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Qualitative and quantitative evaluation of flavanols in green tea [*Camellia sinensis* (L) O Kuntze]

Arti Ghabru and RG Sud

Abstract
Plants are commonly used in treating or preventing specific ailments or diseases and are playing valuable role in health care. Probably, around 60% of world's population is relying on medicinal plants for their primary healthcare. Bioactive plant extracts are considered as a promising source of biological friendly antibacterial agents. The relationship between the quality and chemical components in green tea have been studied, and have shown that free amino acids, caffeine and polyphenols are qualitatively important components. Especially, catechins, the main component of polyphenols, are well known for their antioxidant properties, which have led to their evaluation in many diseases associated with free radicals. Significant seasonal variations of phenolic content were observed. The order of variations of flavan-3-ols was (–)-epigallocatechin gallate > (–)-epigallocatechin > (–)-epicatechin gallate > epicatechin > (+)-catechin.

Keywords: Epigallocatechin, Green tea, Epigallocatechin gallate, HPLC, TLC

Introduction
Tea originated in China where its legendary iive and history dates to as far back as 2737 B. C. Several centuries later, tea was brought to Japan in the 6th century and to Europe in 1559 A. D. (Wickremasinghe 1978) [30]. All teas are derived from the tea shoots (two leaves and a tender apical bud) of *Camellia sinensis* plant; different processing methods produce tea with diverse attributes. Based on the extent of fermentation the tea shoots undergo during processing, teas may be divided into three major groups: unfermented tea (green tea), semi-fermented tea (oolong tea) and fully fermented tea (black tea). Some factors are more important than others; for example, the highest quality green teas are plucked during the first flush in late April and early May and quality declines in later harvests (Le Gall *et al*., 2004) [15]. Usually, the buds and the first two to three leaves are plucked by hand or a mechanical tea plucker for processing. This process is generally repeated every one to two weeks. These basic types of tea have different quality characteristics, including appearance, flavor, taste, and color (Ho *et al*., 2008) [13]. The relationship between the quality and chemical components in green tea have been studied, and have shown that free amino acids, caffeine and polyphenols are qualitatively important components (Cao and Cao 1999; Muktar and Ahmad 2000; Yang *et al*., 2002; Pfeffer *et al*., 2003; Chyu *et al*., 2004; Mandel and Youdim 2004) [5, 19, 31, 23, 7, 17]. Fresh tea leaves are rich in flavonoids - a group of phenolic compounds known as catechins. These catechins and polyphenol oxidase enzyme which are present in separate compartments in the leaf comes in contact with each other during rolling step in tea processing and ferment to transform catechins into dimeric theaflavins and polymeric thearubigins (Roberts and Williams 1958) [24]. Steaming or firing of green tea leaves inactivates polyphenol oxidase enzyme and the fermentation process is stopped. The five major flavonoids (flavan-3-ols) in fresh green tea shoots, classified as catechins, which have been reported to exhibit bioactive properties (Gramaza *et al*. 2005; Zaveri 2006) [10, 33] are: (–) - epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), (–)-epicatechin (EC) and (+)-catechin (C). The antioxidant properties of catechins are mainly related to the number and position of hydroxyl group in the molecules and consequently binding and neutralization of free radicals by these hydroxyl groups (Millic *et al*. 1998; Guo *et al*.,1999; Farkas *et al*., 2004) [18, 11, 9]. Previous studied have shown that tea catechins are excellent electron donors and effective scavengers of physiologically relevant reactive oxygen species *in vitro*, including superoxide anions, peroxyl radicals, and singlet oxygen (Gou *et al*., 1999; Nakagawa and Yokozawa 2002; Nanjo *et al*., 1993; Velayutham *et al*., 2008) [11, 20, 21, 29].

Most studies on the antioxidant effects of green tea are directly related to the total phenolic extracts, without considering the contributions of individual molecules, although various catechins, such as EGCG, ECG and EGC, have been linked to strong antioxidant activity in green tea extracts. Therefore, in this study, the qualitative and quantitative determination of phenolic compounds in green tea was determined.

Materials and Methods

The present investigations were carried out to explore fresh green tea shoots of Kangra local for their total polyphenols and flavan-3-ols profiles during four harvesting flush seasons (first flush: April to mid May; second flush: mid May to June; rainy flush or main flush: July to mid September and winter flush: mid September to October). Samples of fresh tea shoots were collected from Wah Tea Estate, Rajpura, Palampur at seven day intervals throughout the plucking seasons (April to October/November). These samples were subjected to microwave heat treatment within twenty minutes of plucking, dried and analyzed for total polyphenols and total catechins (flavan-3-ols), using standard techniques. (+)-Catechin (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG) and (–)-epicatechin gallate (ECG) were estimated in tea powders obtained by lyophilizing the aqueous extracts of fresh green tea shoots using chromatographic techniques.

Sampling

Samples of fresh green tea shoots (two leaves and a bud) were collected from Wah Tea Estate, Rajpura, Palampur during four flush seasons (first flush: April to mid May; second flush: mid May to June; rainy flush or main flush: July to mid September and winter flush: mid September to October). Owing to low temperature during winters, the tea plant experience complete dormancy for about 120-150 days. The samples of fresh green tea shoots were always subjected to heat treatment in a microwave oven for three minutes.

Preparation of tea extracts

All quantitative estimations were always carried out in freshly prepared tea extracts. Dried tea sample (300 mg) was taken in a 250 mL Erlenmeyer conical flask and 100 mL of pre-boiled hot double distilled water was poured into the flask. The flask was covered with aluminum foil and kept in a water bath shaker maintained at 60±5 °C for 20 minutes. The contents of the flask were allowed to cool to room temperature and then filtered through Whatman Grade 1 filter paper in a 100 mL measuring flask. The final volume was made with the help of double distilled water.

Estimation of total polyphenols

Total polyphenols were always estimated in freshly prepared tea extracts by the method of Makkar (2003) [16]. The concentration of total polyphenols (TP) was finally expressed in terms of g kg^{-1} of fresh green tea shoots on dry weight

Estimation of total catechins

Total catechins were always estimated in freshly prepared tea extracts by the method of Sun *et al.* (1998) [28]. The concentration of total catechins (TC) was finally expressed in terms of g kg^{-1} in fresh green tea shoots on dry weight basis.

Qualitative evaluation of flavan-3-ols

Qualitative evaluations of flavan-3-ols in tea powders were carried out using chromatographic techniques. The tea powders were subjected to size-exclusion chromatographic separation using Sephadex G-25 (Sigma, USA) as stationary phase and 50% ethanol (AR) as mobile phase by the method of Cutler (2008) [8]. Various fractions collected were subjected to thin-layer chromatography along with standard flavan-3-ols by the method of Sherma and Fried (1996) [25].

1. Size-Exclusion chromatography

Swollen Sephadex G-25 gel was degassed and packed in a glass column (48 x 2.5 cm) to a height of 30 cm (bed volume: 147.32 cm^3 and void volume: 49.11 cm^3). The column was equilibrated with 50% ethanol. Fresh solution prepared by dissolving 1 g tea powder into 6 mL of 50% ethanol and applied on the top of the equilibrated column. Elution at a flow rate of 0.8-1 mL per minute was done with 50% ethanol. The eluted fractions, each of 5 mL, were collected in clean and dry test tubes. These fractions were further subjected to thin-layer chromatography and quantitative estimation of total polyphenols and total catechins by standard techniques.

2. Thin- layer chromatography

Thin layer chromatography was done on glass plate coated with silica gel G by the method of Stahl (1969) [27].

Requirements

1. Glass plate (20 x 20 cm)
2. Silica gel G (Merck, Mumbai) for thin-layer chromatography
3. Standard solutions (0.5 mg mL^{-1}): 0.001 g of each standard flavan-3-ols [(+)-catechin, (–)-epicatechin, (–)-epigallocatechin gallate, (–)-epicatechin gallate and (–)-epigallocatechin (Sigma, USA)] was dissolved in 2 mL methanol (AR).
4. Solvent system: Chloroform: Ethyl acetate: Acetic Acid:: 25:75:0.5 (By volume)
5. Detection reagent: Iodine vapours
6. Samples: fractions eluted from Sephadex G-25 column

Procedure

Washed and dried glass plates were cleaned with acetone. Slurry was prepared by mixing 10 g of silica gel G (for each plate) in 20 mL double distilled water. Plates were coated with thin-layer (thickness 0.2 mm) of silica gel G slurry with the help of spreader. The plates were allowed to dry at room temperature and then activated at 110°C in hot air oven for 1 h.

5 µL of catechin, epicatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate solutions and fractions eluted from Sephadex G-25 column, were spotted on silica plate. The plate was developed in the solvent system. The developed plate was dried at room temperature. Detection was done by exposing the plate to iodine vapours in an iodine saturated chamber.

Preparation of tea powders

Tea powders were prepared by lyophilizing aqueous extracts of representative samples. A known weight (7 g) of the representative sample was taken in a 250 mL Erlenmeyer conical flask. To this was added 100 mL of pre-boiled hot double distilled water and the flask along with its contents was kept on water bath shaker (RSB-12 REMI) maintained at

50±5°C for 60 minutes. The contents of the flask were allowed to cool to room temperature; filtered through muslin cloth and then centrifuged at 764 x *g* for 15 minutes and finally filtered through Whatman Grade 1 filter paper to obtain absolutely transparent/clear aqueous extract. The aqueous extracts were finally dried to powder form with the help of Edwards EF_4 Modulyo freeze dryer and Heto Power Dry LL 3000 freeze dryer. The powders so obtained were immediately transferred into glass tubes fitted with air-tight stoppers which were stored in vacuum desiccator.

High performance liquid chromatography (HPLC)

Samples of tea powder were characterized for flavan-3-ols profiles and their quantification by HPLC techniques by the method of Zhu and Chen (1999) [34]. Acetonitrile (ACN) and double distilled water both acidified with 0.025% H_3PO_4 was filtered using Durapore (0.47 µm) membrane filter. The sample solutions and standard solutions were filtered through Whatman stainless steel syringe assembly using 0.22 µm Durapore membrane filter. 20 µL aliquot was used for HPLC injection. The chromatograms were monitored at 220 nm. The flow rate was 1 mL min^{-1}. The elution was done by the following gradient system:

Time duration (minutes)	ACN* (%)	Water* (%)
0	10	90
40	14	86
50	100	0

*acidified with 0.025% orthophosphoric acid (H_3PO_4)

Figure 1 represents the HPLC profile of standard catechins: catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG)

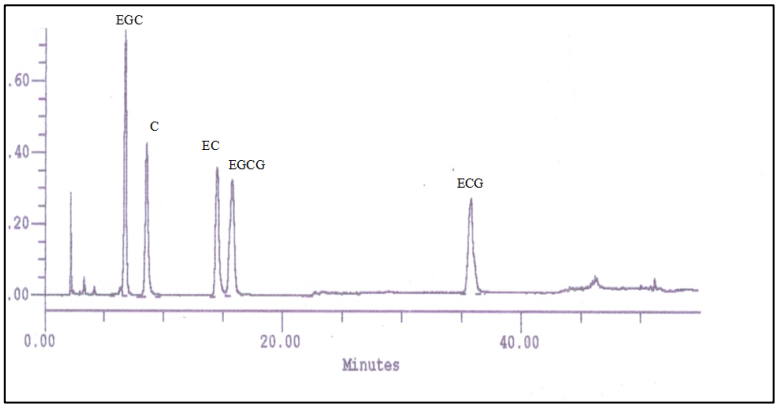

Fig 1: High performance liquid chromatogram of standards: catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG)

Results and Discussion

Qualitative and quantitative evaluation of tea powders

The tea powders obtained by lyophilizing aqueous extracts of fresh green tea shoots, bud, first leaf and second leaf were evaluated qualitatively and quantitatively using chromatographic techniques. Tea powders were subjected to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) to elucidate flavan-3-ols profile of Kangra tea.

Figure 2 represent the percent recovery of tea powder in green tea shoots (on dry matter basis) and comparison of mean total polyphenols (TP) and total catechins (TC) in tea powders and pooled samples of fresh green tea shoots. Figure 2 indicate that TP and TC were more in tea powder than fresh green tea shoots sample.

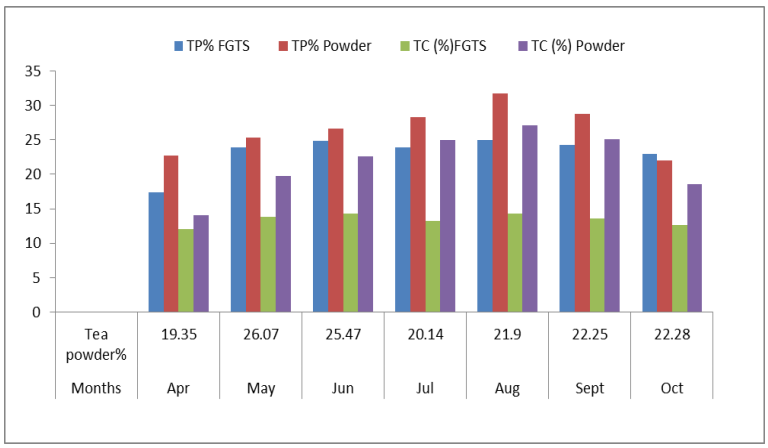

Fig 2: Tea powder recovery per cent green tea shoots (on dry matter basis) and comparison of mean total polyphenols (TP) and total catechins (TC) in tea powders and pooled samples of fresh green tea shoots

Months - Apr- April; Jun- June; Jul-July; Aug- August; Sep- September; Oct- October. FGTS – Samples of fresh green tea shoots; [c]Powders – Samples of tea powders obtained by lyophilizing aqueous extracts of fresh green tea shoots.

Table 1 indicate the percent recovery of tea powder in bud, first leaf and second leaf (on dry matter basis) and comparison

of mean total polyphenols (TP) and total catechins (TC) in tea powders and pooled samples of bud, first leaf and second leaf.

Qualitative evaluation

Figure 3 give graphical representations of absorbance at 725 nm (corresponding to TP) and to absorbance at 500 nm (corresponding TC) of fractions eluted from 50% ethanolic solutions of tea powders obtained from fresh green tea shoots harvested. It was of interest to note that a bell-shaped (normal) curve was always obtained, indicating an increased trend of concentrations of both TP and TC up to fraction numbers 20th, 22th and 19th, after which a steady decrease was always observed. All fractions eluted from column were subjected to thin layer chromatography.

Table 1: Tea powder recovery per cent bud, first leaf and second leaf (on dry matter basis) and comparison of monthly mean total polyphenols (TP) and total catechins (TC) in tea powders and pooled samples of bud, first leaf and second leaf

Months[a]	Tea Powders (%)			TP (%) (FGTS)[b]			TP (%) (powders)[c]			TC (%) (FGTS)[b]			TC (%) (Powders)[c]		
	Bud	*I[st] leaf	**II[nd] leaf	Bud	*I[st] leaf	**II[nd] leaf	Bud	*I[st] leaf	**II[nd] leaf	Bud	*I[st] leaf	**II[nd] leaf	Bud	*I[st] leaf	**II[nd] leaf
Apr	16.90	18.92	20.32	18.60	21.00	20.12	29.84	30.31	28.75	11.59	12.66	11.59	16.23	18.41	14.51
May	23.60	25.50	20.24	23.18	24.69	20.54	28.04	28.36	25.66	13.87	13.70	12.31	20.50	23.34	13.41
Jun	4.90	21.40	24.35	22.92	25.34	18.62	37.25	40.41	35.36	14.93	15.02	14.94	28.68	30.98	28.62
Jul	15.31	16.00	22.35	15.01	19.26	19.30	25.72	29.61	25.79	13.59	14.10	11.22	23.26	26.45	13.44
Aug	26.48	19.82	22.45	19.24	22.72	21.10	30.36	36.41	32.23	15.45	15.63	15.43	24.96	29.29	25.37
Sept	22.70	18.85	21.58	18.48	17.40	18.96	26.24	28.25	27.78	12.92	14.27	11.29	24.08	22.28	14.98
Oct	11.50	14.92	15.88	17.17	19.06	17.31	20.57	27.85	21.08	12.32	12.38	11.06	17.84	22.80	15.55

[a]Months - Apr- April; Jun- June; Jul-July; Aug- August; Sep- September; Oct- October.
[b]FGTS – Samples of fresh green tea shoots; [c]Powders – Samples of tea powders obtained by lyophilizing aqueous extracts of fresh green tea shoots.
*I[st] leaf – Samples of first leaf; **II[nd] leaf – Samples of second leaf.

Thin layer chromatographs (TLC) of standard flavan-3-ols [(+)-catechin (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG) and (–)-epicatechin gallate (ECG)], tea powders and fractions obtained from column are presented in Plate 1. It is evident from a comparison of the chromatograms that the tea powders and column fractions contain all the five flavan-3-ols of interest.

The column fractions which contained significant amount of TC were pooled and lyophilized into powder form for further elucidation of flavan-3-ols.

Quantitative evaluation

In Figure 4, 5, 6 and 7 are given quantitative data on C, EC, EGC, EGCG and ECG estimated by high performance liquid chromatography (HPLC) in various tea powders obtained from samples of fresh green tea shoots, bud, first leaf and second leaf.

The flavan-3-ols in tea powders obtained by lyophilizing aqueous extracts of fresh green tea shoots varied in the range of C: 1.43 to 1.07%, EC: 5.26 to 3.64%, EGC: 33.28 to 23.78%, EGCG: 49.52 to 39.67% and ECG: 24.17 to 20.00%.

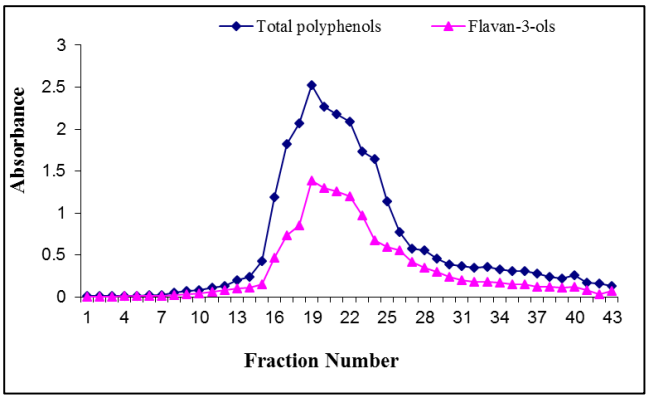

Fig 3: Total polyphenols and flavan-3-ols profile of fractions eluted with 50% ethanol from tea powder obtained by lyophilizing aqueous extract of sample of fresh green tea shoots

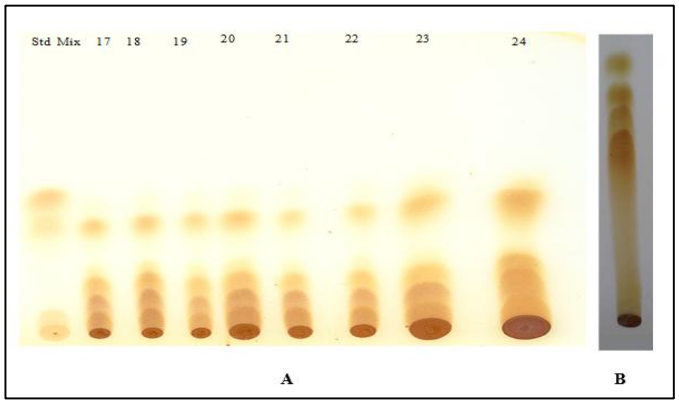

Plate 1: Thin layer chromatogram of tea powder (B) and column fractions (A) containing significant amounts of flavan-3-ols along with standard catechins mixture

Solvent system: Chloroform: Ethyl Acetate: Acetic Acid:: 75:25:0.5

Green tea has been reported to contain approximately 59% EGCG, 19% EGC, 13.6% ECG, EC 6.4% and 0.4% C of the total catechins (Bokuchava and Skobeleva 1969; Cabrera *et al.* 2006) [2, 4]. TC varied in the range of 87.92 to 72.17% (Figure 3). The catechins and catechin gallates have been reported to represent about 80% of the TP in green tea and fresh tea leaves (Opie *et al.* 1988) [22]. A perusal of Figure 3 indicates that the levels of flavan-3-ols in tea powders were invariably higher in the samples of summer and rainy flush seasons. EGC and C were higher in samples of summer flush season; EGCG was always higher in samples of rainy flush season and ECG was higher in samples of summer flush season.

Figures 4, 5 and 6 represent variations of C, EC, ECG, EGCG and EGC in tea powders obtained by lyophilizing aqueous extracts of samples of bud, first leaf and second leaf separately. In the tea powders obtained from bud the levels of flavan-3-ols varied in the range of C:

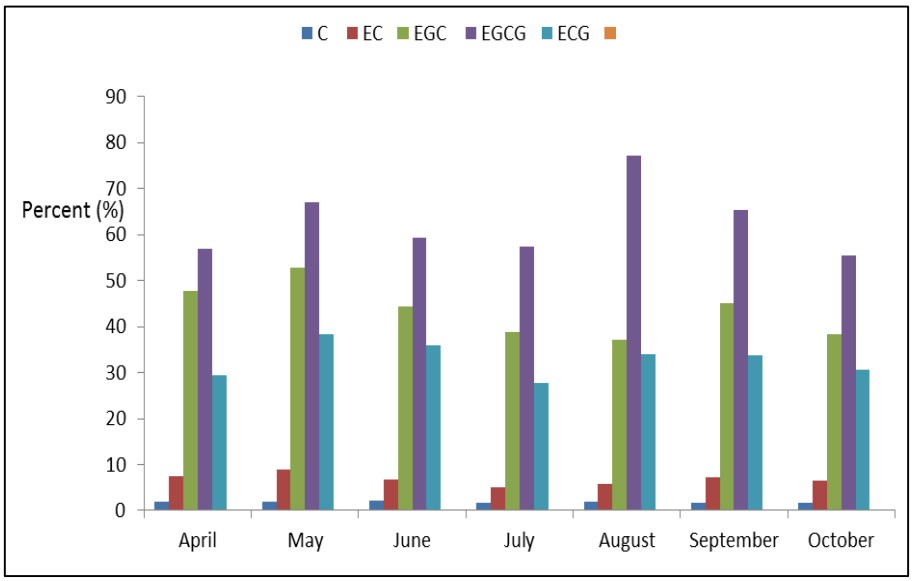

Fig 4: Variations of catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) in tea powders from samples of fresh green tea shoots

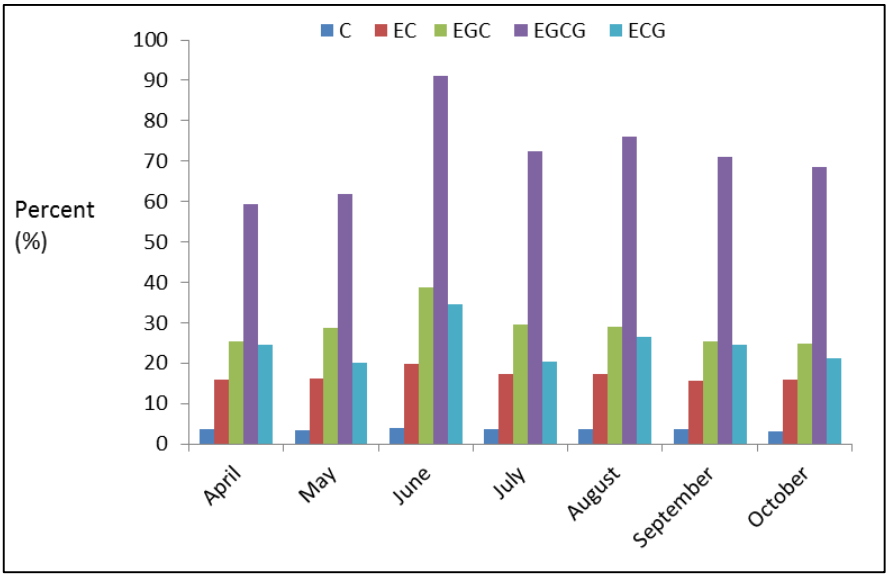

Fig 5: Variations of catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) in tea powders from samples of bud

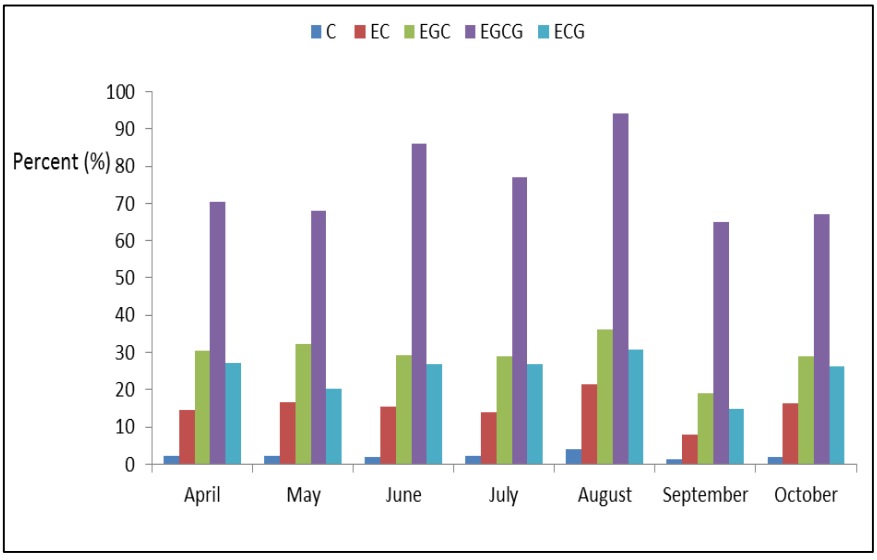

Fig 6: Variations of catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) in tea powders from samples of first leaf

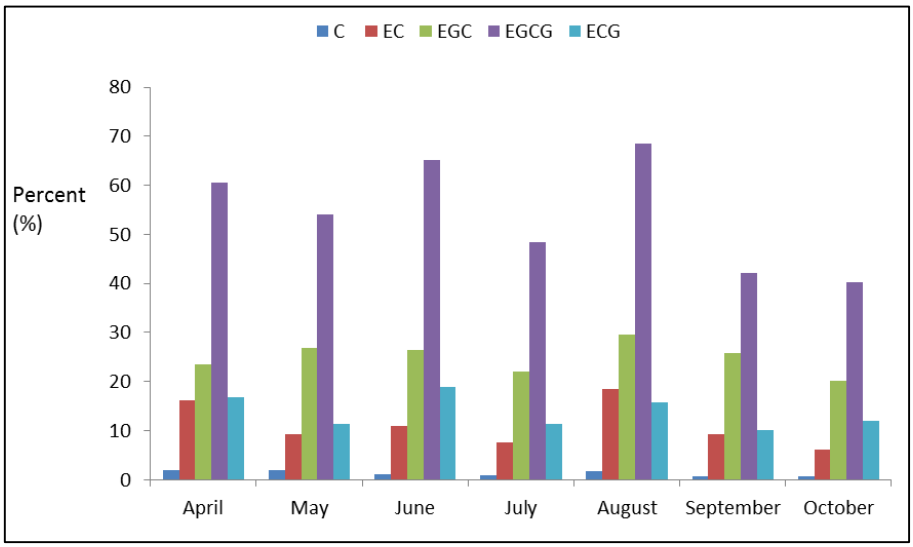

Fig 7: Variations of catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) in tea powders from samples of second leaf

2.84 to 2.09%, EC: 12.38 to 10.47%, EGC: 22.11 to 18.06%, EGCG: 51.27 to 46.05% and ECG: 19.02 to 14.15%. In the tea powders obtained from first leaf C: 2.18 to 1.23%, EC: 12.06 to 7.44%, EGC: 23.08 to 17.62%, EGCG: 59.94 to 47.68% and ECG: 18.80 to 13.73% and in the tea powders obtained from second leaf the variations were in range of C: 1.98 to 0.71%, EC: 13.78 to 7.69%, EGC: 29.34 to 19.78%, EGCG: 53.57 to 47.84% and ECG: 15.46 to 10.96%.

The tea powders obtained from samples of buds during summer flush season recorded higher flavan-3-ols content. In first leaf the tea powders obtained from samples of rainy flush season recorded highest flavan-3-ols contents; however, the tea powders obtained from samples of second leaf had highest flavan-3-ols content during summer and rainy flush seasons. It was of interest to note that whereas the levels of C and EC were higher in samples of tea powders obtained from buds, however, the levels of EGC, EGCG and ECG were higher in tea powders obtained from first leaf. The contents of tea powders obtained from second leaf were intermediate between bud and first leaf. The bud and first leaf of tea have been reported to rich in EGCG content (Bhatia and Ullah 1968) [1].

The EGCG, ECG and CG contents of green teas shoots have been reported to be higher during warmer months compared to cooler months (Chu and Juneja 1997; Singh *et al*. 1999; Yao *et al*. 2005) [6, 26, 32]. This could be due to the active synthesis of EGCG and ECG during summers which in turn may be related to the length of daytime or stronger sunlight (Harbowy and Balentine 1997) [12].

It was of interest to note that the five major flavan-3-ols always varied in order EGCG>EGC>ECG>EC>C. These observations are in agreement with the earlier results (Bronner and Beecher 1998; Karori *et al.* 2007) [3, 14].

Conclusion

Significant seasonal variations of total polyhenols, total catechins and flavan-3-ols in fresh green tea shoots and leaves with increase age (bud, first leaf and second leaf). Fresh green tea shoots and leaves with increasing age of summer and rainy flush seasons invariably had higher contents of total catechins and flavan-3-ols. The per cent composition of flavan-3-ols: (+)-catechin = 2.51 to 0.69%, (–)-epicatechin = 14.05 to 3.54%, (–)-epigallocatechin = 36.01 to 19.65%, (–)-epigallocatechin gallate = 51.88 to 38.08% and (–)-epicatechin gallate = 24.38 to 13.23% in fresh green tea shoots of Kangra local were lower than the reported values. The order of variations of flavan-3-ols was (–)-epigallocatechin gallate > (–)-epigallocatechin > (–)-epicatechin gallate > epicatechin > (+)-catechin.

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