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ANTIOXIDANT ACTIVITY ASSESSMENT OF *CAMELLIA SINENSIS* LEAVES EXTRACT IN GUINEA PIG PLASMA

Neelam Singh [*1, 2], Giriraj T. Kulkarni [3] and Yatendra Kumar [1]

Department of Pharmaceutics [1], ITS College of Pharmacy, NH - 58, Ghaziabad - 201206, Uttar Pradesh, India.
A. P. J. Abdul Kalam Technical University [2], Lucknow - 226031, Uttar Pradesh, India.
Department of Pharmaceutics [3], Amity Institute of Pharmacy, Amity University, Sector - 125, Noida - 201303, Uttar Pradesh, India.

Keywords:

Oxidative stress, ORAC assay, Free radicals, Antioxidant capacity, Dose dependent, MDA and GSH level

Correspondence to Author:
Neelam Singh

Assistant Professor,
Department of Pharmaceutics,
ITS College of Pharmacy, NH - 58,
Ghaziabad - 201206, Uttar Pradesh,
India.

E-mail: singhneelam16@gmail.com

ABSTRACT: This study planned to screen preliminary phytoconstituents, total phenolic- flavonoids content and *in-vitro*, *in-vivo* antioxidant activities of aqueous crude extract of *Camellia sinensis* leaves. We assessed phytochemical constituents in extract using previously reported methods for tannins, phenols, flavonoids, glycosides, alkaloids and saponins. Quantitative estimation of flavonoids and phenolic compounds was carried out by aluminium chloride and Folin Ciocalteu method. ABTS, DPPH and FRAP methods are used for *in-vitro* assessment of antioxidant potential; meanwhile extract was tested for antioxidant capacity in Guinea pig plasma using ORAC, MDA and GSH assay in dose dependent manner. Total phenolic and flavonoid content of the extract was found to be 251.77 ± 6.59 µg/mg and 246.56 ± 2.58 µg/mg respectively. Extract shown 93.76 ± 12% antioxidant activity with ABTS and 95.13 ± 17.89% when tested with DPPH hydrogen reacting radicals. While, it was calculated 92.82 ± 8.72% for FRAP assay. The extract shows dose dependent decrease in MDA level ($P<0.05$, ANOVA) in plasma. Although, the extract produced dose depended increase in GSH value 20.97 ± 2.541 µg/mg protein and ORAC antioxidant potential 23.771 ± 1.926 mM Trolox Eq/L, when compared to control group. Our findings suggest that, extract contain phenolic and flavonoids content and showed *in-vitro* free radical scavenging activity. *In-vivo* results of selected biochemical parameters of oxidative stress in Guinea pig plasma, will serve as a benchmark for further pharmacological screening of *Camellia sinensis* constituents, which makes this as a therapeutic adjuvant agent against free radicals mediated diseases.

INTRODUCTION: Free radicals are the by-products of biochemical processes occurring in living cells and these radicals can cause chronic and degenerative diseases in humans [1].

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Plants are the potential source of natural antioxidants which lower the risk of free radicals mediated deadly diseases [2]. It is well known that tea is second most consumed drink worldwide after water. Several studies have been done on natural antioxidants present in tea and it was reported that, beneficial effect of green tea is higher than black tea due to its high antioxidant capacity. Catachin compounds such as epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallo-catechin (EGC) and epicatechin (EC) are powerful antioxidants present in green tea leaves extract.

Green tea is obtained from *Camellia sinensis*, an evergreen plant of the family Theaceae [3]. Although, level of various chemical constituents of tea, depends on growing environment and region of production [4]. A number of studies have shown that, antioxidant compounds present in green tea have anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities [5]. The present study is concerned to assess qualitative phytochemical analysis and *in-vitro, in-vivo* antioxidant potential of Indian Green tea leaves extract.

MATERIAL AND METHODS:

Plant Material and Chemicals: *Camellia sinensis* (family Theaceae) leaves were collected in the month of March from Kangra district of Himachal Pradesh, India and authenticated from Department of Botany (voucher no. MCM/ Bot-3/2012), Chaudhary Charan Singh University, Meerut, Uttar Pradesh. All chemicals used were of analytical grade and purchased from Fluka Research Chemicals, India.

Preparation of Extract: Green tea leaves were washed under running tap water to remove adherent impurities, and then leaves were boiled with distilled water at 80 ºC for 20 min with stirring. After that, obtained solution was cooled and vacuum filtered. The filtrate was concentrated at 40 ºC, followed by freeze drying to obtain dry extract which was then scrapped off and stored in air tight container [6, 7, 8].

Phytochemical Screening of Crude Extract: The dried extract was dissolved in water to make stock solution and used for phytochemical screening for tannins, flavonoids, terpenoids, cardiac glycosides, alkaloids, steroids, and saponins using reported quailtative methods with some modifications [9-13].

Estimation of Total Flavonoids Content: The total flavonoids content was determined using by aluminium chloride ($AlCl_3$) method [14]. Quercetin standard solution (1 mg/mL) in methanol was prepared. 1 ml standard Quercetin solution was taken in 10 ml volumetric flask and mixed with 0.3 ml 10% $NaNO_2$. After standing for 5 min at room temperature, 0.3 ml of 10% $AlCl_3$ and 2 ml of 1 M NaOH were added and volume made up to 10 ml with water. The mixture was allowed to rest for 15 min at room temperature for color development. The absorbance of the resulting orange-yellowish solution was measured at 430 nm in UV-Vis spectrophotometer (Shimadzu UV1800, Japan). Distilled water was used as blank [14]. All determinations were performed in triplicate, and Quercetin standard curve was plotted. Same procedure was adopted for tea extract and absorbance was taken [15]. The total flavonoids content was expressed as mg Quercetin equivalent per g dry weight of the extract [16].

Estimation of Total Phenolic Content: Total phenolic content was determined by Folin Ciocalteu reagent [16]. Gallic acid was used as a reference standard. Volume of 1 ml extract (100 µg/mL) was mixed with 2 ml of Folin Ciocalteu reagent (diluted 1:10 with distilled water) and were neutralized with 4 ml of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of resulting blue color was measured at 756 nm using double beam spectrophotometer (Shimadzu UV1800, Japan). The content of total Phenolic content expressed as mg Gallic acid equivalent of per gram dry weight of extract [16, 17].

***In-vitro* Antioxidant Assay:**

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay: Antioxidant activity was determined by DPPH (1, 1- diphenyl-2-picryl Hydrazyl) radical scavenging method [18, 19]. To prepare standard and test samples, required quantity of standard Gallic acid and test (Tea extract) were dissolved in distilled water separately to obtain the concentrations of 10, 20, 30, 40 and 50 µg/mL. A freshly prepared 0.1 mM solution of DPPH in ethanol was used. Solution was protected from light by covering the test tubes with aluminium foil. 0.5 ml DPPH solution was diluted upto 3 ml with blank, and absorbance was taken immediately at 517 nm (UV spectro-photometer) for control reading.

A mixture of absolute alcohol and distilled water (1:1) was used as blank [20]. 1 ml sample was taken from 10 µg/mL. concentration standard solution. 0.5 ml DPPH solution was added in this solution, resulting mixture was diluted up to 3 ml with blank. After incubation for 30 min at 25 ºC, absorbance was taken at 517 nm. Same procedure was repeated for rest of standard and tea extract samples [21].

The DPPH free radical scavenging activity was calculated using the following formula:

Percent scavenging = Absorbance of control – Absorbance of test / Absorbance of control × 100

2, 2' -azino -bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Scavenging Activity: ABTS has a characteristic absorbance at 734 nm, which disappears after its reduction by an antiradical compound and the reduction, can be monitored by measuring the decrease in absorbance at 534 nm during the reaction. Antioxidant activity of extract was estimated according to the previously reported methods with some modifications [22, 23]. ABTS was dissolved in distilled water to obtain a 7 mM solution. The ABTS radical cation solution was prepared by allowing the 7 mM ABTS solution to react with 2.45 mM potassium persulfate solution (1:1). ABTS radical cation solution was stored for 12-16 h in the dark at room temperature before use. ABTS cation solution was diluted with methanol to obtain an absorbance of 0.7 ± 0.01 at 734 nm. 10 µl of extract was added to 4 ml of diluted ABTS solution and incubated for 30 min at room temperature. Absorbance was read at 734 nm by using UV spectrophotometer (Shimadzu UV1800, Japan) Gallic acid was used as standard. ABTS percent scavenging activity was calculated using same formula as described for DPPH.

Ferric Reducing Antioxidant Power (FRAP) Assay: The method is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+} - tripyridyl triazine (blue colored complex) formed by the action of electron donating antioxidants at low pH, measured spectrophotometrically at 593 nm [24]. The FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer with pH 3.6, 2.5 ml of 10 mM 2, 4, acetate 6- tri- (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3.6H_2$ at 37 ºC. Freshly prepared working FRAP reagent was mixed with 10 µl diluted extract and incubated for 30 min at 37 ºC. An intense blue color was obtained which was analyzed at 593 nm against a reagent blank by using UV spectrophotometer [25]. FRAP value of samples were calculated from Gallic acid standard curve and percent scavenging activity was calculated.

Determination of Antioxidant Capacity in Guinea Pig Plasma: The experiment was approved by the Institutional Animal Ethics Committee with protocol number (1044/PO/Re/S/07/CPCSEA, ITS/ 01/IAEC/2017) I.T.S College of Pharmacy, Ghaziabad.

Twelve healthy male Dunkin-Hartley albino Guinea pigs having average weight 552 g were obtained from I.T.S College of Pharmacy and kept in the Animal House of the Department. Guinea pigs were housed in standard cages at room temperature and light-dark cycles. The animals left to acclimatize for two weeks before the start of the experiment with feed and water provided *ad libitum*. Animals were handled as per Guide for the care and use of laboratory animals [26]. Guinea pigs were randomly divided into three groups of four animals each. Group 1 kept as the control and fed with 10 ml of distilled water. Group 2 received extract as 200 mg/kg body weight of guinea pig and group 3 received 400 mg/kg of body weight. The animals were dosed once daily for 14 days and were observed throughout the period of study. The blood was withdrawn from retro orbital plexus on the fifteenth day and used for estimation of selected biochemical parameters of oxidative stress [27]. Blood was collected in EDTA coated sample tube and centrifuged at 8000 rpm for 20 min at -4 ºC, resultant plasma was separated and transferred in eppendrof tubes then immediately stored at -20 ºC ± 2 ºC until analysis [27].

ORAC (Oxygen Radical Absorbance Capacity) Assay: Total antioxidant capacity (TAC) has been used to assess antioxidant status of biological samples; ORAC is one of the methods to measure TAC. This is relatively simple, sensitive and reliable method for quantification of ORAC of antioxidants present in serum or plasma. The assay evaluates the loss of fluorescein fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH (2, 2'azobis-2 -methyl-propanimidamide, dihydrochloride) [27, 28].

Trolox a water soluble vitamin E analog, used as a positive control inhibiting fluorescein decay in a dose dependent manner. Plasma samples stored at -20 ºC were first thawed at room temperature and shaken in vortex to perform ORAC assay by using Zen-Bio ORAC kit (Life technologies, Delhi, India) and procedure was adopted as per instruction manual.

Readings were taken on Synergy HT fluorescence multi- plate reader (Bio-Tek Instruments) at 493 nm excitation wavelength and 515 nm emission wavelength. The final ORAC value was calculated as follows [28, 29];

ORAC value = (AUC sample - AUC blank) / (AUC standard-AUC blank) × (Molarity of Trolox / Molarity of sample)

Where, AUC is area under curve, ORAC value is the direct measure of the antioxidant capacity of sample, higher ORAC value corresponds to higher antioxidant capacity. Total antioxidant capacity or ORAC value is expressed as mM Trolox Eq/L.

Lipid Peroxidation (Thiobarbituric Acid-Reactive Substances) Assay: 1 ml plasma was shaken with 5 ml of phosphate buffer pH 7.4 and 75 µl of 200 mM EDTA then homogenized at 2000 rpm for 10 min, followed by cold centrifugation (Remi CM-12 Plus, India) at 6500 rpm for 10 min. 100 µl of 8.1% sodium dodecyl sulfate, 750 µl of 20% acetic acid and 750 µl of 0.8% thiobarbituric acid were added to the mixture. The mixture was heated over boiling water bath at 95 ºC for 60 min, took out and cooled in ice bath for 10 min.

After that, centrifuged again at 10,000 rpm for 10 min and analyzed spectrophotometrically (UV-1800 Shimadzu, Japan) at 532 nm keeping distilled water as a blank [30, 31]. Malondialdehyde (MDA) concentration in the plasma was calculated by using linear regression $y = 0.0022x + 0.0005$, $r^2 = 0.999$ obtained from the standard curve.

Measurement of Plasma Glutathione Level: Plasma samples were processed in the same way as described in MDA estimation. For glutathione (GSH) estimation, 500 µl of plasma was taken and vortexed (Remi CM-101 Plus, India) with 500 µl of 5% ice cold sulfosalicylic acid followed by centrifugation at 10,000 rpm for 10 min. 50 µl of liquid was separated and shaken with 450 µl of phosphate buffer pH 7.4.

Subsequently, resulting mixture was vortexed with 1500 µl of Ellman's reagent then allowed to stand for 5 min. Absorbance was read at 412 nm spectro-photometrically using phosphate buffer pH 7.4 as a blank [32, 34]. GSH concentration was calculated from linear regression equation $y = 0.014x \pm 0.081$, $r^2 = 0.999$.

Total Plasma Protein Estimation by Lowry's Assay: 50 µl of plasma was taken and the volume was made up to 500 µl with distilled water. Lowry's solution (2.5 ml) was added to the liquid, vortexed and incubated for 10 min at 37 ºC. Latterly, 250 µl of Folin's reagent was mixed and again incubated at 37 ºC for 30 min. Absorbance was read at 660 nm by UV Spectrophotometer keeping distilled water as a blank [33, 35]. Standard solution of protein with different concentration of bovine serum albumin (200-1200 µg/mL) was prepared and treated in the similar manner to get regression equation $y = 0.074x + 0.008$, $r^2 = 0.981$.

Statistical Analysis: The correlations between biochemical parameters were statistically analyzed by GraphPad Prism Version 7.04 software with level of significance and confidence interval 5% and 95% respectively. The data of control group are compared with group 1 and group 2 using ANOVA.

RESULTS AND DISCUSSION:

Phytochemical Screening of Crude Extract: Phytochemical analysis carried out on green tea extract notified the presence of constituents which are known to have herbal medicinal values. The results for qualitative phytochemical screening are shown in **Table 1**.

TABLE 1: PHYTOCHEMICAL TESTS OF *CAMELLIA SINENSIS* EXTRACT

Constituents	Test	Conclusion
Tannins	Gelatin test	+
	Lead acetate test	+
Phenols	Ellagic acid test	+
	Ferric chloride tests	+
Flavonoids	NaOH tests	+
	Shinoda test	+
Glycosides	Keller-killiani	+
	Liebermann's test	+
Alkaloids	Wagner's test	+
	Dragendorff's tests	+
	Mayer reagent	+
Saponins	Foam test	-

+ Positive, - Negative

Total Phenolic and Flavonoid Profile: The compounds come from plants in the form of phenolic, are mainly responsible for antioxidant activity. Total phenolic compounds in green tea extract are determined by Folin Ciocalteu method and reported as Gallic acid equivalents (mg/g) by reference to standard curve.

Total phenolic and flavonoid content was estimated as 251.77 ± 6.59 mg gallic acid equivalent per g dry weight and 246.56 ± 2.58 mg Quercetin equivalent per g dry weight of extract respectively. The results showed noticeable level of phenolic and flavonoids which plays a great role in scavenging free radicals. Previously, Yang J also carried out extraction of green tea by using boiling water and quantified phenolic content as 128.7 ± 1.7 mg/g [36]. The Folin-Ciocalteu method gives a crude estimation of the total phenolic compounds present in a sample. It is not specific to polyphenols, but many interfering compounds present in crude plant extract may react with the reagent, skewing the result of total phenolic content [37, 38].

***In-vitro* Evaluation of Antioxidant Activity:** Reactive oxygen species (ROS) generated endogenously or exogenously are associated with pathogenesis of various diseases. Thus antioxidants which can scavenge ROS are expected to improve these disorders [39]. In this study, three different assays were used to evaluate antioxidant activity of green tea extract as there is no simple universal antioxidant assay by which antioxidant potential can be measured accurately and quantitatively. The DPPH assay is one of the most frequently used method to determine the antioxidant activity of most of the substances tested. The total antioxidant activity measured by different methods varied because different mechanism and reaction involved in different *in-vitro* assays.

Extract shown 93.76 ± 12% antioxidant activity with ABTS and 95.13 ± 17.89% when tested with DPPH hydrogen reacting radicals. While, it was calculated 92.82 ± 8.72% for FRAP assay. The result of antioxidant assay is given in **Fig. 1**. Thus, antioxidant activity of crude extract is not limited to phenolic compounds only; presence of other components may contribute to the overall observed antioxidant potential.

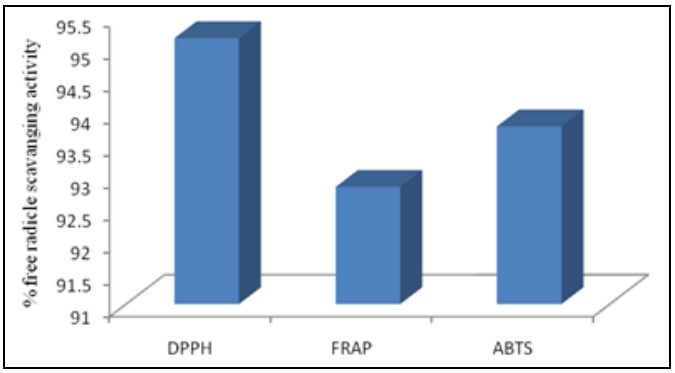

FIG. 1: FREE RADICAL SCAVENGING ACTIVITY OF GREEN TEA EXTRACT

Plasma Antioxidant Measurements: The results of the *in-vivo* antioxidant effect of extract on guinea pigs are presented in **Table 2**. The extract shows dose dependent decrease in MDA level in plasma. Although, the extract produced dose depended increase in GSH value and ORAC antioxidant potential. Extract dosages were chosen from earlier *in-vivo* studies on guinea pigs and rats at 100-500 mg per kg body weight [40].

MDA has been identified as an index for lipid peroxidation, the decrease in MDA level in plasma may indicate increased activity of antioxidant enzyme glutathione peroxidase [40]. Green tea is one of the most commonly consumed beverages in the world with no reported side effects, this study results shows that extract modulate GSH level in plasma and improved antioxidant defense system. GSH is the most abundant intracellular thiol, produced naturally by liver. It is an important anti-oxidant preventing damage to cellular components caused by reactive oxygen species. An increased level of GSH is an indicative of decreased oxidative stress.

TABLE 2: BIOCHEMICAL PARAMETERS VALUES IN GUINEA PIG PLASMA (n=4, MEAN ± SEM)

Group	MDA (µg/mg protein)	GSH (µg/mg protein)	ORAC Value (mM Trolox Eq/L)
Control (Distilled water)	0.013 ± 0.002	12.722 ± 1.918	6.771 ± 1.023
Group 1 (Extract 200 mg/kg)	0.009 ± 0.001	16.36 ± 1.835*	11.771 ± 2.633*
Group 2 (Extract 400 mg/kg)	0.006 ± 0.001*	20.97 ± 2.541*	23.771 ± 1.926*

*P<0.05 are statistically significant when compared to control.

Plasma ORAC value is also significantly higher (P<0.05, ANOVA) in guinea pigs received 200 mg/kg and 400 mg/kg extract, when compared to control group. This suggests dose dependent increase in total antioxidant capacity in the plasma.

CONCLUSION: The findings of this study reveal that, green tea extract shows *in-vitro* antioxidant capacity as well as there is significant variation in MDA, GSH and ORAC value among different groups of Guinea pig in dose dependent manner.

We expect that our results will serve as a benchmark for further pharmacological screening of *Camellia sinensis* constituents, which makes this as a therapeutic adjuvant agent against free radicals mediated diseases.

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CONFLICT OF INTEREST: Declared none.

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