Investigation of Organic Constituents of Commercial Tea Brands and Fresh Tea Leaves by High-Performance Liquid Chromatography

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Summary: To date, the organic constituents such as caffeine, pyrogallol, gallic acid, and polyphenols (catechin and epicatechin) of the various tea brands and fresh tea leaves commercially available in Pakistan have not been investigated or published elsewhere. Therefore, the current study was undertaken to investigate the contents of these important organic constituents of the tea samples. A simple, fast and reliable isocratic reverse-phase high-performance liquid chromatographic method with slight modifications was applied for the determination of selected ingredients. The results show high level of caffeine and gallic acid in black teas compared to green teas and fresh tea leaves. Similarly, in green teas, enormous quantity of pyrogallol, catechin and epicatechin was found. Epicatechin was detected only in green teas and fresh tea leaves. Fresh tea leaves contain all the selected components but at lower concentrations compared to other tea samples. It is speculated that the remarkable increase and decrease in the concentration of the selected analytes is due to the fermentation processes used for manufacturing black and green teas and the elevated levels can be controlled using the optimal fermentation and manufacturing conditions.

Introduction

Tea (*Camellia sinensis*) originated in South China and is consumed by over two third of the world's population. Tea has an attractive aroma, good taste and health promoting effects, and these benefits make it one of the most popular drinks in the world. It is used in almost every country around the world and has reached ceremonial status in many places. For example, in UK, one liter of tea in average is consumed per person per day [1]. Tea is an excellent beverage and has effective biological and pharmaceutical usefulness. Tea plants are now widely cultivated in Southeast Asia including China, India, Pakistan, Japan, Taiwan, Sri Lanka, Malaysia, Bangladesh, Indonesia and Central African countries [1].

The beneficial effects of tea are; reduction of cholestrol, neurodegeneratuive diseases, *i.e.,* Alzheimer's and Parkinson's, preventing the degradation of cell membrane, decreasing tension, antioxidant and antimicrobial activities, protection against cardiovascular diseases and cancer [2-4]. All types of *C. sinensis* have stimulant properties. Pregnant and nursing mothers, anxiety disorder and insomnia patients limit their daily intake to 1 or 2 cups with the last cup taken at least 3 hours before bed time [5].

Keeping in view the importance of tea bioactive constituents, it is important to establish a simple and reliable analytical method for the determination of these compounds in commercial tea samples in order to produce and select good quality tea products. In reported methods, the tea catechins and caffeine have been analyzed using paper chromatography [6], thin layer chromatography [7], gas liquid chromatography [8], high-performance liquid chromatography [9, 10] and various spectroscopic methods [11], with emphasis on individual catechin. But little work has been reported on the determination of free phenolic compounds, which are also important tea constituents with antioxidative properties [9].

In the previous studies, we have reported the physico-chemical characteristics and determined heavy metals [12] and vitamin C [13] in the commercial tea brands and fresh tea leaves. There is no data available on the organic constituents of various tea brands and fresh tea leaves marketed in

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Pakistan. The present study was conducted to investigate the organic constituents, *i.e.*, caffeine, pyrogallol, gallic acid and polyphenols (catechin and epicatechin) in tea samples.

Results and Discussion

From the literature survey and to the best of our knowledge, among the various methods used for the analysis of tea constituents, high-performance liquid chromatography finds numerous advantages due to its simplicity, accuracy and precision. Tea samples were extracted with a mixture of aqueous ethanol and analyzed by an isocratic reverse-phase high-performance liquid chromatographic (RP-HPLC) method [14] with some modifications for the determination of organic constituents, *i.e.*, caffeine, pyrogallol, gallic acid and polyphenols (catechin and epicatechin). The chemical structures of the compounds studied are given in Fig. 1. It was observed that when the tea samples were extracted with a mixture of distilled water and ethanol in the concentration range of 65-75% (v/v), high amount of the selected compounds were obtained. When the tea samples were extracted with 75% (v/v) aqueous ethanol mixture, the amount of these components were 4.7% higher than those extracted with boiling distilled water (data not shown). This might be due to the lack of significant water to assist tea leaves in spreading the matrix structures. We therefore, continued to use 75% (v/v) aqueous ethanol mixture for the extraction of selected analytes from all tea samples. The standard solutions of selected compounds were injected into HPLC and from the retention times, the analytes were identified and from the peak areas, the compounds were quantitatively determined. The mobile phase (methanol/ deionized double distilled water/ formic acid, 19.5 : 82.5 : 0.3, v/v/v) was eluted isocratically at a flow rate of 2.3 mL/min while in the earlier work, 1.0 mL/ min was used [14]. In the current study, it was observed that with higher flow rate the retention time was decreased while the resolution was increased, which could account for the advantage over the existing method.

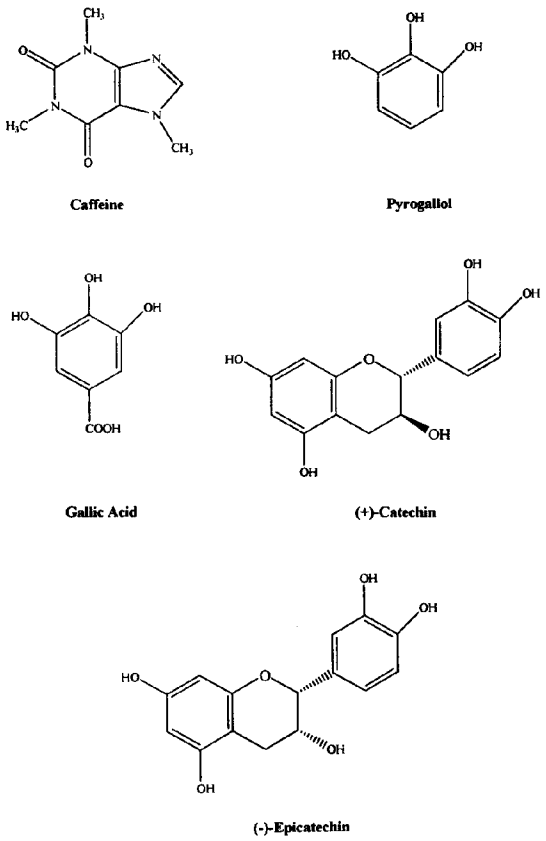

Fig. 1: Chemical structures of the compounds studied.

Table-1: Concentration (mg/g) of organic constituents in commercial tea brands and fresh tea leaves.

S. No.	Tea Brands	Caffeine	Pyrogallol	Gallic acid	Catechin	Epicatechin
1.	Supreme[bb]	90.96 ± 5.20	65.68 ± 5.00	9.30 ± 1.15	7.09 ± 0.59	ND
2.	Lipton[bb]	52.29 ± 3.33	60.33 ± 4.46	7.38 ± 0.99	5.06 ± 0.37	ND
3.	Tapal[bb]	59.86 ± 4.00	116.88 ± 8.76	10.34 ± 1.25	4.43 ± 0.26	ND
4.	Tetley[bb]	82.06 ± 4.81	74.16 ± 6.72	10.55 ± 1.87	6.32 ± 0.45	ND
5.	Kenya Plane[ub]	92.29 ± 6.11	108.59 ± 7.31	12.13 ± 2.09	4.99 ± 0.22	ND
6.	Kenya Danidar[ub]	55.66 ± 4.90	94.03 ± 7.05	9.63 ± 0.97	11.76 ± 1.27	ND
7.	Bangladesh[ub]	76.76 ± 5.20	63.17 ± 4.91	9.87 ± 1.03	4.87 ± 0.38	ND
8.	Indonesia[ub]	123.83 ± 11.75	171.71 ± 15.34	16.73 ± 1.92	9.65 ± 1.00	ND
9.	India[ub]	57.88 ± 3.92	91.63 ± 6.88	9.02 ± 0.89	4.67 ± 0.17	ND
10.	Brazil[ug]	10.95 ± 1.33	282.86 ± 25.22	4.00 ± 0.27	30.83 ± 2.36	23.84 ± 2.77
11.	India[ug]	5.90 ± 0.61	418.86 ± 38.03	5.97 ± 0.32	15.17 ± 1.14	38.02 ± 3.56
12.	Indonesia[ug]	5.90 ± 0.70	132.82 ± 12.47	12.70 ± 1.50	32.54 ± 2.66	34.52 ± 3.09
13.	Vietman[ug]	14.66 ± 1.99	325.51 ± 34.69	0.71 ± 0.02	19.35 ± 2.13	52.97 ± 6.04
14.	Fresh Tea Leaves	3.63 ± 0.21	26.56 ± 2.39	5.02 ± 0.20	5.13 ± 0.31	22.01 ± 1.99

Each reading is the mean of triplicate analysis ± SD.
bb: Branded black
ub: Unbranded black
ug: Unbranded green
ND: Not detected
SD: Standard deviation

Caffeine

The concentration of caffeine in all the tea samples was in the range between 3.63 ± 0.21 to 123.83 ± 11.75 mg/g as shown in Table-1. Among the black branded and unbranded tea samples, maximum concentration of caffeine was found in the Sample-8 (123.83 ± 11.75 mg/g) followed by

Sample-5 (92.29 ± 6.11 mg/g) and minimum was observed for the Sample-2 (52.29 ± 3.33 mg/g). Previously, the amount of caffeine reported in the black teas marketed in China was in the range of 35.5 to 58.7 mg/g [15]. In green tea leaves, higher amount of caffeine was detected in Sample-13 (14.66 ± 1.99 mg/g) followed by Sample-10 (10.95 ± 1.33 mg/g) while Sample-11 and Sample-12 contained the same amount of caffeine, *i.e.*, 5.90 mg/g. In case of fresh tea leaves collected directly from the tea plant, the concentration of caffeine was recorded as 3.63 ± 0.21 mg/g, which is lowest among all the tea samples. Low levels of caffeine in tea shoots collected in the field were also reported previously [16]. Thus, as a whole the concentration of caffeine in the tea samples was in order of black teas > green teas > fresh tea leaves.

The amount of caffeine determined in the black tea samples was much higher compared to green tea and fresh tea leaves (Table-1). It could be therefore, speculated that the concentration of caffeine increased tremendously with the fermentation and oxidation processes. Similar results were also reported elsewhere [14, 17]. There is also much variation of caffeine levels within the black tea brands but in case of green tea, this variation is much lower. There are three possible reasons for this, *i.e.*,

1. Most of the tea samples purchased from the market are of import quality.
2. The difference in the degree of fermentation during the manufacturing processes.
3. The third possible reason may be due to the difference in the origin of tea plant and their environmental and ecological conditions.

The major concern with drinking too much tea is the caffeine it contains. Too much caffeine can cause nausea, insomnia or frequent urination and encourage the formation of scabs if applied directly to a wound.

Pyrogallol

Pyrogallol contents were investigated in all the tea samples and its concentration was from 26.56 ± 2.39 to 418.86 ± 38.03 mg/g as presented in Table-1. In black teas, its concentration was in the range from 60.33 ± 4.46 (Sample-2) to 171.71 ± 15.34 mg/g (Sample-8), while in green teas, the amount of pyrogallol was from 132.82 ± 12.47 (Sample-12) to 418.86 ± 38.03 mg/g (Sample-11). In fresh tea leaves, its concentration was found to be 26.56 ± 2.39 mg/g, which is lowest value among all the tested tea samples. The results show a general trend of pyrogallol concentration in the tea samples in order of green teas > black teas > fresh tea leaves.

Slight variations in the concentration of pyrogallol among the same brand may be associated with different environmental and climatic changes, horticulture and fermentation conditions [6]. However, in case of green tea samples, the amount of pyrogallol was increased to its maximum value among all the tea samples. It could be concluded that the manufacturing processes are responsible for the increase in the amount of pyrogallol in the tea samples.

Gallic Acid

In case of gallic acid, minimum amount was recorded in Sample-13 (0.71 ± 0.02 mg/g) while maximum was found in Sample-8 (16.73 ± 1.92 mg/g) and the other levels were within these values (Table-1). In black tea brands, its concentration ranges from 7.38 ± 0.99 (Sample-2) to 16.73 ± 1.92 mg/g (Sample-8) and in green teas, its amount varies from 0.71 ± 0.02 (Sample-13) to 12.70 ± 1.50 mg/g (Sample-12). The level of the gallic acid in fresh tea leaves was 5.02 ± 0.20 mg/g.

From the results, is clear that higher amount of gallic acid was found in the black tea samples compared to green teas and fresh tea leaves. Although, the variation in the concentration of gallic acid among the black tea brands was not much higher compared to the green tea samples as shown in Table-1. Thus, the overall concentration level of gallic acid in the tested tea samples was in the order of black teas > green teas > fresh tea leaves. Similar results were also reported by Zuo *et al* [17].

Polyphenols

Polyphenols including catechin and epicatechin were also analyzed in all the tea samples. Catechin was found in all the tested tea samples while epicatechin was detected only in the green teas and fresh tea leaves (Table-1). The effects of tea consumption on health have received a great deal of attention. Many laboratory studies have demonstrated clearly and repeatedly the inhibition of tumorigenesis by tea and tea polyphenols [18,19].

Catechin

The concentration of catechin determined in the tea samples was in the range 4.43 ± 0.26 to 32.54 ± 2.66 mg/g as shown in Table-1. As can be seen from these results that catechin was present in black and green teas as well as in the fresh tea leaves but the quantity recorded was found to be high in green teas as compared to black teas and fresh tea leaves. In black teas, the concentration of catechin was in the range 4.43 ± 0.26 (Sample-3) to 11.76 ± 1.27 (Sample-6) mg/g, while in green tea its amount was from 15.17 ± 1.14 (Sample-11) to 32.54 ± 2.66 (Sample-12) mg/g. Catechin concentration in the fresh tea leaves (Sample-14) was found to be 5.13 ± 0.31 mg/g (Table-1). Variations in the concentration of catechin were found even among the same tea brands. The general trend of catechin level in the tea samples was in order of green teas > fresh tea leaves > black teas. Similar findings were also reported elsewhere [14, 20].

Catechins constitute about 25% of the dry weight of fresh tea leaves, although total catechin content varies widely depending on clonal variation, growing location, seasonal/light variation and altitude. Catechins are very sensitive and heating them above their point of decomposition releases pyrocatechol and converts them into more complex structures such as caffeine, thearubigin, theaflavin, bisflavanols, etc. [9]. In case of black and oolong teas, the leaves are allowed to oxidize (ferment), during which enzymes present in the tea convert some or all of the catechins to larger molecules. However, green tea is produced by steaming the fresh-cut leaf, which inactivates these enzymes, and oxidation does not significantly occur [21].

Epicatechin

Epicatechin was not detected in black teas and found only in the green teas and fresh tea leaves (Table-1). The amount of epicatechin in the green tea samples was found to high compared to the fresh tea leaves. In green teas, its concentration was from 23.84 ± 2.77 to 52.97 ± 6.04 mg/g in which maximum amount was recorded for Sample-13 (52.97 ± 6.04 mg/g) followed by Sample-11 (38.02 ± 3.56 mg/g) and Sample-12 (34.52 ± 3.09 mg/g) and minimum in Sample-10 (23.84 ± 2.77 mg/g). The amount of epicatechin in the fresh tea leaves (Sample-14) was found to be 22.01 ± 1.99 mg/g, which is the lowest concentration of this analyte among all the tea samples. As a whole the amount of epicatechin found in the tea samples was in the order of green teas > fresh tea leaves > black teas. Lower and higher concentration of epicatechin in black and green teas respectively, was also reported in the literature [14, 17, 20].

The total contents of polyphenols in green teas were higher than the fresh tea leaves and black tea samples. It appears that the catechin biosynthesis has continued during the manufacturing process, increasing the amount of total polyphenols. Another explanation is that the mechanical operation in the withering process may rupture the outer membrane of the tea leaves, liberating the intracellular polyphenols and rendering them available for extraction [14].

In conclusion, the present study is the first of its kind which provides new reference data for the major organic constituents in the commercial tea brands and fresh tea leaves available in Pakistan. This research work will also create awareness and provide useful information to the public and health related institutions in Pakistan, regarding the beneficial effects of tea and its daily intake.

Experimental

Chemicals and Reagents

All the chemicals and reagents used in the present work were of analytical grade. Caffeine, pyrogallol, gallic acid and polyphenols (catechin and epicatechin) were purchased from Sigma Chemical Co. (St. Louis Mo, USA). Methanol, ethanol, formic acid, dimethyl sulphoxide (DMSO) and other solvents were of HPLC grade and were obtained from Merck (Darmstadt, Germany). De-ionized double distilled water was used through out this work.

Instrument

A Shimadzu (Japan) HPLC system equipped with techosphere ODS 5 μm column (250 mm x 4.6 mm i.d., Welwyn Garden City, Herts, USA) and a UV-visible detector (Uvis-204, Techno World) was used for the analyses of selected analytes.

Collection of Tea Samples

A total of 14 tea samples including branded and unbranded black and green teas were collected,

in which 13 tea samples were of commercial grade and purchased from the local markets in Peshawar, N.W.F.P., Pakistan. Majority of these tea brands are imported from various countries including Kenya, Bangladesh, Indonesia, India, Brazil and Vietman. The remaining one tea sample (fresh tea leaves) was obtained directly from tea plants at the National Tea Research Center, Mansehra, N.W.F.P., Pakistan (Table-1).

Preparation of Extracts from Commercial Tea and Fresh Tea Leaves

Tea samples were first dried in oven at 100 ^{0}C till a constant weight. Each of the dry tea sample (1 g) was steeped in 75% (v/v) aqueous ethanol (100 mL) at 60 ^{0}C for 30 min. The infusion was filtered through a 0.22 μm GV Durapore membrane filter (Millipore, Ireland). The filtrate was dried under reduced pressure by rotary evaporator to give a powdered crude extract which was kept in a refrigerator until use. In most experiments, the crude extract was dissolved in DMSO (50 mg/mL) and diluted to desired concentrations [14]. In case of fresh tea leaves, the tea sample was first shade dried and then extracted with the desired solvent system as mentioned above.

RP-HPLC Analyses of Tea Caffeine, Pyrogallol, Gallic Acid and Polyphenols

The chemical composition of tea caffeine, pyrogallol, gallic acid and polyphenois (catechins and epi-catechin) (Fig. 1) in 13 different commercial tea samples and fresh tea leaves were determined by an isocratic RP-HPLC method [14] with slight modifications. The tea extracts were filtered through a 0.22 μm filter disk and was injected into the HPLC system. The concentration of all the analytes working solution was 100 mg/L. Five hundred nanograms of each standard compound (caffeine, gallic acid, pyrogallal catechin, epi-catechin) was injected. The mobile phase used was comprised of methanol/de-ionized double distilled water/formic acid (19.5 : 82.5 : 0.3, v/v/v) and was degassed by sonication (Branson 2210). The mobile phase was eluted isocratically at a flow rate of 2.3 mL/min and the analytes were detected at 280 nm using a UV-visible absorbance detector. In the previous study [14], mobile phase was eluted at a flow rate of 1.0 mL/min. The increase in the mobile phase flow rate increased the resolution while it decreased the retention time of each constituent, as observed in the present study. The analytes were identified by comparing the retention times of the sample peaks to those of reference standards and from the peak areas, the amount of compounds were determined. Each sample was analyzed in triplicate and reported as the mean of three readings. All measurements were taken at room temperature.

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