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Radical Scavenging Conserves from Unused Fresh Green Tea Leaves

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Green teas were made by inactivating the enzymes present in fresh leaves of coarse/pruned (unused) and normal (used for tea) grades using different sources of thermal energies. Green teas were extracted in a Soxhlet using different solvents. The obtained miscella was subjected to concentration to give the extract. The extract was subjected to solvent−solvent extraction. Solvent extract was concentrated to obtain conserve. The yields of conserves are 17 ± 0.8 and 15 ± 0.8% from green teas of normal and coarse tea leaves, respectively. The radical scavenging activity of these extracts was evaluated using a DPPH in vitro model system. The total polyphenol content was also determined and found to be higher in conserves from normal tea leaves. However, radical scavenging activity of conserves from coarse and normal green tea leaves was found to be >90% at 15 ppm concentration. The HPLC profiles of these conserves were used to quantify the total catechin content with the help of calibration curves prepared using authentic samples at known concentrations. The total catechin content is found to be in the range of 55−85%. Results indicated that the extracts from coarse leaves also possess potential biological activity and could be used as nutraceuticals as well as for preservation purposes in food formulations.

KEYWORDS: Tea; *Camellia sinensis*; Theaceae; coarse leaves; infrared dryer; crossflow dryer; green tea and radical scavenging activity

INTRODUCTION

Tea obtained from processed shoots of *Camellia sinensis* is one of the most popular nonalcoholic beverages in the world. The shoots, consisting of the tender apical bud and subtending two leaves, are processed to give the tea beverage. The main constituents, catechins, which constitute up to 30% on a dry weight basis (*1*), possess medicinal properties, namely, antioxidative, anticancerous, and antibacterial (*2*−*6*). The main compounds present in tea leaves are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), (+)-gallocatechin gallate (GCG), (+)-gallocatechin (GC), and (+)-catechin (C). During the green tea manufacturing process, some of the catechins undergo isomerization at the C-2 position of flavan-3-ol. For example, (−)-EGCG, (−)-EGC, (−)-ECG, and EC isomerize to (+)-GCG, (+)-GC, (+)-catechin gallate (CG), and (+)-C, respectively. These catechins, belonging to the flavanol group of phenols, remain unoxidized in the green tea, as the enzymes are deactivated during the processing. An antioxidant can be defined as any substance that when present at low concentration compared to that of substrate significantly delays or inhibits the oxidation (*7*). Green tea extracts are powerful antioxidants, mainly due to the presence of the above flavanols (*8*, *9*). These compounds are believed to have physiological effects by acting as free radical scavengers, which are generated by metabolic pathways within body tissue or introduced by external sources such as foods, drugs, and environmental pollutants (*8*, *10*, *11*). Tea catechins are effective scavengers of free radicals with more effective catechins having a galloyl moiety at C-3 (*12*) and a trihydroxy structure in ring B (*13*). Chlorophyll present in organic extract from green tea also affects the antioxidant activity of the extracts (*14*). Besides these, caffeine, theophylline, and theobromine are the main methyl xanthines constituting the tea alkaloids and are important factors in determining the quality of green teas.

Many epidemiological and preclinical studies strongly suggest that drinking green tea may lower the risks of cancer and cardiovascular disease. Moreover, other health beneficial effects including anti-inflammation and antiobesity have been reported (*15*, *16*).

Despite several reports on the radical scavenging activity of green tea from two leaves and a bud, the radical scavenging activity of green tea from coarse and pruned leaves in particular is not studied, and this material is presently being used as compost. The pruned and coarse tea leaves are tea plantation waste, after the two leaves and bud are used for the manufacture of various types of teas. A huge quantity of this waste is available in India for value addition, because India is one of the largest producers of tea. Therefore, testing of its radical

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scavenging 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 plantation wastage. Use of natural antioxidants as food additives for inactivating free radicals has received much attention recently due to the health consciousness of recent generations, as these consumers are more exposed to environmental pollutants such as those released from various types of vehicles and industries.

The present paper describes the preparation of green teas from the pruned or coarse tea leaves and optimization of extraction conditions to obtain radical scavenging conserve (*17*). Here, this waste (unused tea leaves) was utilized to prepare the catechin-rich conserve, and its radical scavenging activity was evaluated. The radical scavenging activities of these conserves are evaluated by using the DPPH model system, and the results are presented along with the HPLC profiles of the active conserves for identification and quantification of composition.

MATERIALS AND METHODS

Chemicals and Reagents. The analytical grade solvents (viz., methanol, ethyl acetate, and formic acid), Folin−Ciocalteu's reagent, and sodium carbonate were procured from Merck (India). Tea catechins [viz., (+)-catechin (Cat, 98%), (+)-epicatechin (EC, 98%), (−)-epigallocatechin (EGC, 98%), (−)-epicatechin gallate (EGC, 98%, (−)-epigallocatechin gallate (EGCG, 95%), (−)-gallocatechin gallate (GCG, 98%)], alkaloids [viz., theophylline (TP, 98%), theobromine (TB, 98%), caffeine (C, 99%), gallic acid, and 1,1-di-phenyl-2-picrylhydrazyl radical (DPPH•) were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Plant Material. Coarse and normal green tea leaves were collected from a tea estate in the Nilgiri-wynad region (Tamilnadu, India). Normal (control) leaves are plucked as one bud and two leaves from the tea plant, whereas coarse material comprised the remaining leaves of the shoot.

Enzyme Inactivation. Enzymes (including polyphenol oxidase) of the fresh tea leaves were inactivated immediately by drying using two different kinds of dryers, namely, crossflow and infrared dryers, having different drying conditions (*18*, *19*).

Moisture. The amount of moisture in the crossflow and infrared dried tea samples was measured using a vacuum oven (*20*).

Size Reduction. The green teas were then subjected to size reduction by grinding in a mixer to a particle size of 30 μm and stored in suitable polypropylene bags at 4 °C under refrigerated conditions until further use.

Extraction Procedure. Dried and powdered green tea leaves (200 g) were extracted with various solvents (e.g., acetone, ethyl acetate, methanol, ethanol, and their aqueous mixtures) for a period of 15 h using the Soxhlet apparatus. The material to solvent ratio used was 1:12. The extract was desolventized using a rotavapor at 50 °C under reduced pressure.

Fractionation of the Extract. The extract was subjected to solvent−solvent extraction using water and ester for 20−25 h using an all glass liquid−liquid extraction unit. Both aqueous and solvent-partitioned portions were desolventized in a rotavapor at 50 °C under reduced pressure.

Determination of Total Phenolic Content. Samples were analyzed for content of total phenolics according to the Folin−Ciocalteu method (*21*). Powdered tea samples and extracts (0.5 g) were introduced in test tubes, and methanol/deionized water (70:30) solution was added, heated on a water bath maintained at 70 °C for 10 min. The samples were cooled to room temperature and subjected to centrifugation. The supernatant was mixed with saturated sodium carbonate and Folin−Ciocalteu's reagent. The mixture was diluted to 10 mL with deionized water; tubes were mixed and incubated in the dark for 60 min for color development. The absorbance of this solution was measured using a UV−visible spectrophotometer (GBC Cintra 10, Australia) at 765 nm. The total phenol content of each extractive was estimated by comparison with a calibration curve generated from the analysis of gallic acid solutions and expressed as mean (±SD) percent of gallic acid equivalents for the extracts in triplicate.

Determination of Radical Scavenging Activity. The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) method, which has been widely used to evaluate the free radical scavenging ability of various samples (*4*), was adopted here.

Tea extract conserve samples were dissolved in distilled methanol, and solutions of different concentrations (25, 50, 100, and 200 ppm) were prepared in different test tubes. Four milliliters of a 0.1 mM methanol solution of DPPH was added to these test tubes and shaken vigorously. The tubes were then incubated in the dark at room temperature for 20 min. A DPPH blank sample was prepared without any extract, and methanol was used for the baseline correction. Changes (or decrease) in the absorbance at 517 nm were measured using a UV−visible spectrophotometer. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4 °C between measurements. All experiments were carried out in duplicate and repeated three times. The percent decrease in the absorbance was recorded for each concentration, and percent quenching of DPPH• was calculated on the basis of the observed decrease in absorbance of the radical. The radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{radical scavenging activity (\%)} = [(\text{control OD} - \text{sample OD})/\text{control OD}] \times 100$$

The radical scavenging activity of BHA was also measured and compared with that of the various green tea leaf extracts.

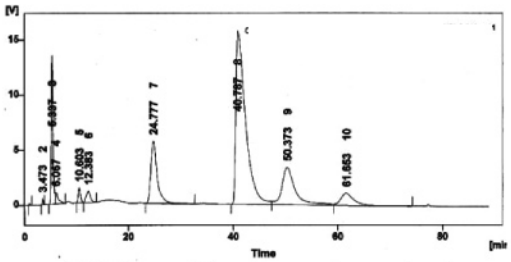
Identification of Catechins in the Solvent−Solvent Extract by HPLC. Several studies were reported for determining tea catechins and alkaloids separately using HPLC following either isocratic or gradient elution methods (*22*, *23*). To determine the composition of the active conserves a simple HPLC isocratic elution method was developed, which efficiently separates various tea biochemicals, namely, catechins and gallic acid.

Preparation of Sample. Tea extract (10 mg) was dissolved in 10 mL of methanol; from this aliquot about 0.25 mL was taken and made up to 4 mL by methanol, and from this, 10 μL of the sample was injected for HPLC analysis under the following conditions (*24*): The column used was a Spherisorb S10 OD52 (4.6 × 250 mm, Waters). The mobile phase was water/methanol/formic acid (19.5:80.2:0.3 v/v) under isocratic conditions at a flow rate of 1.0 mL/min using the Waters 515 HPLC pump. Peaks were observed at a wavelength of 280 nm using a UV-2487 dual wavelength absorbance detector (Waters).

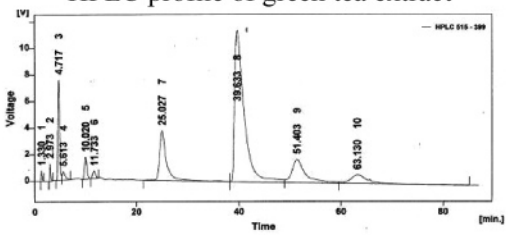
The chromatograms of green tea extracts/conserves are shown in **Figure 1**, whereas **Table 4** shows the retention time, percentage area identification, and quantification of particular compounds in the chromatogram.

RESULTS AND DISCUSSION

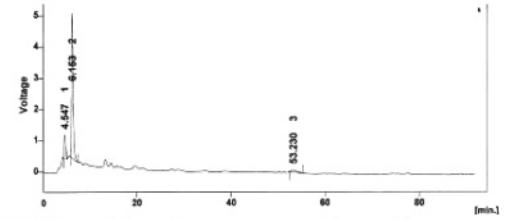
Fresh tea leaves of normal and coarse grades were procured and subjected to enzyme inactivation within 4 h of plucking. These were dried and preserved at low temperature to avoid any changes. The normal tea leaf samples were subjected to solvent extraction using various solvents (e.g., acetone, ethyl acetate, methanol, ethanol, and their aqueous mixtures). The solvents were removed from these extracts under vacuum, and the yields were calculated on a moisture-free basis. The radical scavenging activity (RSA) of these extractives at 50 and 100 ppm concentrations (which were found to be optimal for these extracts in the preliminary study varying from 10 to 1000 ppm concentrations) were evaluated using the DPPH model system. The yields and activities of the extractives are presented in **Table 1**. The results indicate that the yields of the extractives were increased with increase in polarity of the solvent used for extraction. Addition of water to solvents further increased the yields of the extractives. Furthermore, the results showed that the radical scavenging activities of the extractives also increased



HPLC profile of green tea extract



HPLC profile of solvent portion of tea extract



HPLC profile of aqueous portion of tea extract

Figure 1. HPLC profiles of green tea extractive/conserves.

Table 1. Yield and Radical Scavenging Activity of Normal Green Tea Extractives Using Different Solvents

		radical scavenging activity (%)	
solvent	yield (%)	50 ppm	100 ppm
ethyl acetate	23.9 ± 0.4	64 ± 0.8	91 ± 0.4
acetone	27.7 ± 0.4	67 ± 0.5	91 ± 0.5
ethyl alcohol	32.7 ± 0.5	76 ± 0.7	92 ± 0.6
methyl alcohol	34.8 ± 0.6	82 ± 0.9	92 ± 0.5
ethyl acetate + water	24.5 ± 0.4	70 ± 1.1	91 ± 0.5
acetone + water	29.2 ± 0.5	75 ± 1.0	91 ± 1.2
ethyl alcohol + water	36.3 ± 0.6	82 ± 0.5	92 ± 0.7
methyl alcohol + water	36.7 ± 0.7	86 ± 0.9	93 ± 0.6
BHA		83 ± 0.5	92 ± 0.6

with the increase in polarity of the solvent. This may be due to the increase in extraction of the active components along with the polarity of the solvent. Hence, alcohols are found to be the best solvents to obtain maximum extractives with high activity. The aqueous alcoholic mixtures were used for further processing.

The extractions for the further work on the normal and coarse tea leaves were carried out using a methanol/water mixture. The green teas from normal and coarse leaves were subjected to solvent extraction, followed by solvent removal. The miscella was subjected to lyophilization for removal of the aqueous portion. The yields were computed on a moisture-free basis (**Table 2**). The extractives were evaluated for their polyphenol content and radical scavenging activities, and the results are presented in **Table 2**. It was found that the yields of the extractives from green teas of coarse leaves are relatively low on the expected lines. However, the radical scavenging activities of the extractives of green teas from coarse leaves are marginally low at different concentrations. This observation indicated that the green teas from coarse leaves could be used for the preparation of radical scavenging conserves by separating/enriching the active components using a suitable technique.

Table 2. Yield, Polyphenol Content, and Radical Scavenging Activity of Normal/Coarse Green Tea Extractives Prepared under Optimized Conditions

			radical scavenging activity (%)			
tea sample	yield (%)	polyphenol content[a] (%)	25 ppm	50 ppm	100 ppm	200 ppm
normal	36.7 ± 0.7	31.0 ± 0.6	50 ± 0.7	83 ± 0.9	93 ± 0.8	92 ± 0.3
coarse	32.5 ± 0.5	20.6 ± 0.8	45 ± 1.0	74 ± 0.2	92 ± 0.4	91 ± 0.8
BHA			82 ± 0.8	92 ± 0.4	93 ± 0.7	93 ± 0.4

[a] As gallic acid equivalents.

The extractives were subjected to liquid−liquid extraction using water and a low molecular weight ester to fractionate the catechins into the solvent fraction. These extracts were analyzed for total polyphenol content and evaluated for RSA (**Table 3**). The polyphenol content of the solvent extracts was found to be 30 ± 2.3% as gallic acid equivalents for coarse leaves, whereas the polyphenol content of the solvent extract of normal leaves was found to be 31 ± 2.4% as gallic acid equivalents. The total polyphenol content in the aqueous portion of these extracts was 23 ± 2.1% as gallic acid equivalents for normal leaves, whereas that for coarse leaf extracts was found to be 18 ± 3.0% as gallic acid equivalents. The yields of the solvent extracts were found to be 15 ± 0.8% for coarse leaves, and for normal leaves the yield of solvent extract was found to be 17 ± 0.8%. The yield of the aqueous extract is 17 ± 0.9% for coarse leaves, and for normal leaves the yield of solvent extract was found to be 19 ± 1.0%. However, the RSA of the solvent extracts from both normal and coarse leaves was found to be same (92 ± 1% at 15 ppm). The RSA of the aqueous extracts was found to be lower.

Antioxidant reacts with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm, and converts it to 1,1-diphenyl-2-picryl hydrazine, due to its hydrogen-donating ability at a very rapid rate (*25*). The degree of discoloration indicates the scavenging potentials of the antioxidant. The activity of the extracts is attributed to their hydrogen-donating ability (*26*). It is well-known that free radicals cause autoxidation of unsaturated lipids in foods (*27*). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of lipid (*28*). The data obtained reveal that the green tea extract/conserve is a free radical inhibitor and primary antioxidant that reacts with the DPPH radical, which may be attributed to its hydrogen-donating ability.

HPLC Profiling of Green Tea Extractives, Chemical Composition, and Quantification. To determine the chemical composition of the green tea extracts/conserves, the calibration curve for each of the catechins was prepared. The concentrations ranges used for the calibration curves were 5−50 μg. The retention time of each of the catechins was noted. Under optimized conditions the green tea extracts, fractionated solvent portion, and aqueous portion were subjected to HPLC. The profiles are presented in **Figure 1**. Quantification was carried out using the external standard method. Solutions of each standard at various concentration levels were injected into the HPLC system, and the peak areas were recorded. Thus, the

Table 3. Fractionation of Green Tea Extracts: Yield, Polyphenol Content, and Radical Scavenging Activity of Solvent and Aqueous Extracts

					radical scavenging activity (%)			
	yield (%)		polyphenol content[a] (%)		conserve (solvent)		conserve (aqueous)	
tea sample	conserve (solvent)	conserve (aqueous)	conserve (solvent)	conserve (aqueous)	15 ppm	100 ppm	15 ppm	100 ppm
normal	17 ± 0.8	19 ± 1.0	31 ± 2.4	23 ± 2.1	92 ± 1	>94	45 ± 1	92 ± 2
coarse	15 ± 0.8	17 ± 0.9	30 ± 2.3	18 ± 3.0	92 ± 1	>94	40 ± 1	85 ± 2
BHA					82 ± 1	>94	82 ± 1	>94

[a] As gallic acid equivalents.

Table 4. Catechin Composition of Radical Scavenging (Solvent) Conserve

compound	retention time (min)	quantity[a] (μg/mg)
caffeine	4.7	104.8 ± 10.4
catechin	10.2	64.5 ± 5.6
epigalloctechin gallate	25.0	177.4 ± 20.1
gallocatechin gallate	39.6	371.0 ± 42.1
epicatechin gallate	51.4	182.3 ± 12.1

[a] Epicatechin and epigallocatechin were found in traces only.

Table 5. Radical Scavenging Activity of Gallic Acid, Alkaloids, and Catechins[a]

standard	25 ppm	50 ppm
gallic acid	90 ± 1.0	92 ± 0.9
theophylline	–	–
theobromine	9 ± 0.3	1 ± 0.2
catechin	79 ± 0.7	89 ± 0.8
epicatechin	61 ± 0.8	83 ± 0.8
epicatechin gallate	84 ± 0.9	90 ± 0.9
epigallocatechin gallate	86 ± 0.8	89 ± 0.7
gallocatechin gallate	85 ± 0.7	89 ± 0.8
epigallocatechin	86 ± 0.9	90 ± 1.0
BHA	92 ± 1.0	92 ± 0.9

[a] Butylated hydroxyanisole (BHA) was used as positive control. Values are mean ± SD ($n = 3$). –, no activity.

calibration curves were prepared and response factors were calculated under the same conditions. The total catechin content in the green tea extract 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%. After fractionation, the solvent extract is enriched with catechin, and the total catechin content was found to be in the range of 55−85% (**Table 4**), whereas the HPLC profile of aqueous extracts showed the presence of only gallic acid and caffeine.

Coarse green tea leaf extract obtained from the solvent−solvent extraction method was analyzed for individual catechins and methylxanthines and for gallic acid by employing the HPLC method; the contents of the above compounds were calculated as micrograms per milligram of dry weight.

To clarify the above finding, the RSA of the pure gallic acid, alkaloids such as theophylline, theobromine, and catechins such as catechin, epicatechins, epigallocatechin, gallocatechin gallate, epicatechin gallate, and epigallocatechin gallate derivatives was evaluated, and the results are presented in **Table 5**. EGC showed the highest activity at 25 ppm concentration among the catechins. EGCG and GCG show the almost identical activity. The activities of EGC, ECG, EGCG, and GCG are nearly equal to that of butylated hydroxyanisole (BHA) at 25 ppm concentrations. However, all of the catechins showed close to 90% activity at 50 ppm concentration, except epicatechin.

In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow diphenylpicryl hydrazine. The method is based on the reduction of an alcoholic DPPH solution at 517 nm in the presence of a hydrogen-donating antioxidant (AH) due to the formation of the nonradical form (DPPH-H), according to the following reaction:

$$\text{DPPH} + \text{AH} \rightarrow \text{DPPH-H} + \text{A}$$

The remaining DPPH, measured after a certain time, corresponds inversely to the radical−radical interaction; the radical A can contribute to the formation of stable molecules. This method is simple, rapid (20 min), and sensitive. No expensive reagent or sophisticated instruments are required.

In the literature, the RSA of the phenolic compound is described as being largely influenced by the number of hydroxyl groups on the aromatic ring. The higher the number of hydroxyl groups, the greater the RSA. The results of this study are in perfect agreement with data. It has already been reported that >70% of the antioxidant activity in green tea extracts can be attributed to tea catechins, and (−)-ECG and (−)-EGCG in particular strongly contributed to the antioxidant activity of green tea (*8*).

Extracts from unused fresh green tea leaves have the potential for large-scale application as natural antioxidants. Extracts of the green tea are becoming increasingly important as functional ingredients in the diet and are being added to a range of foods and beverages (*29*).

Conclusions. The results of the present work indicate that the solvent−solvent extract portion from the green tea extract possesses high free radical scavenging activity and shows the presence of highest catechin content. It is considered to be a radical scavenging conserve. The radical scavenging activity of solvent conserves from both coarse and normal green tea leaves was found to be >90% at 15 ppm concentration. The HPLC profiles of these conserves were used to quantify the total catechin content with the help of calibration curves prepared using authentic samples at known concentrations. The total catechin content was found to be in the range of 70 ± 15.0%. Results indicated that the extracts from coarse leaves also possess potential biological activity and could be used as nutraceuticals as well as for preservation purposes in food formulations. The activity of the individual compounds was marginally lower than that of the solvent conserves, which may be due to the synergisms among the catechins in the solvent conserves.

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