

## HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY ANALYSIS AND FREE RADICAL SCAVENGING POTENTIAL OF SOUTH INDIAN ORTHODOX BLACK TEA

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### ABSTRACT

**Objective:** The objective of this study was to evaluate the free radical scavenging potential and high-performance thin-layer chromatography (HPTLC) fingerprinting of the ethanolic extract of south Indian orthodox black tea (OBT).

**Methods:** Phytochemical analysis was carried out using standard methods, and free radical scavenging activity of the extract was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), superoxide anion (SOD), and hydroxyl radical scavenging capacities. The ethanolic extract of OBT was loaded in the pre-coated HPTLC plates (silica gel 60 F 254) E-MERCK KGaA. HPTLC was carried out with toluene: ethyl acetate: diethylamine (7:2:1), chloroform: methanol: formic acid (8.5:1.0:0.5), and butanol: isopropyl alcohol (1:1) as mobile phase for alkaloids, flavonoids, and terpenoids, respectively.

**Results:** HPTLC results confirmed that the extract contained several potential active components such as flavonoids, alkaloids, and terpenoids as the slides revealed multicolored bands of varying intensities. Extract of OBT reliably showed the total phenolics 132.27 mg/g, flavonoids 72.52 mg/g, and alkaloids 66.01 mg/g of dry matter. The IC<sub>50</sub> value of OBT for DPPH was found to be 372.22 µg/ml, SOD 311.93 µg/ml, NO 362.17 µg/ml, hydroxyl radical 342.14 µg/ml, and reducing power 178.54 µg/ml.

**Conclusion:** The HPTLC fingerprinting profile developed for ethanolic extract will help in proper identification and quantification of marker compounds. The ethanolic extract of OBT was found to possess a wide range of phytochemicals with excellent antioxidant properties. This information may help to choose the best beverage to be consumed in the future.

**Keywords:** Orthodox black tea, High-performance thin-layer chromatography, Alkaloids, Flavonoids, Terpenoids.

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### INTRODUCTION

Plants are the richest source of phytochemicals. The world is overwhelmed with the great variety of natural vegetation, of which few are used in traditional medicine, in curing various ailments. Secondary metabolites possess toxicological pharmacological and ecological importance [1]. In recent years, phytotherapeutics play vital role in concern with health aid due to the presence of good radical scavengers which exhibit a great variety of therapeutic effects [2].

Tea is reported to contain nearly 4,000 active principles, of which one-third is contributed by polyphenols. The natural polyphenols present in tea are the flavonoids which are commonly branded as total catechins. The flavonoids (and their fraction, catechins) are the essential phenolic compounds in tea responsible for antioxidant activities such as neutralizing the free radicals which have been produced during the process of metabolism [3]. All the varieties of teas are produced from the same plant. The variety and quality of the tea are decided based on the level of fermentation, i.e., the processing techniques involved. Unfermented teas are rapidly dried, which quickly inactivate the enzyme polyphenol oxidase. This possesses a very high quantity of polyphenolic compounds, when compared to semi-fermented tea, in which polyphenols undergo partial oxidation followed by complete oxidation in black tea [4,5].

Throughout the world, about 78% of completely fermented tea (black tea) was consumed by the people in Western and Asian countries. 20% of unfermented tea (green tea) was consumed by the population in Asian countries. 2% of semi-fermented tea was consumed mainly by

China and Taiwan [6]. It was found that the infusion of tea holds more water-soluble polyphenols, particularly flavonols, flavonol gallate, and flavonol glycosides. It also contains catechins which consist of epigallocatechin, epicatechin, epigallocatechin gallate, and epicatechin gallate [7].

The present study was focused on screening the bioactive principles present in the ethanolic extract of orthodox black tea (OBT). High-performance thin-layer chromatography (HPTLC) fingerprint profile was also carried out to mark the signature of classes of secondary metabolites such as alkaloids, flavonoids, and terpenoids followed by the determination of free radical scavenging activity.

### MATERIALS AND METHODS

#### Collection and preparation of tea powder

The South Indian OBT (BOP grade) was purchased from the local market. About 250 g of OBT powder was first defatted with petroleum ether (40–60°C), and then, it is extracted in Soxhlet apparatus with ethanol. The extract was dried under reduced pressure at low temperature (40–50°C).

#### Determination of total phenolic content

Total phenolic content of the extract was determined by the Folin-Ciocalteu reagent method [8]. About 1 ml of the extract/standard solutions with different concentrations was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with water [1:10, v/v]) and 4 ml of sodium carbonate (7.5%). The mixtures were vortexed for a few seconds and allowed to stand for 30 min at 20°C for color development.

Absorbance of samples and standard was measured at 765 nm using a spectrophotometer against blank. The total phenolic content of the extract was calculated as the gallic acid equivalent (GAE).

#### Determination of total flavonoid content

The total flavonoid content in the ethanolic extract of OBT was determined colorimetrically using a slightly modified method [9]. 0.5 ml of diluted extract was mixed with 0.5 ml of methanol, 50 µl of 10% aluminum chloride, 50 µl of 1 M potassium acetate, and 1.4 ml of water. The mixtures were allowed to stand at room temperature for 30 min. After incubation, absorbance was read colorimetrically at 415 nm and the total flavonoid content in the extract was reported as quercetin equivalent (QE).

#### Determination of alkaloid

The plant extract about 1 mg was dissolved in dimethyl sulfoxide and added 1 ml of 2 N hydrochloric acid and filtered. The filtrate was transferred to a separating funnel to which 5 ml of bromocresol green, 5 ml of phosphate buffer solution, and 5 ml of chloroform were added. After shaking, the chloroform fraction alone was collected in a 10 ml standard flask and made up to 10 ml with chloroform. Caffeine was used as the standard. The absorbance of the test and standard solution was quantified against blank at 470 nm. The total alkaloid content was expressed as mg of caffeine equivalent/g of extract [10].

#### 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging capacity of the extract was determined using DPPH [11]. The mixture of DPPH (0.1 mM) in methanol was prepared, and 4 ml of this solution was added to 1 ml of sample solution in methanol at different concentrations (50–250 µg/ml). 30 min later, the absorbance was measured at 517 nm at room temperature using a Lambda UV-Vis spectrophotometer (PerkinElmer). Lower absorbance of the reaction mixture indicates a higher free radical scavenging activity. Ascorbic acid was used as a standard.

$$\text{DPPH scavenged (\%)} = \frac{[\text{Abs control} - \text{Abs test}]/\text{Abs control}}{\text{Abs control}} \times 100\%$$

Where Abs control is the absorbance of the control reaction, and Abs test is the absorbance of the extract/standard.

#### Hydroxyl radical scavenging activity

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid unit phospholipids of the cell membrane and injures the cell [12]. Samples of various concentrations were taken in the tube and added 1 ml of 1.5 mM ferrous sulfate, 0.7 ml of 6 mM hydrogen peroxide, and 0.3 ml of 20 mM sodium salicylate. The solutions after mixing were incubated at 37°C for 1 h. After incubation, the absorbance was read colorimetrically at 562 nm, against a blank sample. Ascorbic acid [13] was used as a positive control. Percentage inhibition was calculated by comparing the test and blank solutions. The effect of scavenging was compared with the standard antioxidant  $\alpha$ -tocopherol.

#### Superoxide radical scavenging activity

The scavenging activity toward the superoxide radical was measured in terms of the inhibition of generation of  $\text{O}_2^{\cdot-}$ . The assay was performed using alkaline DMSO method [14]. Potassium superoxide and DMSO were allowed to stand in contact for 24 h, and the solution was filtered immediately before use. Filtrate (200 µl) was added to 2.8 ml of an aqueous solution containing NBT (500 µM), EDTA (10 µM), and potassium phosphate buffer (10 mM). Test compounds (1 ml) at various concentrations (1–1000 µg/ml) were added, and the absorbance was recorded at 560 nm against a control in which pure DMSO was added instead of alkaline DMSO. The results were compared with the reference antioxidant quercetin.

#### Nitric oxide (NO) radical scavenging activity

NO produced from sodium nitroprusside in aqueous solution at physiological pH acts together with oxygen to produce nitrite ions,

which were measured using the Griess reaction reagent [15]. 2 ml of 10 mM sodium nitroprusside and 0.5 ml phosphate buffer are added to 2 ml of extract and reference compound in different concentrations (20–100 µg/ml). The resulting solutions are then incubated at 25°C for 60 min. To 5 ml of the incubated sample, 5 ml of Griess reagent was added and the absorbance of the chromophore formed is measured at 540 nm. A similar procedure is repeated with methanol as blank, which serves as control. The percentage inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test preparations. Ascorbic acid was used as a positive control.

#### Reducing power assay

Reducing power was carried out using the method reported by Yildirim et al. [16]. 1 ml of the extract and its subfractions (1–1000 µg/ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. To this mixture, 2.5 ml of trichloroacetic acid was added and centrifuged at 3000 rpm for 30 min. Finally, 2.5 ml of the supernatant solution was collected and mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride, and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard and phosphate buffer as the blank solution.

#### HPTLC fingerprint profile

The HPTLC analysis of the ethanolic extract of OBT was carried out to confirm the presence of a variety of phytochemicals present. The ethanolic extract of OBT was loaded in the pre-coated HPTLC plates (silica gel 60 F 254) E-MERCK KGaA and plate size 10×10 cm. The TLC plates loaded with sample were kept in twin trough chamber which was saturated with solvent vapors. HPTLC was carried out with toluene: ethyl acetate: diethylamine (7:2:1), chloroform: methanol:formic acid (8.5:1.0:0.5), and butanol: isopropyl alcohol (1:1) as a mobile phase for alkaloids, flavonoids, and terpenoids, respectively. The developed plates were dried by hot air oven at 60°C for 5 min to make the solvent evaporate from the plate. After drying, the plates were taken for photo documentation (CAMAG TLC Scanner) chamber. The images were taken at UV 254nm and UV 366 nm

#### Statistical analysis

Statistical analysis was carried out in triplicates (n=3), and standard error (SE) was calculated. All the data were analyzed using analysis of variance with the statistical software Prism 7.0 version. The analyses were made with 95% confidence. The significance of differences (p<0.05) between mean values obtained from the experiments was determined by Tukey's test.

## RESULTS

A thorough understanding of their chemical composition is essential for conducting a safety risk assessment. Chemical constituents of processed made tea were performed by many researches, but they are not always exactly similar due to their geographic location. The chemical constituents and their amounts in processed made tea can be different, due to growing conditions, such as climate, soil fertility, harvest season, age of the leaves, degree of fermentation, and the drying process.

#### Total phenolic content

The total phenolic content of the ethanolic extract was determined using the Folin-Ciocalteu reagent and expressed as GAE per gram of plant extract. The total phenolic content of the extract was calculated using the standard curve of gallic acid ( $y=15.885 \times +10.133$ ;  $R^2=0.0998$ ). Ethanolic extract of OBT was found to have 132.27±1.84 mg of GAE/g of dry matter.

#### Total flavonoid content

Aluminum chloride colorimetric methods were used to determine the total flavonoid content of the ethanolic extract of OBT. Total flavonoid content was calculated using the standard curve of quercetin ( $y=0.461 \times -0.141$ ;  $R^2=0.977$ ) and expressed as QE/g of the plant extract. Ethanolic extract of OBT was found to have 72.52±2.87 mg of QE/g of dry matter.

### Determination of total alkaloid content

The alkaloid contents were examined in plant extracts and expressed in terms of atropine equivalent as mg of AE/g of extract. The highest concentration of alkaloid was measured  $66.01 \pm 0.049$  mg of extract in ethyl acetate fraction and  $32.14 \pm 0.23$  mg of AE/g of dry matter.

### HPTLC analysis

#### Alkaloids

The results of phytochemical screening showed that the ethanolic extract of OBT possesses phytoconstituents such as alkaloids,

**Table 1: Peak table with Rf values, height, and area of alkaloids and unknown compounds**

Track	Peak	Rf value	Height	Area	Assigned substance
Standard	1	0.14	3.5	17438.6	Colchicine
Sample	1	0.07	25.7	23583.2	Alkaloid 1
Sample	2	0.11	20.5	713.9	Unknown*
Sample	3	0.14	0.6	208.5	Colchicine
Sample	4	0.38	5.6	8940.9	Alkaloid 2
Sample	5	0.46	111.6	2463.2	Unknown*
Sample	6	0.57	12.7	28578.2	Alkaloid 3
Sample	7	0.74	2.1	367.8	Unknown*
Sample	8	1.11	3.3	3465.4	Unknown*

Rf: Retention factor

**Table 2: Peak table with Rf values, height, and area of flavonoids and unknown compounds**

Track	Peak	Rf value	Height	Area	Assigned substance
Standard	1	0.45	10.9	31681.7	Quercetin
Sample	1	0.06	456.4	27562.7	Flavonoid 1
Sample	2	0.25	43.8	30624.9	Flavonoid 2
Sample	3	0.38	69.8	6244.3	Unknown*
Sample	4	0.43	49.5	2145.4	Flavonoid 3
Sample	5	0.47	42.9	1399.1	Flavonoid 4
Sample	6	0.55	43.1	2210.2	Unknown*
Sample	7	0.61	104.3	3131.4	Unknown*
Sample	8	0.73	33.6	10656.0	Flavonoid 5
Sample	9	0.80	124.0	3785.8	Unknown*
Sample	10	0.94	21.7	37333.1	Flavonoid 6
Sample	11	1.01	15.4	895.8	Unknown*
Sample	12	1.22	0.8	8441.5	Unknown*

Rf: Retention factor

flavonoids, and terpenoids which was determined with the help of HPTLC chromatogram. Various solvent systems were used to examine the secondary metabolites such as alkaloids, flavonoids, and terpenoids. Colchicine, quercetin, and lupeol were the standards used for the analysis.

Table 1 shows the presence of alkaloids in the ethanolic extract of OBT with retention factor (Rf) values such as 0.07, 0.11, and 0.14. The Rf value of standard colchicines was found to be 0.14. Chromatogram and the densitogram results (Fig. 2) of the extract of OBT in HPTLC analysis for alkaloids under daylight, 254 nm, and 366 nm are shown in Fig. 1. Comparing the Rf values of both standard and sample, it reveals that the sample possesses the colchicine. The peaks showed the presence of other alkaloids in the ethanolic extract of OBT.

#### Flavonoids

Six different types of flavonoid compounds were found in the ethanolic extract of OBT with the Rf values of 0.06, 0.25, 0.43, 0.47, 0.73, and 0.94 (Table 2). The standard quercetin has an Rf value of 0.45. The chromatogram and the densitogram (Figs. 3 and 4) of the extract illustrate that the sample possesses the compound quercetin and other flavonoids with higher concentration. The HPTLC chromatogram was examined under daylight, UV 254 nm, and UV 366 nm.

#### Terpenoids

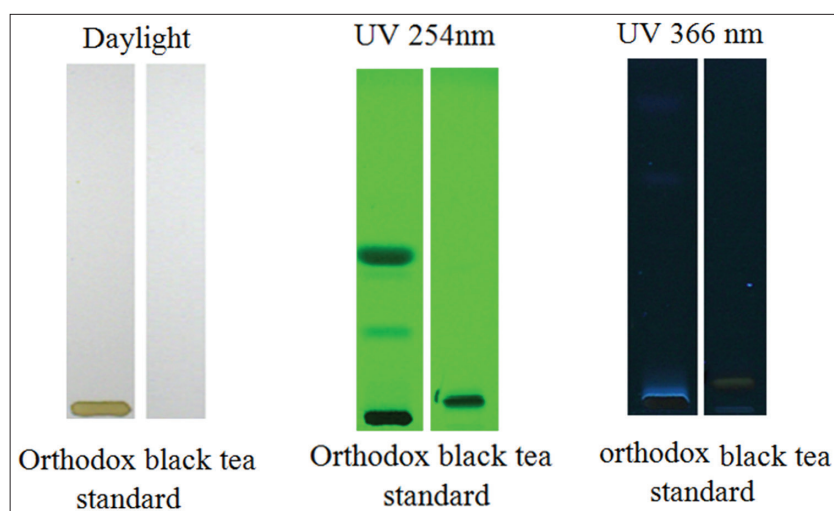
HPTLC slides developed at UV 254 nm and UV 366 nm in the Figs. (5 and 6) showed that four different terpenoid compounds were found to be present in the ethanolic extract of OBT, with the Rf values of 0.04, 0.36, 0.49, and 1.06. Lupeol was used as a standard with the Rf value 1.08. Apart from the Rf values, the peak areas were compared. The peak areas in the Table 3 are as follows: 15289.6, 23745, 18329, 15224, 19486, 44392, 17441, 15841.6, and 60872.4, and this showed that flavonoids other than lupeol were also present with very higher concentration.

#### DPPH scavenging assay

The antioxidant activity of OBT was evaluated using an alcoholic solution of the stable free radical DPPH. The free radical scavenging activity of the extract was found to be dose-dependent, and the results were compared with positive control ascorbic acid. The percentage of DPPH scavenging capacity of the extract indicated the sensitivity of the assay toward water-soluble antioxidants. The  $IC_{50}$  value of OBT and ascorbic acid for DPPH scavenging activity was found as 372.22 and 80.78  $\mu$ g/ml, respectively (Fig. 7).

#### Superoxide scavenging activity

Ethanolic extract of OBT displayed a dose-dependent effect in scavenging superoxide radicals, and the results were compared with



**Fig. 1: Chromatograms of extract in high-performance thin-layer chromatography for alkaloid analysis: Under daylight, UV 254 nm, and UV 366 nm**

known antioxidant quercetin. The  $IC_{50}$  value for superoxide scavenging activity for OBT was found as 311.93  $\mu\text{g/ml}$  (Fig. 8). At 500  $\mu\text{g/ml}$ , the percentage of inhibition was found as 74.15%.

#### NO radical scavenging activity

The OBT showed a dose-dependent inhibition of NO with an  $IC_{50}$  of 52.0  $\mu\text{g/ml}$  (Fig. 9). Quercetin was used as a reference compound and 362.17  $\mu\text{g/ml}$  quercetin was needed for 50% inhibition. At 500  $\mu\text{g/ml}$ , the percentage of inhibition was found as 65.42% for the extract.

**Table 3: Peak table with Rf values, height, and area of terpenoids and unknown compounds**

Track	Peak	Rf value	Height	Area	Assigned substance
Standard	1	1.08	6.6	3368	Lupeol
Sample	1	0.02	468.5	7434.5	Unknown*
Sample	2	0.04	421.7	15289.6	Terpenoid 1
Sample	3	0.16	376.6	23745.5	Unknown*
Sample	4	0.24	375.0	18329.0	Unknown*
Sample	5	0.30	365.3	15224.0	Unknown*
Sample	6	0.36	616.5	19486.0	Terpenoid 2
Sample	7	0.49	200.6	44392.6	Terpenoid 3
Sample	8	0.62	201.4	17441.2	Unknown*
Sample	9	0.76	225.6	15841.6	Unknown*
Sample	10	1.06	98.8	60872.4	Terpenoid 4

Rf: Retention factor

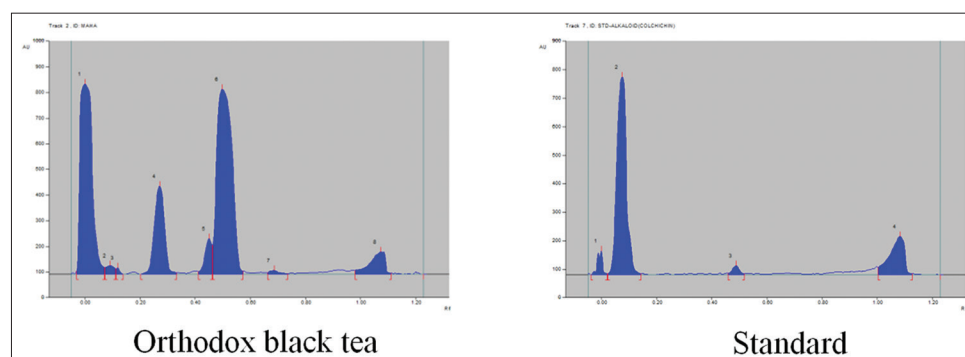
The hydroxyl radical scavenging activity of the extract correlated well with increasing concentration. The effect was compared with the standard antioxidant  $\alpha$ -tocopherol and the  $IC_{50}$  values of the extract and  $\alpha$ -tocopherol were 342.14 and 236.41  $\mu\text{g/ml}$ , respectively (Fig. 10). However, as anticipated, the activity of  $\alpha$ -tocopherol was relatively more pronounced than the extract.

#### Reducing power

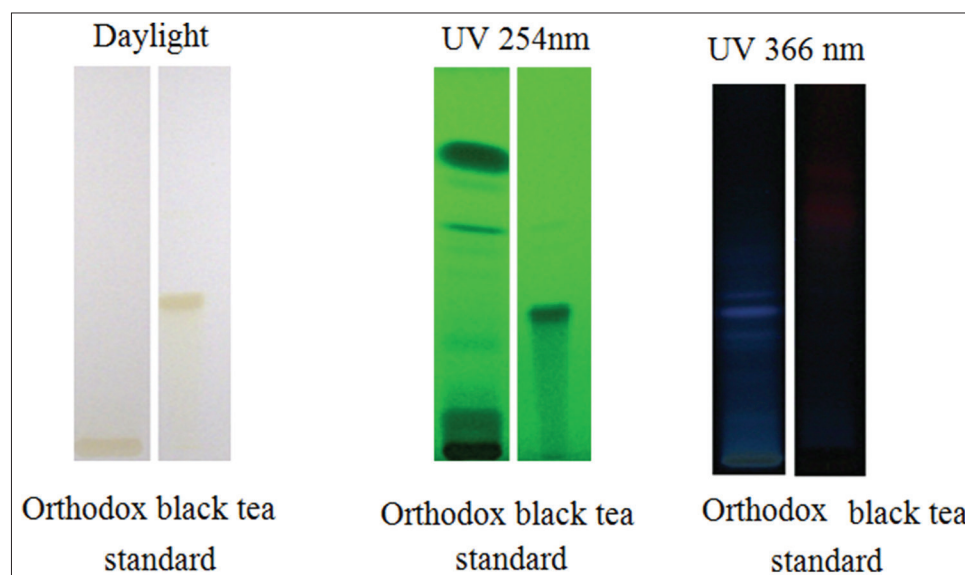
The effect of OBT on reducing power was studied according to the reaction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The results were compared with the reference compound ascorbic acid (Fig. 11). The  $IC_{50}$  values of the extract and ascorbic acid were 178.54 and 120.38  $\mu\text{g/ml}$ , respectively.

#### DISCUSSION

The components of tea possess various bioactive principles which act as antioxidants antimutagenic and anticarcinogenic agents, and hence, it has magnetized the interest of people due to its enormous health benefits. These bioactive principles protect us from various environmental factors which cause diseases [17]. Those diseases become a serious public health issues. A huge amount of synthetic drugs exist in the market against various diseases and involve in adverse drug effect. An alternate is the consumption of natural compounds elucidated from plants and food supplements (functional foods). Such natural compounds have isolated and determined for its therapeutic property [18].



**Fig. 2: High-performance thin-layer chromatography densitogram of alkaloids in standard colchicine and orthodox black tea**



**Fig. 3: Chromatograms of extract in high-performance thin-layer chromatography for flavonoid analysis: Under daylight, UV 254 nm, and UV 366 nm**



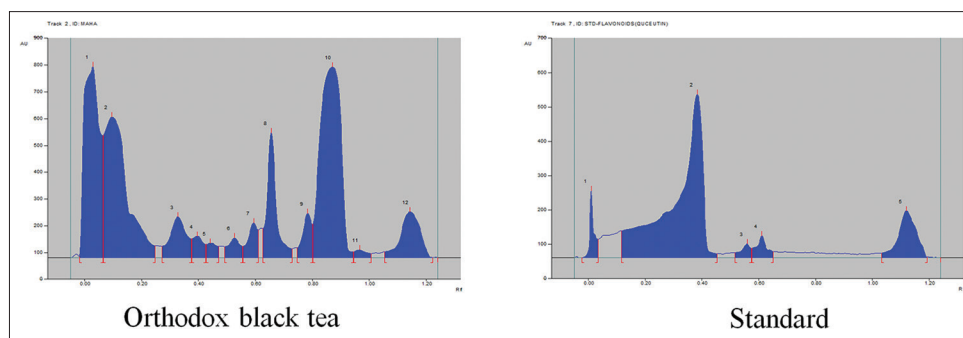


Fig. 4: High-performance thin-layer chromatography densitogram of flavonoids in standard quercetin and orthodox black tea

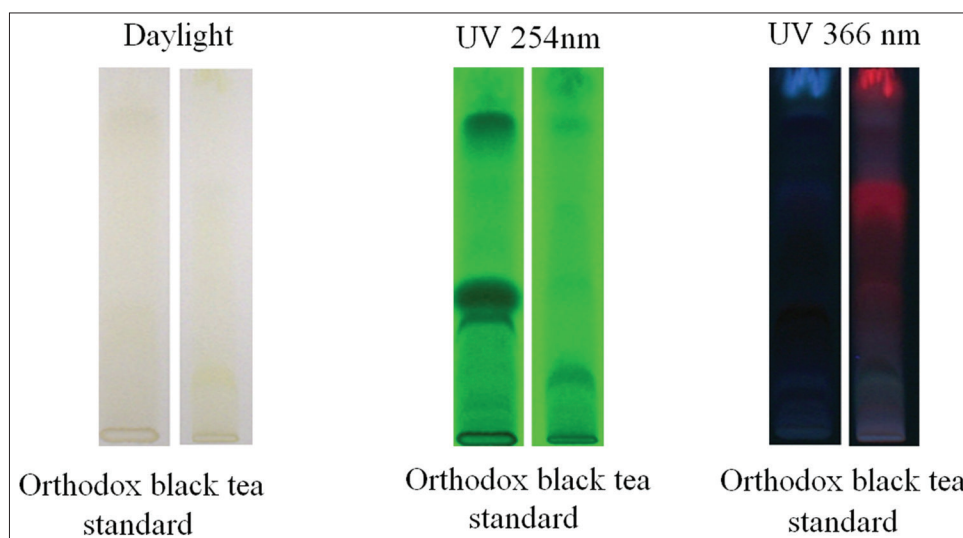


Fig. 5: Chromatograms of extract in high-performance thin-layer chromatography for terpenoids analysis: Under daylight, UV 254 nm, and UV 366 nm

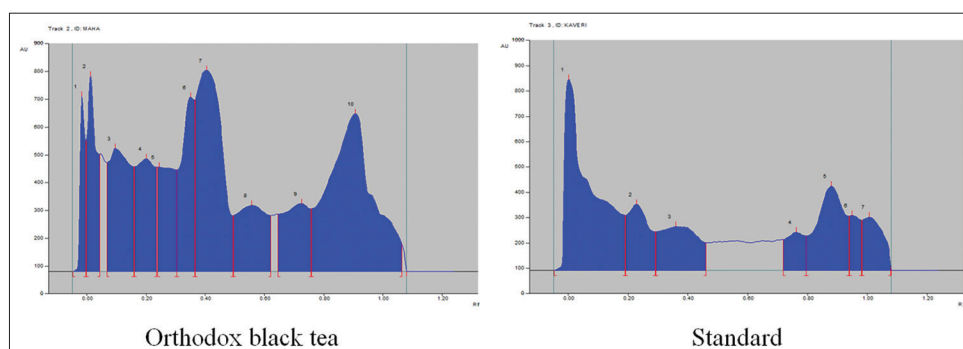


Fig. 6: High-performance thin-layer chromatography densitogram of terpenoids in standard lupeol and orthodox black tea

Various parts of the plant were used traditionally for the treatment of several diseases. The development in the technology increased the interest among the researchers to know the active principles (phytochemicals) present in the medicinal plants. The present investigation revealed the antioxidant property of an unexplored variety of tea using *in vitro* assessment [19]. The total phenolic and flavonoid content was carried out in the OBT and compared with the standard antioxidants. This present study revealed the antioxidant property of the extract.

The DPPH radical scavenging activity of OBT clearly showed that it possesses the property in a dose-dependent fashion with ascorbic acid as standard. It is illustrious that phenolic compounds are potential antioxidants, and hence, there should be a close connection between the content of phenolic compounds and its antioxidant activity [20].

Huge biological damage was caused by the major active oxygen species, hydroxyl radical. Free radicals initiate the polymerization reaction and other oxidizing reaction. The free radical scavengers in the plant extract inhibit the oxidation process initiated by radicals [21]. Free radical was found to be removed by the addition of OBT, which was compared against the standard  $\alpha$ -tocopherol. Harmful reactive oxygen species were formed from superoxide radical such as hydrogen peroxide and hydroxyl radical. These radicals, in turn, damage the biomolecules which lead to dreadful diseases. The OBT had greater superoxide radical scavenging activity. The substance which involved in scavenging the radical might be the polyphenolic compounds present in the extract [22]. These antioxidants contribute significantly toward the biological activities such as hypoglycemic, antidiabetic, antimicrobial,

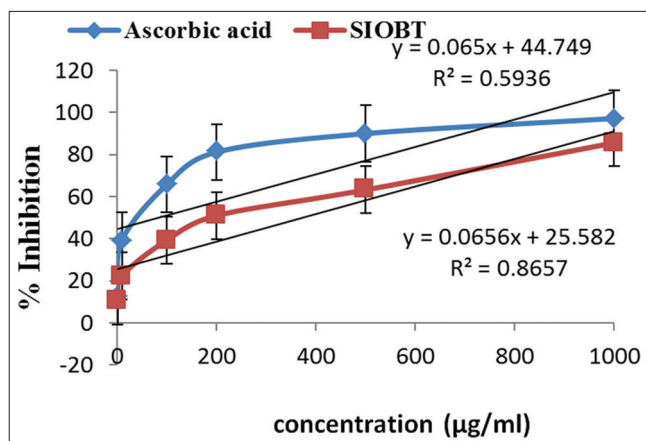


Fig. 7: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of orthodox black tea

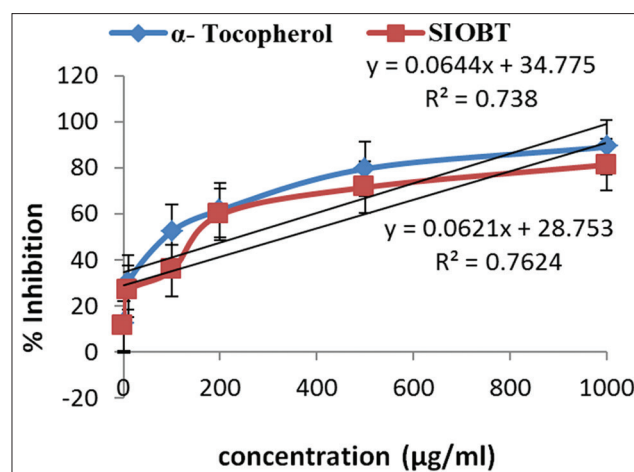


Fig. 10: Hydroxy radical scavenging activity of orthodox black tea

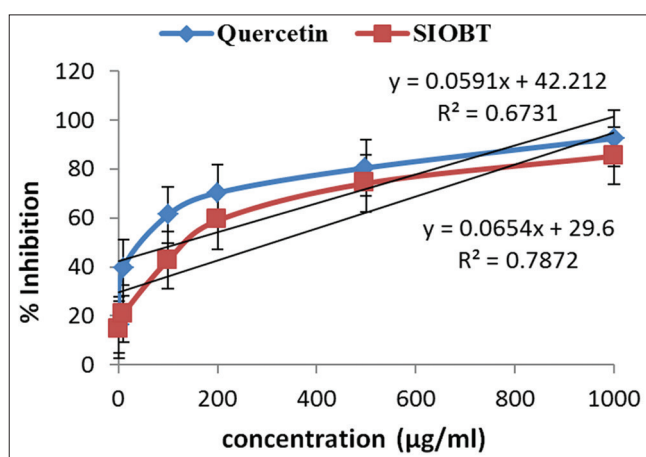


Fig. 8: Superoxide radical scavenging activity of orthodox black tea

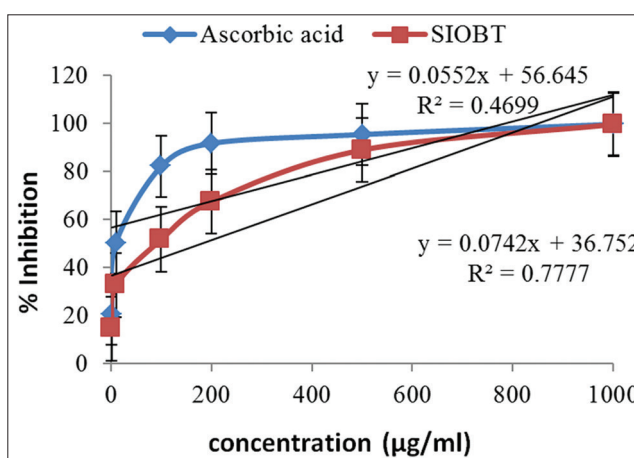


Fig. 11: Reducing power activity of orthodox black tea

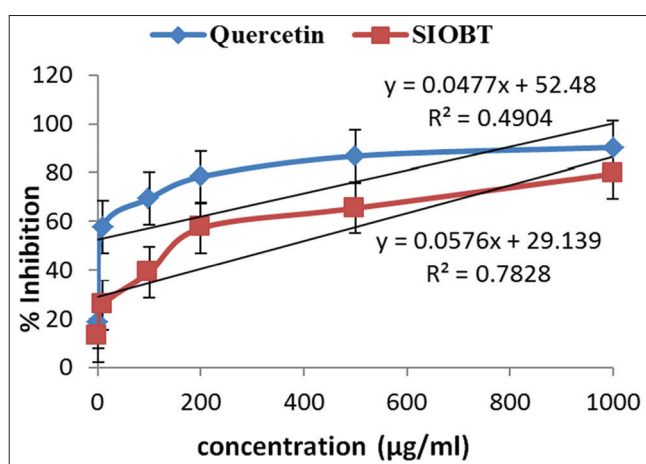


Fig. 9: Nitric oxide radical scavenging activity of orthodox black tea Hydroxyl radical scavenging activity

antioxidant, anti-inflammatory, anticarcinogenic, antimalarial, and anti-leprosy activities [23].

Mammalian cells involve in the regulation of enormous physiological process which ends up with the production of NO radical; furthermore, the amount of NO radical increases even during ailments. Thus, the production of radicals can be prevented with the aid of free radical

scavengers [24]. In the present study, we deduced the NO scavenging activity of OBTE and also compared with standard quercetin. The compound with the characteristics of reducing the radical is the good sign of antioxidants. The reducers lend electron to the radicals and thereby cease the radical chain reaction forming stable compounds. The study revealed that the extract possesses similar reducing power compared with ascorbic acid as standard [25].

#### CONCLUSION

The existence of the phytochemical constituents was identified using HPTLC analysis in ethanolic extract of OBTE. The presence of alkaloids, flavonoids, and terpenoids was identified for its presence with the peaks and the retardation factor (R<sub>f</sub> value) obtained for the extract and compared with the peaks obtained for the standard. The HPTLC analysis of the OBTE suggested the presence of potential therapeutic substances in preventing and curing the disease.

#### AUTHOR'S CONTRIBUTION

This work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. R. Mahalakshmi carried out the experiments and had written the manuscript. Dr. A. Nishadh helped in all the laboratory work. Dr. K. Kalaivani designed the work, proofread, and validated the manuscript.

#### CONFLICTS OF INTEREST

The authors declare no conflicts of interest of this study.

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