyphenols. Frozen plant material was macerated with methanol to obtain the extracts. Crude methanol extract was purified with methylene chloride and ethyl acetate using liquid phase separation. Finally, total polyphenol was condensed in a rotary evaporator under a temperature range of 50−70 °C depending on the boiling point of the solvent used. Condensed polyphenol powder was stored under vacuum at 4 °C for further biochemical estimations and electrophoretic studies. Total polyphenols and catechins were quantified spectrophotometrically as per the described procedure.

Estimation of Polyphenols and Catechins. Total polyphenols and catechins were quantified conventionally using a spectrophotometer (model Genesys 10UV) adopting the method reported by Dev Choudhary and Goswami (*16*) and Swain and Hillis (*17*), using Folin−Ciocalteu and vanillin reagents. Phenols react with phosphomolybdic acid in Folin−Ciocalteu reagent in alkaline medium to produce a blue complex. The optical density of the compound was measured at 700 nm.

Extraction of Individual Catechin Molecules. *Sample Preparation for Agarose Gel Electrophoresis.* Two grams of extracted powder was dissolved in Milli-Q water and incubated at 65 °C for 30 min in a water bath. The solution thus obtained was centrifuged at 14000 rpm (Sigma Z14) for 10 min, and the supernatant was reserved for agarose gel electrophoresis. Twenty percent glycerol was added to the sample mixture in the ratio 1:3 before loading onto the gel. Individual catechin standards procured from Sigma were prepared according to the same procedure and electrophoretic separation was attempted to migrate along with the samples.

Electrofocusing of Samples. Separations of the compounds were attempted on a vertical mini gel of dimension of 6 × 10 cm using 0.7% agarose. The electrophoresis buffer was modified by the removal of EDTA from TAE buffer (Tris base, 50.4 g, and glacial acetic acid, 11.4 mL, in 1 L of water). The samples were run on the gel and electrophoresed for a period of 4 h at 80 V. Individual catechin standards were used for comparison in each replicate. The separate fractions were visualized on a transilluminator under UV light (360 nm). Gels representing individual bands were cut under UV light and subjected to gel elution using the standard protocol. Gel pieces containing individual fragments were macerated in methanol and incubated at 65 °C for 30 min in a water bath before they were centrifuged at 14000 rpm for 10 min (Sigma Z14). The supernatant methanol solution was collected repeatedly in a new tube, and the sample was concentrated in a rotary evaporator at 55 °C. Finally, the concentrate was dissolved in 70% methanol. The solution was subjected to 0.2 μm filtration prior to HPLC analysis using Acrodisc syringe filters (PAL Co., Ann Arbor, MI).

Determination of Individual Catechin by HPLC. The individual catechin fractions were analyzed according to the method of Jibu Thomas et al. (*18*) in HPLC (Hewlett-Packard series 1100) fitted with a Phenomenex column with an autosampler. Acidified acetonitrile (18%) and acetonitrile (80%) were used as mobile phases A and B, respectively. Authentic standards of individual catechins procured from Sigma were used in the present study as standards for the spiking test. Relative distribution of these constituents was expressed in percentage of individual component (w/w) according to ISO method (*19*).

All of the above-mentioned experiments were repeated three times with replicates for all of the parameters. The mean of the data obtained is represented.

RESULTS AND DISCUSSION

Crop shoots containing two leaves and a bud of UPASI 17 were known to be high in polyphenol content among the UPASI-released tea clones (*4*). Totals of 31.62 and 21.72% of total polyphenol and catechin contents were confirmed, respectively, by spectrophotometric method (**Table 1**). In HPLC, EGC (2.73%) was eluted first followed by EC (2.04%), EGCG (13.85%), and ECG (1.77%). It may be noted that the values of total catechins and their fractions are not tallying with each other; this may be due to the variation in the precision of the spectrophotometric and HPLC methods followed in the present study. Variations in the catechin content are due to biochemical and metabolic functions of tea clones under stipulated conditions (*3*, *20*).

Table 1. Content of Individual Catechin Fractions, Total Catechin, and Total Polyphenol Obtained from Green Tea Leaves and Methanol-Extracted Sample

	phenolic compounds (%)	
	green leaves	polyphenol extract
epigallocatechin	02.73	08.20
epicatechin	02.04	06.84
epigallocatechin gallate	13.85	41.25
epicatechin gallate	01.77	05.43
total catechins[a]	20.39	62.47
total polyphenol[a]	31.62	79.30

[a] Spectrophotometric analysis.

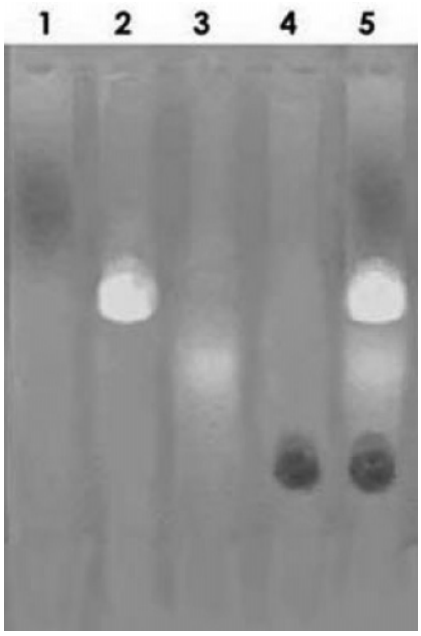

Figure 1. UV fluoresced agarose gel showing the distinct bands of individual catechin molecules: lane 1, EGC; lane 2, EC; lane 3, EGCG; lane 4, ECGl ;ane 5, extracted sample.

Methanol was found to be a promising solvent in complete extraction of total polyphenols from fresh green tea leaves for purification. Repeated extraction completely elutes the polyphenolic compounds from the crop shoots. Data on the polyphenolic content corroborate the findings of Saravanan et al. (*4*). Methylene chloride efficiently removed the alkaloids and pigments contaminating the extract (*14*). Extracted powder subjected to estimation of total polyphenol and catechin by the spectrophotometric method revealed 79.3 and 62.47% of total polyphenol and catechin, respectively. In HPLC it was EGC (8.2%) eluted first followed by EC (6.84%), EGCG (41.25%), and ECG (5.43%), respectively (**Table 1**).

The methanol extract was electrofocused on 0.7% agarose gel at 80 V for separation. Addition of 20% of glycerol with the water-suspended sample made the sample dense. The individual catechin fractions moved under electrophoresis on the basis of their mass ratio and molecular sizes, because each of the catechin fractions possesses its own individual retention factor. Hence, they tend to separate into individual components under the applied electric field of 80 V. After 4 h of run, when visualized under UV transilluminator, these were distinct bands.

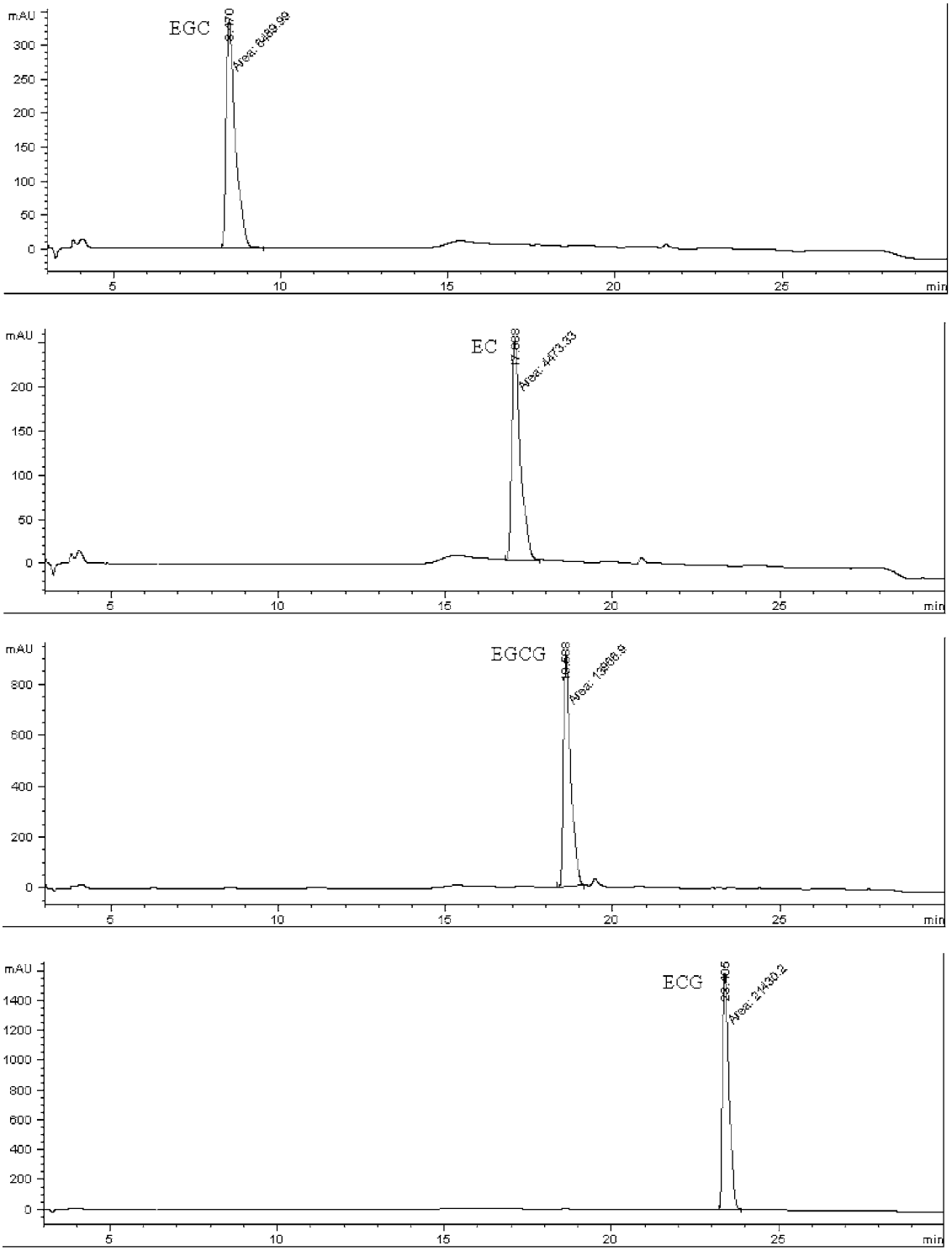

Figure 2. High-performance liquid chromatograph of the gel-eluted catechin molecules.

The smaller and lesser negatively charged molecule immigrates more quickly as the components pass through the gel (*21*). This corroborates the earlier reports regarding the fluorescent property of secondary metabolites under UV transillumination (*22*). Four different molecules migrated toward positive charge on the basis of their mass ratio, and they showed different color emissions such as dark blue, fluorescent green, pale yellow, and violet (**Figure 1**) under UV florescence. Simultaneously, the individual standards of catechin molecules were also run under similar conditions, and the four molecules were revealed to be EGC, EC, ECG, and EGCG. Among the four catechins the relative mobility of EC was highest, followed by EGC, ECG, and EGCG. The gel-eluted solvents were subjected to HPLC analysis. The results confirmed the presence of similar compounds corresponding to the spiked authentic standards on the basis of their retention times (**Figure 2**). The quantities of these molecules were found to be EGC, 6.2%; EC, 5.34%; EGCG, 31.85%; and ECG, 4.23%. After quantification, the peaks obtained revealed the purity of isolates was ~90%. Comparison was carried out with the total available in the extracted leaf before separation on agarose gel to find the percentage of extractability. The results indicated that quantity was reduced by ~25% (**Table 2**), which may be due to the loss of some amount of molecules in the gel elution process.

Table 2. High-Performance Chromatography Analyses of Gel-Eluted Samples

	gel-eluted catechins fractions		
	molecule (%)	purity (%)	extractability (%)
epigallocatechin	06.20	97.20	75.61
epicatechin	05.34	96.10	78.07
epigallocatechin gallate	31.85	96.87	77.21
epicatechin gallate	04.23	95.90	77.90

Soluble phenolic compounds are generally extracted using water, methanol, ethanol, and acetone. The presence of attached sugars tends to render phenolic compounds more water-soluble, and combinations of the above solvents with water are thus better solvents for glycosides. In contrast, less polar aglycones such as isoflavones, flavonols, and highly methoxylated flavones and flavonols tend to be more soluble in nonaqueous solvents (*1*, *23*, *24*). A number of methods have been developed in the past for the determination of polyphenols in plant extracts such as Folin−Ciocalteu and Price and Butler methods, but they were mostly nonspecific and cannot distinguish flavonoid classes in general and are best used for the determination of total soluble polyphenols (*25*−*27*). Likewise, other oxidants used volumetrically for the same purpose such as ferric sulfate and potassium permanganate are also nonspecific (*26*). From the above experiment attempted, extraction with methanol was found to be suitable for the isolation of individual flavonols in tea. About 70−80% of methanol gave a superior yield.

Agarose gel electrophoresis and elution have been found to be extremely successful for obtaining pure samples of individual catechins. On comparison, it was found that the time involved could be considerably reduced for the separation of individual catechin fractions using this method with a reduction in the cost as well. Until now, no method has been employed in the isolation of individual molecules in tea using agarose gel electrofocusing. Exploitation of this method is possible for the successful isolation of important biomolecules in commercial crops. Considering the commercial value of biomolecules with regard to purity, the methods developed can be suitably modified for the isolation of any compound of interest. The method developed describes the isolation of valuable molecules in tea.

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