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ORIGINAL ARTICLE

In vitro Accumulation of Polyphenols in Tea Callus Derived from Anther

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ABSTRACT

Background: Tea is an economic important crop with high medicinal value due to rich polyphenols content. In the present research we studied the accumulation of polyphenols of *in vitro* regenerated callus from anthers. **Objective:** Callus induction of tea anthers and *in vitro* accumulation of phenolic compounds from the anther-derived callus. **Materials and Methods:** Standardization of callus induction for tea anthers. *In vitro* generated callus was screened for *in vivo* accumulation of catechins and its isomers were screened by FC reagent staining technique. The methanol extract of dry and green callus obtained were estimated qualitatively by Fourier transform infrared spectroscopy (FTIR)–alternative total reflection (ATR) and quantitatively by HPLC method. **Results:** Anthers inoculated on half strength MS media fortified with 2,4-dichloro acetic acid (2 mg/L), Kn (1 mg/L), and BAP (1 mg/L) induced callus under photoperiod of 9:15 h light. The *in vivo* histochemical studies revealed the accumulation of polyphenols in the callus. The *in vitro* generated fresh and dry callus were used for extraction and screened for accumulated polyphenols [galic acid, (+)-catechin (C), (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-gallocatechins, (−)-epicatechin gallate] were estimated qualitatively by FTIR–ATR method and quantitatively by HPLC method. **Conclusion:** The FC staining technique used here helps in localization of polyphenol compounds accumulation in the tissues by instant microscopic studies. The study have scope in large-scale isolation of various medicinally important flavonol by using anther culture.

Key words: Anther, catechins, callus, PGR, polyphenols

Abbreviations used: HPLC: high pressure liquid chromatography; FTIR: Fourier transform infrared spectroscopy; 2,4-D: 2,4-dichloro acetic acid; BAP: N^6-benzyl amino purine; kn: kinetin

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INTRODUCTION

Tea is the most widely consumed beverage in the world, which ranks after water,[1] and is especially popular in Asian countries.[2] The chemical composition of tea is complex and includes polyphenols, alkaloids (caffeine, theophylline, and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements, and other unidentified compounds.[3] Green tea is known to its high medicinal properties contains nearly 4000 bioactive compounds, among all the chemical constitutes major polyphenolic / flavonol compounds in tea are the flavan-3-ols (i.e., catechins), which include (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-gallocatechins (GC), (−)-epicatechin gallate (ECG), and (−)-gallocatechin gallate (GCG).[4,5] Number of studies recorded the beneficial effects of tea, which include antioxidant properties of tea,[6-8] anticarcinoma,[9-11] anti-inflammatory,[12-14] antidiabetic,[15] and antimicrobial properties.[16,17] In this study, the accumulation of these biochemical constituents were studied in the *in vitro* generated callus derived from anther culture.

MATERIALS AND METHODS

Explant material

Flower buds were collected from UPASI-9 cultivar belongs to *Camellia sinensis* grown at United Planters' Association of Southern India (UPASI experimental farm; 11°59'0.69"N, 76°57'2.32"E and 1050 m above mean sea level). Flower buds size of ≥0.5 ± 0.1 cm were preferred for the culturing.[18]

Surface sterilization

Floral buds were sterilized in 0.1% mercuric chloride solution for 60 s, followed by rinsing with the absolute alcohol for 30 s, and finally washed thrice with autoclaved double distilled water to remove the alcohol traces.

Culture media preparation

Full and half strength Murashige and Skoog (1962)[19] 0.8 % agar medium (pH 5.8) containing carbon source 1.5 % of sucrose (HI Media, Mumbai, India), supplemented with 2 mg/L 2,4-dichloro acetic acid (2,4-D) and varied concentrations of kinetin, BAP, and NAA (0.5, 1.0, and 1.5 mg/L). The culture medium devoid of auxins and cytokinins served as control as shown in Table 1. The medium was autoclaved at 120°C under 15 lbs pressure for 15 min. All the cultures were maintained in replicates of three.

Culture maintenance and incubation condition

Anther cultures were maintained at 22 ± 1°C, 9:15 h photoperiod under 40 μmol/m^2/s photoperiod intensity provided by white fluorescent light and a relative humidity maintained around 65–70 %. The cultures were subcultured every 15 days interval and increase in biomass was recorded with respective medium.

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Table 1: The growth medium supplemented with MS and 0.5 MS varied proportionate of PGRs

Treatments with growth medium (MS and 0.5 MS)	Plant growth regulator (mg/L)			
	2,4-D	Kin	BAP	NAA
T1	0	0	0	0
T2	2	0.5	0	0
T3	2	1	0	0
T4	2	1.5	0	0
T5	2	0	0.5	0
T6	2	0	1	0
T7	2	0	1.5	0
T8	2	0	0	0.5
T9	2	0	0	1
T10	2	0	0	1.5

Tissue localization studies from anther derived callus

The callus size of 1.35 cm and weighs 22 mg was used for tissue localization studies. Callus was decolorized in absolute ethanol for 30 min. The decolorized callus was washed thrice with sodium phosphate buffer, pH 7.4. The sections were fixed with 4% (w/v) paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.4) for 1 h. The callus was microsectioned and stained with Folin–Ciocalteu reagent. The Folin–Ciocalteu treated slides were dipped in 20% Na_2CO_3 solution. The stained specimens were washed thrice with 50 mM sodium phosphate buffer and placed on clean microscopy slide. A drop of 50% glycerol was added on the specimen and coverslip was placed, images were captured using microscope (Nikon Eclipse TE2000-5).

Determination of callus fresh weight

The callus were removed from medium washed with sterile double distilled water. Moisture was removed by wrapping in autoclave sterilized blotting paper. Fresh weight for three callus was recorded by placing in a sterile petri plate. Fresh weight of callus was enumerated by using the formula: Fresh weight of callus = Weight of petri plate with callus – Weight of empty petri plate.

Determination of callus dry weight

After determining fresh weight, callus was placed on petri plate and kept in an oven for 24 h at 45°C for drying or to complete dryness. Dry weight for three callus was recorded by placing in a sterile petri plate. Dry weight of callus was enumerated by using the formula:

Dry weight of callus = Weight of petri plate with callus – Weight of empty petri plate.

Determination of callus moisture content

The moisture content was determined using the fresh and dry weight of callus. Moisture content % of fresh green was determined by following formula: $\text{Moisture content}\,\% = (B-A)-(C-A)/(B-A)\times 100$,

where A is the weight of empty petri plate, B is the weight of petri plate with fresh callus, and C is the weight of petri plate with dry callus.

Sample preparation and extraction

The fresh and dry callus material was carefully weighed and ground in 70% methanol (v/v). Total polyphenols were extracted by modified methods of Hara (2001) and John *et al.*[20,21] The ground plant samples (0.75 g) were separately placed in a stopped conical flask and macerated with 25 mL methanol (70%, v/v) at room temperature (22–25°C) for 60 min with occasional stirring. The solvent was filtered with Whatmann filter paper No. 1 and evaporated in a vacuum rotary evaporator (Buchi) at 45°C. Total polyphenols were extracted with equal volumes (10 mL) of ethyl acetate. The ethyl acetate phase kept on dry bath for condensation at 80°C and freeze dried. The freeze dried polyphenols were liquefied at room temperature and the final volume was made up to 4 mL with 70% methanol and analyzed through FTIR and HPLC methods.

FTIR qualitative estimation method

Fourier transform infrared spectroscopy (FTIR)–alternative total reflection (ATR; Manf. Bruker) was calibrated by using polystyrene film. Sample extract of three to four drops was placed on the zinc selenium disc at ATR module. Samples were measured at given range 400–4000 cm^{-1} absorbance, after 16 scans spectrum was generated, base line correction was done to generate spectrum, and two times smoothing was done. Then sample spectras were compared with the standard spectras.

Quantitative estimation method by HPLC

The purified extract was analyzed for individual catechin fractions, according to the method described in John *et al.*,[21] by using reversed-phase HPLC method (HPLC Agilent 1100 series with diode array detector system) with a Phenomenex column. Acidified acetonitrile (18%) and acetonitrile (80%) were used as mobile phases A and B, respectively. Sigma-Aldrich HPLC grade chemicals GA (97%), Cf (99%), C (97%), EC (98%), EGC (99 %), EGCG (95 %), GC, and ECG (98%) were used in the present study as internal standards. Three mixture standard-A, standard-B, and standard-C where Std-A, Std-B, and Std-C exponential increased concentrations of each component in the mixture as follows [GA (5, 10, and 25 μg/mL), Cf (50, 100, and 150 μg/mL), C (50, 100, and 150 μg/mL), EC (50, 100, and 150 μg/mL), EGC (100, 200, and 300 μg/mL), EGCG (100, 200, and 400 μg/mL), ECG (50, 100, and 200 μg/mL)]. Relative distribution of these constituents were expressed in ppm of individual component (w/w) modified ISO 14502-2:2005 (E) method.[22] Linear calibration graphs were made between the peak area and concentrations of the standards (μg/mL), from which slope of the intercept were obtained. Using the slope and intercept, values for catechins

Table 2: Concentrations of metabolites present in green callus and dry callus with their respective peak areas.

Metabolites quantified	Green callus extract peak area	Dry callus extract peak area	Conc. of metabolite in green callus (ppm)	Conc. of metabolite in dry callus (ppm)	LOD in ppm	LOQ in ppm
Gallic acid	28.177	26.717	4.65	4.41	0.61	1.87
EGC	93.681	6.073	206.98	13.42	11.20	33.96
Catechin	278.38	5.614	195.89	3.95	4.33	13.15
Epicatechin	2568.865	40.526	1853.14	29.23	4.45	13.50
EGCG	8.757	60.372	2.96	20.41	13.00	39.41
ECG	62.161	30.129	17.35	8.41	4.17	12.64

and its isomers concentrations sample were obtained. For precision of the HPLC analysis for calculating LOD and LOQ, six determinations covering the identified range of three concentrations with three replicates were analyzed according to ICH guidelines[23] and included in Table 2. The individual component, w_c expressed as percentage by mass of samples either fresh or dry matter is given by the formula.

$$w_c = (A_{sample} - A_{intercept}) \times F_{std} \times v_{sample} \times d \times 100 \div S_{std} \times m_{sample},$$

where A_{sample} is the peak area of the individual component in the test sample; $A_{intercept}$ is the peak area at the point the standard calibration line intercepts the y-axis; S_{std} is the standard calibration line slope; F_{std} is the relative response factor, measured with respect to caffeine for the individual component; V_{sample} is the sample extraction volume, in milliliters; and d is the mass, in grams of the sample test portion.

RESULTS AND DISCUSSION

Callus induction and biomass accumulation

The auxin and cytokinin ratio was standardized according to Nagapavan *et al.*,[18] which induced callus from anthers of UPASI-9 tea clone. The percentage of callus was calculated by number of anthers responded after inoculated on to MS and 0.5 MS medium fortified with auxin 2 mg/L 2,4-D and cytokinin (Kn, BAP, and NAA at 0.5, 1.0, and 1.5 mg/L, respectively). The MS medium containing full strength of organic and inorganic salts and vitamins with respective treatments (T1–T10) did not show any response. The anthers incubated on full strength MS medium turned brown to black and lost its viability within 15 days, whereas the medium supplemented with half strength MS fortified with respective PGR treatments (T2–T7) produced callus induction. In this study, the auxin:cytokinin ratio was optimized as 2 mg/L 2,4-D with respective PGR's 1 mg/L Kn/BAP showed best callus induction rates were depicted in Figures 1 and 4C, D, and E. Among all the formulations studied, 0.5 MS supplemented with treatment T2 (0.5 MS + 2 mg/L 2,4-D + 1 mg/L Kn) was found to be best formulation which recorded 90% of callus induction. The increase in biomass was observed at 15 days intervals for respective treatments (T2–T7) were depicted in Figure 2. Moisture content of callus ranged from 47 to 60% [Table 3] under the influence of various PGRs, which supports the growth of the callus.

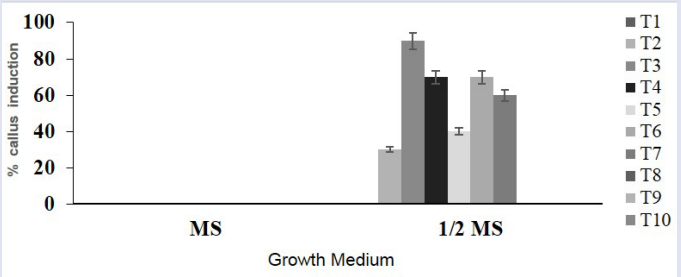

Figure 1: Percentage of callus induction on different media studied

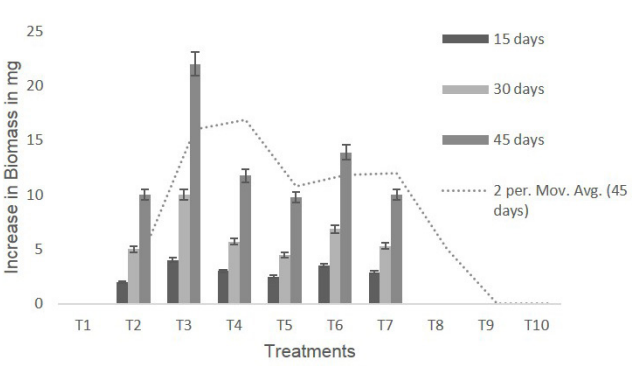

Figure 2: Increase in biomass of callus at 15 days interval periods on different treatments studied

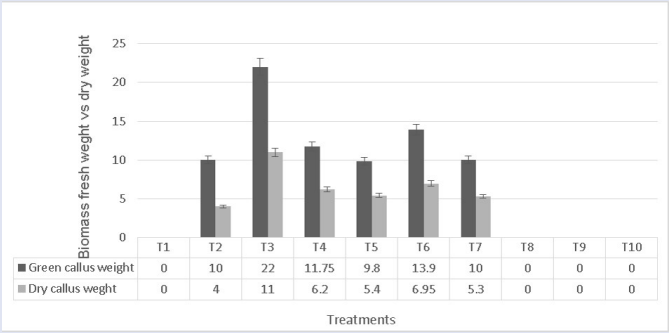

Figure 3: Biomass of callus at 45 days for fresh and dry callus periods on different treatments studied.

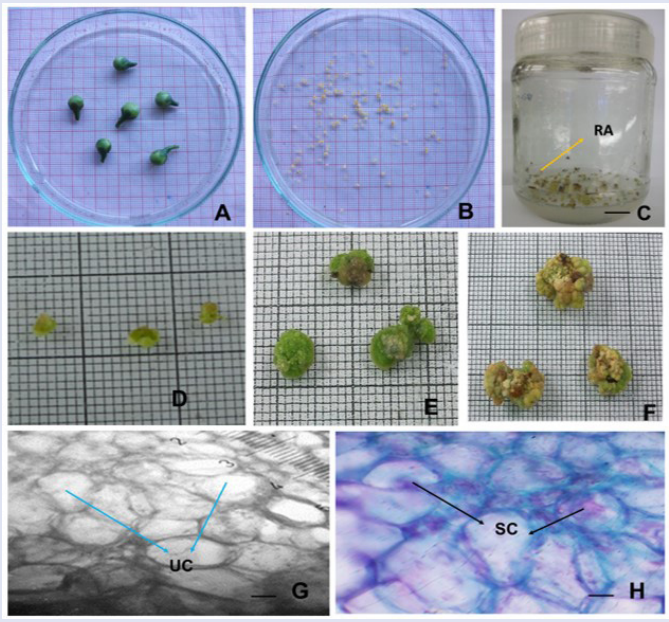

Figure 4: (A) Flower buds; (B) dissected anthers from the flower buds; (C) responding anthers (RA) depicted by arrow; (D, E, and F) increase in biomass at 15 days interval; (G) unstained cells (uc) and (H&I): stained cells (sc) by FC reagent for poly phenolic compound accumulation in cells. (Bars: C, 0.3 cm; G, 40 µm; H, 40 µm)

Increase in size of callus

Increase in size was observed by photographing (placing the callus on graph sheet), which aids in measuring the increase in callus size at 15 days intervals. The increase in callus size are depicted in Figure 4D, E, and F for 15, 30 and 45 days, respectively. Initially anther was 0.1 mm in size at the time of inoculation, due to callus induction increase in biomass and the size was noticed [Table 3]. Anther induced callus culture increased its height and width by 0.3 mm after 15 days of incubation

period. It obtained the height and width of 0.5–0.75 cm after 30 days. At the end of 45 days, height and width 1.2–1.35 cm was measured [Figure 4D, E, and F].

Histochemical localization of polyphenols

The polyphenol localization for anther derived callus was established by sectioning fresh green callus followed by treating with FC reagent. The results revealed the presence of polyphenols compounds inside the cellular vacuoles as shown in Figure 4G and H.

Qualitative analysis of polyphenols

The qualitative analysis of various polyphenols present in green and dry callus was compared with the standards of GA, Cf, C, EC, EGC, EGCG, GC, and ECG (procured from Sigma Aldrich, Bangalore). The chemical structures of the biochemical metabolites screened were given in Figure 5. Chemical finger print of dry callus and green callus extracts were matching to the spectrum of the mixed standard solution containing standard metabolites mentioned earlier. This shows qualitative inference of the accumulated polyphenols in the *in vitro* induced callus. The FTIR–ATR module generated spectrum of known standards and extracts generated from green and dry callus were shown in Figure 5.

Quantitative analysis of polyphenols

Considering the chromatographic method for analysis of polyphenols, the present study shows that quantitative estimation of GA, Cf, C, EC, EGC, EGCG, and ECG was carried out in the fresh green callus and dried callus using HPLC method.[21] Three standard solutions were prepared by mixing them in proportion mentioned as per ISO method.[22] The

Table 3: Growth biometrics and morphology of callus grown under different treatments

Treatments	% of callus induction	Degree of callus induction	Green callus weight (mg)	Dry callus weight (mg)	% of moisture content	Size of callus (cm) at 45 day	Morphology and nature of callus
T1	0	–	0	0	0	–	
T2	30	++	10.00	4.00	60.00	0.85	Green friable
T3	90	++++	22.00	11.00	50.00	1.35	Green Compact
T4	70	+++	11.75	6.20	47.23	1.25	Green friable
T5	40	++	9.80	5.40	44.89	0.80	Green friable
T6	70	+++	13.90	6.95	50.00	1.0	Green compact
T7	60	+++	10.00	5.30	47.00	0.84	Green friable
T8	0	–	0	0	0	–	–
T9	0	–	0	0	0	–	–
T10	0	–	0	0	0	–	–

"+" degree of callus induction and growth with respective treatment and "–" nonresponsive cultures.

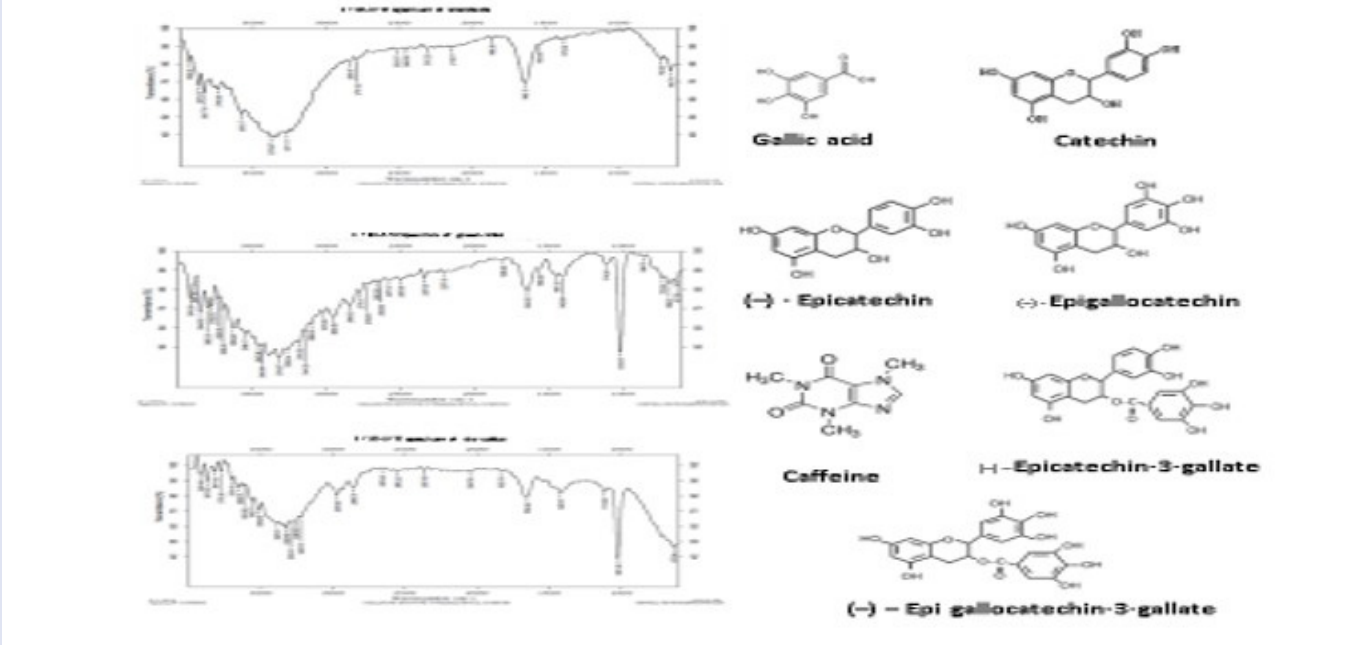

Figure 5: Qualitative comparison of FTIR spectrum of the metabolites from fresh green callus and dried callus with known standard solution containing GA, Cf, C, EC, EGC, EGCG, and ECG, and their chemical structures are given

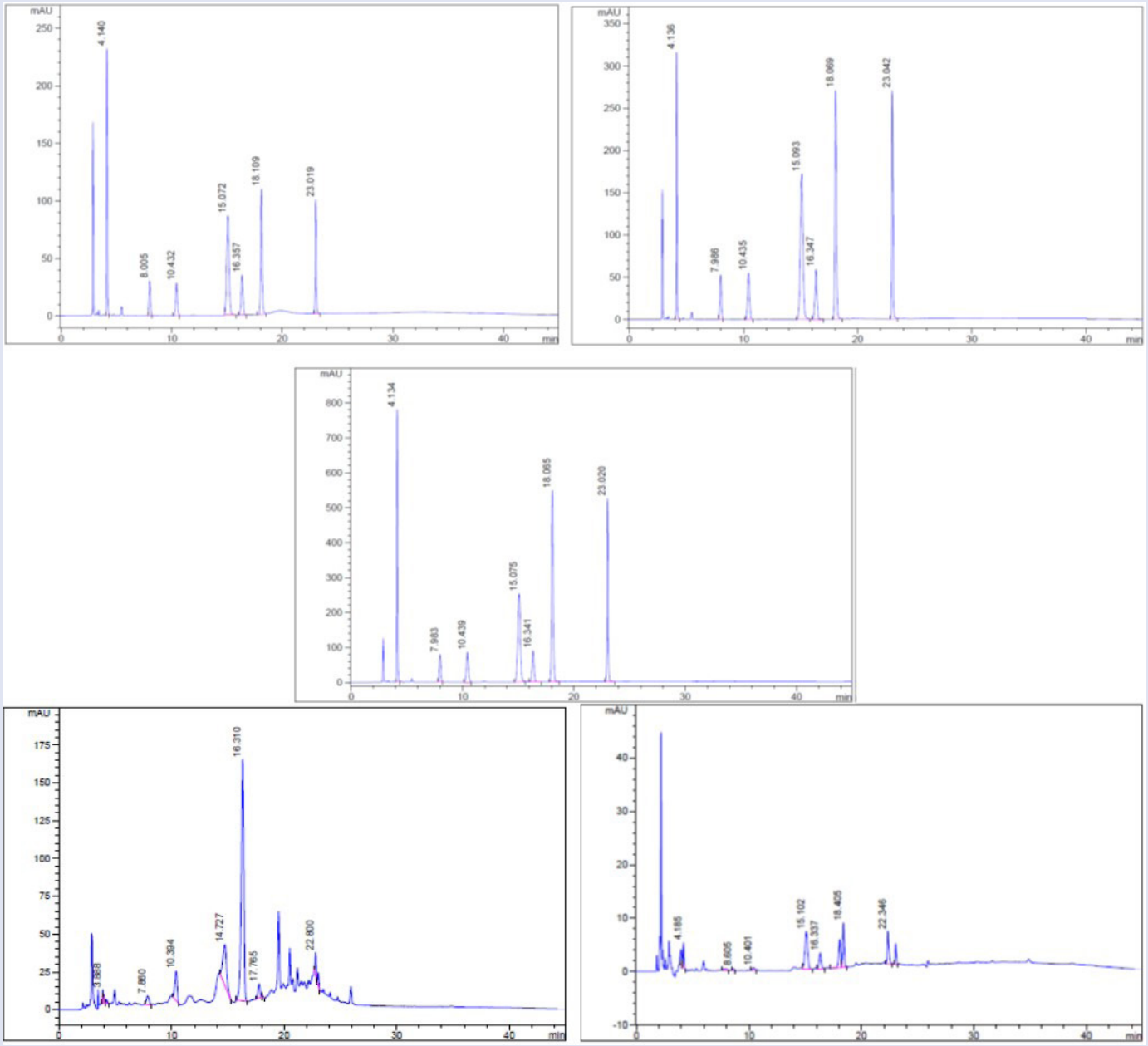

Figure 6: Quantitative comparison of secondary metabolites by reversed-phase HPLC method and its chromatographic separation of known mixed standard catechin solutions Std-A, Std-B, and Std-C along with fresh and dry callus of tea

identified chemical finger print of compounds derived chromatogram of different concentrated known mixed standard catechin solutions Std-A, Std-B, and Std-C were confirmed by the retention times and their UV absorption. The marker compounds assessed were GA, Cf, C, EC, EGC, EGCG, and ECG are depicted in Figure 6 with respective retention times and peak area defining concentrations of standards (Std-A, Std-B, and Std-C) by deriving the slope and intercept value, as shown in Figure 7. The results showed that the catechins and its isomers were rich in extracts of green friable callus than dried callus. Metabolites accumulated were expressed in ppm levels for green callus extract and dry callus extract which were elucidated in Table 2.

Fresh callus had high amounts of polyphenols when compared to dry callus because metabolites level may decrease or may be converted to isomers during drying process. The estimated polyphenols concentration are depicted in Table 2.

In green callus: EC > EGC > C > Cf > ECG > GA> EGCG.

In dry callus: EC > Cf > EGCG > EGC >ECG > GA > C.

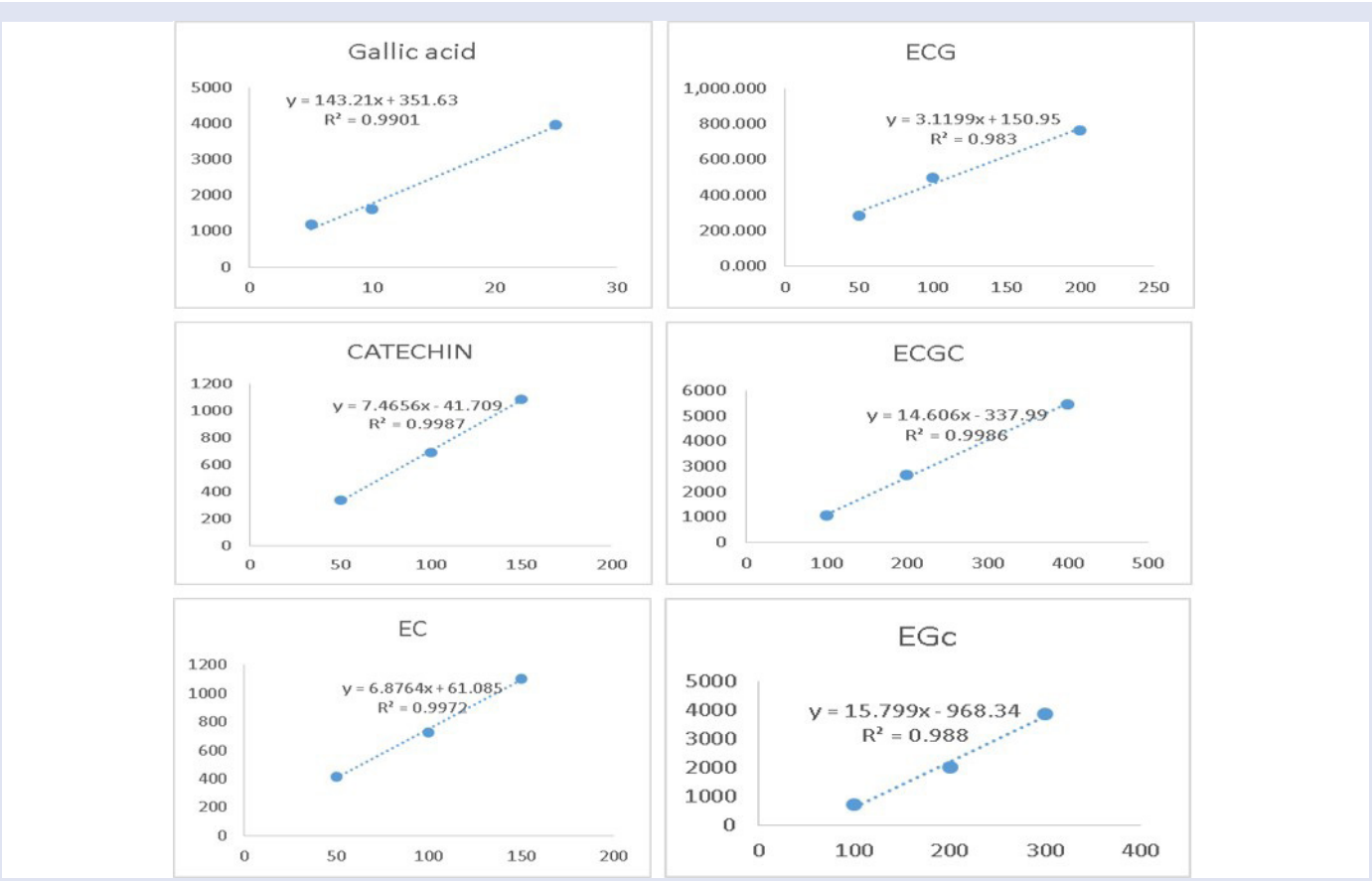

Figure 7: Linear caffeine calibration graphs for each standard concentrations (µg/mL) against the peak areas GA, Cf, C, EC, EGC, EGCG, and ECG to obtain the slope and intercept value

CONCLUSION

In present study, callus was successfully induced from anthers of UPASI-9 cultivar and the methods elucidated here is useful in defining the flavanols chemical metabolites by qualitative as well as quantitative assays, which are having medicinal importance. The staining technique developed by using FC reagent here is useful in localization of polyphenolic compounds in the tissues by instant microscopic observations. The study have scope in large-scale isolation of various medicinally important flavonol compounds such as EGCG, EC, ECG, and ECG, etc.

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Conflicts of interest

There are no conflicts of interest.

Authors' contributions

N.P.C. designed and executed the work; N.T.C. and K.D. carried out FTIR and HPLC analysis, respectively; R.R.N. supervised the FTIR work; and C.K.N. conceived and designed the experiments, drafted manuscript, and supervised the work. All authors read and approved the final manuscript.

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