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QUANTITATIVE ESTIMATION OF TERPENOID CONTENT IN SOME TEA CULTIVARS OF NORTH EAST INDIA AND THEIR *IN VITRO* CELL CULTURES USING AN OPTIMIZED SPECTROPHOTOMETRIC METHOD

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ABSTRACT

Terpenoids, which constitute one of the most diverse groups of bioactive compounds in plants, contribute to about 60% of the known natural compounds. Tea (*Camellia sinensis*), which is a highly preferred beverage throughout the world, is also rich in these flavor-imparting bioactive compounds. In this study, a simplified spectrophotometric method has been developed for the quantitative estimation of terpenoids in polyphenol-rich tea plant. Furthermore, terpenoid content in leaves and flowers of tea plant and their respective *in vitro* cultured cells have also been estimated. Terpenoid content was found to be up to five folds higher in flowers (123.9 mg/g DW) than in leaves (24.2 mg/g DW), and up to 1.7 folds higher in cultured flower cells (184.8 mg/g DW) as compared to *in vitro* cultured leaf cells (110.1 mg/g DW). Additionally, comparison of terpenoid content in tea leaves across the four different plucking seasons revealed a unique pattern wherein second (42.2 mg/g) and fourth flush (42.6 mg/g) tea were found to have the highest terpenoid content. First of its kind, this study provides an inexpensive method for screening of cultivars with better flavour and defense attributes. Furthermore, it also paves a way for future research in tea flowers which have high terpenoid content and the utilization of these therapeutic bioactive terpenoids in the development of diverse products for human health and wellness.

Keywords: Terpenoid, Quantitative estimation, Tea, *Camellia sinensis*, Cell culture, Spectrophotometry.

1. INTRODUCTION

Tea, the highly popular non-alcoholic beverage, is the second most consumed in the world, next to water. The attractiveness and appeal of tea comes from its rich flavor, gentle stimulatory effects, and health beneficial properties. Tea flavor can be divided into two forms: aroma, which consists of the volatile compounds, and taste, which consists of the non-volatile compounds. Both volatile and non-volatile compounds have various roles to play in the lives of the plant as well as humans. One of the most important compounds involved in flavour formation in tea are terpenoids.

Terpenoids consist of myriad of structurally diverse group of compounds amounting to about 40,000 individual compounds [1-2]. They are classified based on the number of isoprene units - hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), sesquarterpenes (C_{35}) and polyterpenes (>C_{40}). Their functions range from their role as phytohormones such as abscisic acid (C_{15}) and gibberellins (C_{20}), to key components of membrane structure such as sterols (C_{30}) and as photosynthetic pigments such as carotenoids (C_{40}).

Terpenes/terpenoids are elemental in plant-plant as well as plant-environment interactions [3-4]. They act in plant defense against pathogens, predators and competitors [5]. In flowering plants, volatile terpenoids (mono- and sesqui-terpenoids) mainly function in attracting pollinators. They also act as defense against

herbivores by either directly acting on them or by attracting their natural predators [6].

In humans, terpenoids play diverse roles ranging from anti-bacterial, anti-fungal, anti-viral [7-9], anti-inflammatory [10], cytotoxic or anti-tumour [11-12] to anti-cancer [8] properties. The objective of this study is to develop a method for quantitative estimation of the vital bioactive components, terpenoids, in the polyphenol-rich tea plant, which would act as a quality index for different cultivars.

2. MATERIAL AND METHODS

2.1. Reagents and chemicals

Linalool standard and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were purchased from Sigma (St. Louis, MO, USA), pectinase, Murashige and Skoog medium, kinetin and 6-benzylaminopurine (BAP) were purchased from Himedia (Mumbai, India). Sulphuric acid was purchased from Qualigens (Mumbai, India). Methanol, chloroform and petroleum ether were purchased from Merck (Bangalore, India). Purified water from Rio's and Synergy system (Millipore, Milford, MA, USA) was used to prepare media and dilutions of standard.

2.2. Plant material

Four cultivars were selected for the study- S.3A/1 and T.3E/3 (Assam-type) and HV 39 and CP1 (China-type). Flowers and shoots (two leaves and apical bud) of the selected cultivars were collected from New Botanical Area (NBA) of Tocklai Tea Research Institute (TTRI), Tea Research Association (TRA), Jorhat, Assam, India (26°43'14''N, 94°11'54''E). Shoots were collected during the different plucking seasons (flushes)-first flush (F1) during the months of February-March, second flush (F2) during the months of May-June, third/rain flush (F3) during the months of July-August and fourth/ autumn flush (F4) during the months of October-November. The experiment was conducted for two consecutive years.

2.3. *In vitro* culture development

Leaves and flowers of the four selected varieties S.3A/1, T.3E/3, HV 39 and CP 1 were sterilized by treating them with 0.3% bavistin for 1 hour followed by 0.1% mercuric chloride for 15-20 minutes with occasional washing. The sterilized samples were then sliced into smaller pieces and used for callus induction in MS [13] and modified MS media containing varying concentrations of 2, 4-D (1-2 mg/L), 6-BAP (0.5-1 mg/L) and/or kinetin (1-1.5 mg/L). Callus growth was found to be highest in modified MS medium containing 1 mg/L 2, 4-D and 1 mg/L BAP. The explants were maintained under dark conditions and repeatedly sub-cultured every 3 weeks for sustainable growth of friable callus. One gram (1 g) of friable callus obtained from each of the explants was added to 10mL of sterilized MS broth. The mixtures were treated with 0.5% pectinase (filter sterilized) to digest the middle lamella of the cells and allowed to stand under laminar hood for 12 hours, with occasional shaking. The cells were then separated by centrifugation at 5,000 rpm for 15 minutes and washed with MS broth thrice by centrifugation at 5,000 rpm for 5 minutes. The harvested cells were re-suspended in 100mL liquid modified MS medium containing 1 mg/L 2, 4-D and 1 mg/L BAP and put in an orbital shaker under dark conditions. Cefotaxime (100 mg/L) was added to each flask to prevent contamination.

2.4. Sample preparation

2.4.1. Leaves and flowers from field-grown plants

Shoots containing a bud, first and second leaves of all the selected varieties were collected and allowed to wither for 16-18 hours. The withered samples were then steamed for 2 minutes for enzyme deactivation and air dried. Samples were dried in an oven at 60°C for 5-8 hours and ground in a ball mill. The terpenoid content was estimated using a modified version of the protocol by Salkowski [14]. Two grams (2 g) of sample powder was added to screw-capped tubes containing 10 mL of 95% methanol and 200 mg polyvinylpolypyrrolidone (PVPP), sonicated for 15 minutes and incubated in dark for 48 hours at 25°C. One gram (1g) of flowers (except sepals) were crushed and added to screw-capped tubes containing 10 mL of 95% methanol and 100 mg PVPP. The mixtures were sonicated for 15 minutes and incubated in dark for 48 hours at 25°C. After incubation, the mixtures were filtered and clear filtrate was obtained. One milliliter (1 mL) of each extract was taken in glass tubes and 3 mL chloroform was added to each tube. The tubes were vortexed thoroughly and cooled using ice packs. Conc. sulphuric acid (400 µL) was added to each tube and the tubes were incubated in dark for 4 hours at 25°C. At the end of the incubation period, reddish brown color appeared at the bottom of the tubes. Supernatant in each reaction tube was carefully decanted and the colored part was made up to a final volume of 4 mL using 95% methanol. Absorbance of the samples was measured at 538 nm using 95% methanol as blank.

2.4.2. In vitro cell cultures

Cell cultures were filtered and the cells and media were separated. Cells were crushed in presence of 100 mg PVPP and added to screw-capped tubes containing 5 mL of 95% methanol. Extraction was carried out using the protocol mentioned earlier. Terpenoids in the culture medium were extracted using petroleum ether in a separatory funnel. The extract was evaporated to dryness and dissolved in 5 mL of 95% methanol. Five milliliters (5 mL) each of cell and media extracts were mixed to obtain a final volume of 10 mL. One milliliter (1 mL) of the methanol extract was used for determination of terpenoid content in each sample using the earlier mentioned protocol and absorbance was measured at 538 nm using 95% methanol as blank.

2.5. Preparation of standard curve

Standard curve was prepared by using varying concentrations (1.29 µM-12.9 µM) of linalool as a standard.

2.6. Optimization of time period for incubation

Optimization of the time period for incubation after addition of concentrated sulphuric acid was carried out for each sample (flowers, leaves, *in vitro* cultured cells) individually by incubating identical samples for 1, 2, 3, 4, 5, 6 and 12 hours in the dark at 25°C and absorbance was measured at 538 nm.

2.7. Determination of dry matter content

One gram (1 g) each of leaves, flowers and *in vitro* cultured cells were taken in pre-weighed moisture basins. The samples were dried at 100±2°C in a hot air oven for 5-6 hours. Dry weight of the samples was taken and dry matter content of each individual sample was calculated.

2.8. Statistical analysis

All experiments were performed in triplicates and the data obtained were expressed as mean ± standard deviation of the three independent replicates. Statistical analysis was performed using IBM SPSS ver. 24 software. All data were analyzed using one – way analysis of variance (ANOVA). The criterion for significance was set at $p \leq 0.05$. Pearson's correlation study was also carried out to test the significant correlations among *in situ* and *in vitro* cultures, different cultivars and the four plucking seasons (flushes) at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

3. RESULTS AND DISCUSSION

Qualitative analysis of terpenoids in various plants has been carried out for a long time [15-16]. In tea, qualitative analysis of terpenoids has also been performed [17-18]. In addition, quantitative estimation of volatiles (monoterpenes and sesquiterpenes) in tea has been carried out in numerous occasions using GC-MS [19-21]. However, no standard spectrophotometric method had so far been developed for the quantitative estimation of tea terpenoids. In this study, a method was optimized to quantify the terpenoid content in tea based on Salkowski's test principles. Here, concentrated sulphuric acid acts as a dehydrating agent removing water molecules and sulphonic acid derivatives of terpenoids in presence of chloroform resulting in a reddish brown colour. Concentrated sulphuric acid also reacts with phenolics and alkaloids present in tea, in addition to terpenoids. However, the resulting colour developed was in the bluish green range, and absorbance occurred at different wavelength than in terpenoids. This is the first reported study where tea terpenoids have been estimated quantitatively through spectro-photometry.

The standard curve was prepared using linalool as standard. Varying concentrations (1.29 µM-12.9 µM) of linalool were used and absorbance was taken at 538nm. The standard curve obtained had an R^2 value of 0.993 with an equation

$$y=0.11x-0.08$$

Where, x is the linalool concentration used and y is the absorbance obtained at 538nm.

Addition of conc. sulphuric acid to the methanol extract of the samples and its incubation under dark conditions is a crucial step for the concentration of terpenoids. After the incubation period, terpenoids get collected at the base of the tubes in the form of a reddish brown color. Lesser the incubation period lower will be the concentration of detectable terpenoids, and higher the incubation period terpenoids might get degraded to other compounds thus hampering its detection. Since the optimum time of incubation varied with plant species, optimization of the time period for incubation is thus crucial for optimal terpenoid detection. In the present study, an incubation period of 4 hours was found to produce the highest intensity of color in tea samples (Fig.1). Ghorai *et al* [22] reported an incubation period of 1.5 to 2 hours for optimum color development in other plant species, however, 4 hours of incubation period was found to be optimum in case of *Camellia sinensis*.

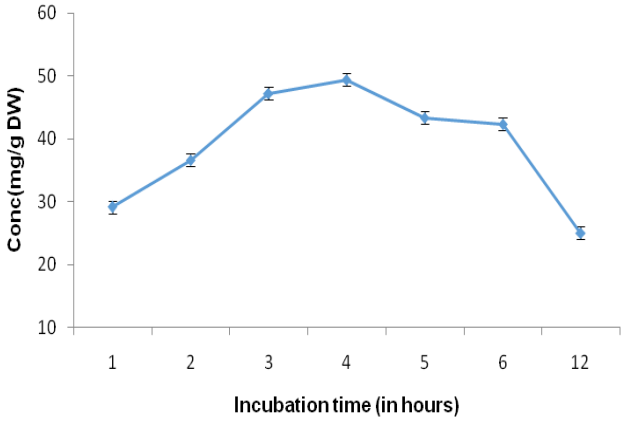
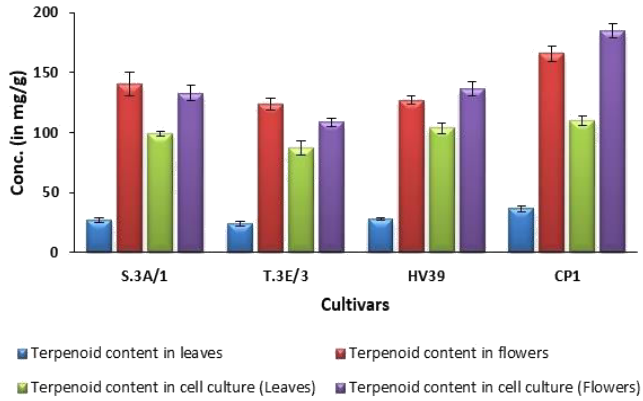


Fig. 1: Effect of incubation time on extraction of terpenoids from leaves of *Camellia sinensis*

3.1. Variation of terpenoid content between *in situ* plant parts and their respective *in vitro* cultures

Terpenoids are secondary metabolites found in different parts of plants which contribute to various flavors and fragrances, in addition to conferring numerous health benefits to its consumers [8, 23]. Variability in terpenoid content may be attributed to factors such as genetic makeup of the plant and different agro-climatic conditions prevailing during the period of growth. Likewise, geographical and clonal variations also affect terpenoid composition of tea. Teas of different varieties and from different countries of origin were observed to have different terpene indices [24].



The different colours indicate different samples of each cultivar

Fig. 2: Variation of terpenoid content in *in situ* and *in vitro* samples

The present study confirmed differential distribution of terpenoid content between leaves, flowers and their respective *in vitro* cultures in the four different cultivars studied. The variation of terpenoids in leaves, flowers and *in vitro* culture of leaves (LC) and flowers (FC) are presented in Fig. 2. In the present study, terpenoids were found to be higher (4.5-5.2 folds) in flowers as compared to leaves of all the cultivars under study. The terpenoid content ranged from 24.2 to 36.5 mg/g DW in leaves and 123.9 to 165.8 mg/g DW in flowers. Terpenoid content in LC ranged from 87.7 to 110.1 mg/g DW and in FC from 108.7 to 184.8 mg/g DW. This indicates 1.2-1.7 folds higher terpenoid content in FC as compared to LC. In general, terpenoid content was found to be higher in flowers (both *in situ* and *in vitro* culture). This is due to the fact that terpenoids have their role in plant defense against biotic and abiotic stresses. According to the Optimal Defense Hypothesis (ODH), defenses will be concentrated to the plant tissues which are of high fitness value to the plant. Reproductive tissues are of higher fitness value to the plant and consequently defensive compounds are present in higher concentrations in the reproductive tissues as compared to vegetative tissues [25].

Floral terpenes also play a vital role in pollination in plants. Floral phyllospheric microbiota also influenced the quality and quantity of floral terpenes emitted and in turn play a possible key role in pollination [26]. Terpenes emitted by flowers also play a role in inhibiting growth of disease-associated microbes in bees [27].

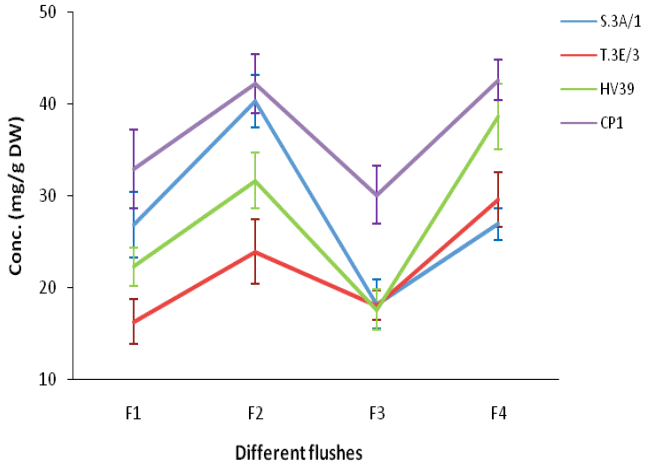
Additionally, China-type varieties were found to contain higher terpenoid content than the Assam-type varieties-both *in situ* and *in vitro*. This is in agreement with earlier studies in which China varieties have been observed to exhibit more terpenoids possessing characteristic floral notes as compared to the Assam varieties [28].

There was a statistically significant difference between tea leaves and flowers of cultivars studied as determined by one way ANOVA, the flower of cultivar CP1 containing the highest terpenoid content. The terpenoid content in flowers of all the cultivars exhibited significant variations at 5% level of significance ($p \leq 0.05$). Similar results were also observed between the *in vitro* samples.

3.2. Variation of terpenoid content in different flushes

The tea harvesting season of northeast India is divided into four different plucking seasons/flushes depending on the periodical dormancy of the shoot growth and climatic condition particularly soil temperature, humidity, rainfall and sunshine hours. These flushes are F1-First flush (March to April), F2-Second Flush (May to June), F3-Rain flush (July to August) and F4-Autumn

flush (October to November). The overall quality of tea varies along the seasons i.e. flushes and, therefore, tea manufactured from different flushes exhibit different flush characters. Tea of every flush has its unique characteristics, distinct aroma, flavor and taste. The aggregate of different terpenoids play a vital role in determining the unique characteristics of every flush and hence the quality of tea. The terpenoid content of four different cultivars and flushes are presented in Fig. 3.



F1-First flush, F2-Second flush, F3-Third/Rain flush, F4-Fourth/Autumn flush

Fig. 3: Variation of terpenoid content in tea shoots during different flushes

It was observed that there is significant variation of terpenoid content within the cultivars as well as between different flushes. Terpenoid content in shoots during different flushes-F1, F2, F3 and F4 varied from 16.2 to 32.9 mg/g, 23.9 to 42.2 mg/g, 17.6 to 30.1 mg/g and 26.9 to 42.6 mg/g dry weight (DW) respectively. The terpenoid content in F2 and F4 leaves was found to be higher as compared to F1 and F3 leaves. The increase or decrease of flavor during different seasons is the consequence of enzymatic reaction sequences controlling the dynamic metabolism system and altering the leaf physiology, which are modified during processing resulting in volatile flavor component (VFC) formation [29]. Hazarika and Mahanta [30] also studied the variation of VFC from April to October and observed that terpenoid compounds are highest in second flush (May to June) followed by first flush tea as compared to rain flush tea. Whereas terpenoid compounds show an upward trend during autumn flush (October to November) tea when atmospheric temperature and humidity fall considerably.

In the present study, F3 was found to contain the lowest terpenoid content as compared to the other three flushes, as suggested by Hazarika and Mahanta [30]. This is also in accordance with the findings of Gulati and Ravindranath [31] who studied seasonal variation of the volatile flavor compounds in Kangra teas and found that aroma compounds were present at their lowest concentration during the months of July-August. This is due to the dilution effect of precipitation during the season on tea quality [32-33].

Significant variation of terpenoids was also observed between the cultivars; CP1 containing the highest terpenoids. The terpenoid content in shoots in all the flushes exhibited significant variations at 5% level of significance ($p \leq 0.05$). Correlation study between the parameters showed that F3 exhibited significant positive correlation with F1 and F4 at 5% level of significance ($p \leq 0.05$) while, F1 showed significant positive relationship with F2 at 1% level of significance ($p \leq 0.01$).

4. CONCLUSION

Terpenoids play a significant role in human health, and as such they can be utilized for the development of diversified health-beneficial products. This study proposes a simple and effective method for the quantitative estimation of terpenoid content in different parts of tea plant, which in turn can be used to estimate the flavor and quality of various cultivars. The study revealed the presence of higher concentration of terpenoids in floral parts as compared to leaves, which can be used as an alternate source for the development of various products for human well-being. Similar biogenesis of terpenoids was also observed in *in vitro* cell cultures obtained from floral parts which proved the sustenance of similar physiology of flowers. Varietal and seasonal difference of terpenoids was also observed which may be a biochemical marker for determining the quality of tea as well as defensive efficiency of a cultivar. This study, being the first report on the quantitative estimation of terpenoid content in tea, serves as a platform for future references and investigation related to screening of economically important tea cultivars with better quality and defense attributes.

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Declaration of competing interest

The authors state that there is no conflict of interest and that all the authors have read and approved the final manuscript for publication. The authors declare that they do not have any competing financial interests or personal relationships that could have influenced the work reported in this paper.

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