In vitro antioxidant effect of *Camellia sinensis* on human cell cultures

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Abstract: *Camellia sinensis*is traditionally used in many polyherbal preparations for the treatment of different diseases and infections. Its action has been associated with its antioxidant activities. In this study, antioxidant effect of *Camellia sinensis* on hydrogen peroxide-induced human lymphocyte cell cultures was estimated. *Camellia sinensis* showed high contents of ascorbic acid, phenols, flavonoids, and flavonols. Good scavenging activity was evident by scavenging assays e.g. 2,2-DiPhenyl-2-Picrylhydrazyl Hydrate (DPPH), 2,2-Azinobis (3-ethyl-BenzoThiazoline-6-Sulfonic acid (ABTS) radical assay and reducing power assay. Moreover, High Performance Liquid Chromatography (HPLC-UV) chromatographs showed many notable peaks of unidentified bioactive compounds. *In vitro* antioxidant actions were determined by the activities of catalase (ELISA kit method), superoxide dismutase, lipid peroxidation and total protein contents on lymphocyte cell cultures. In vitro experimental trial showed strong antioxidant repair mechanism of plant against oxidative stress. Results of extraction with solvent methanol showed the highest antioxidant activity. *Camellia sinensis* is promising source of natural antioxidants and further studies might be a likely source of its use in remedy of different diseases.

Keywords: Antioxidant, Free radicals, Camellia sinensis, ABTS, Lymphocytes, HPLC.

INTRODUCTION

During organic evolution aerobic organisms have evolved certain defence mechanisms against free radical cellular oxidation (Kumar, 2009). Naturally present antioxidants in human body act by prevention, removal and repair of oxidative damage (Sen *et al.,* 2010). However, natural antioxidants can be incompetent so dietary intake of antioxidant compounds becomes imperative. Recently, therapeutic potentials of medicinal plants have gained a lot of interest. Side effects of synthetic drugs make them more dangerous to use than the diseases they aver to cure. However natural substances in plant-derived formulations attest them to be safe and pharmacologically potent. The regain of natural remedies is own due to amelioration power of plants (Muanda *et al.,* 2010; Pourmorad *et al.,* 2006; Özgen *et al.,* 2004). The preclude role of plant phytochemicals as natural antioxidants has engrossed their screening for therapeutic components (Nam *et al.,* 2012; Adhikarimayum Haripyaree, 2010). Recently, many antioxidants have been isolated from plants (Kalim *et al.,* 2010). Therefore, the development and commercialization of novel functional compounds derived from these plants must be pursued to improve the functionality of these phytoconstituents as phytomedicine (Turkog Lu *et al.,* 2010). *Camellia sinensis* is the second most consumable drink after water (Ziaedini *et al.,* 2010). Its leaves contain many constituents and biological important are polyphenols. Three main constituents namely dihydroactinidiolide, phenylethyl alcohol and benzyl alcohol are present in following concentration 8.32µg/g, 6.56µg/g and 4.49µg/g respectively (Nam *et al.,* 2012). Among polyphenols, catechins are famous for anti-mutagenicity, anti-tumorogenicity and anti-carcinogenicity (Ziaedini *et al.,* 2010). It has been shown its role in improving the antioxidant capacity of plasma and some organs (Antonios *et al.,* 2006)**,** curing diabetes (Chen *et al.,* 2009), lowering the content of cholesterol and maintaining the ratio of good (HDL) cholesterol to bad (LDL) cholesterol. Iinsomnia due to presence of caffeine is the only negative side effect reported so far (Fukushima *et al.,* 2009). The leaves have long-established medical importance to systems to treat asthma, angina pectoris and cardiac disorders (Koutelidakis *et al.,* 2009).

The main objectives were to: (1) compare total antioxidant capacity by three commonly used antioxidant activity methods (2) determine the effect of extraction solvent on activity of plants (3) determine the *in vitro* antioxidant activity in lymphocytes (4) determine the correlation between antioxidant activity and phenolic compounds.

MATERIALS AND METHODS

Plant material

The *Camellia sinensis* leaves were collected from local area based on their basic information available and authenticated using references and authorized books (Pharmacopoeia Committee of Ministry of Health of China, 1995). Dried extracts were stored at 4°C in air-tight bottles.

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Preparation of plant extract

Three different extraction systems were used to evaluate and compare the antioxidant potential of plants. These extraction solvents were aqueous, methanol and ethanol (Preethi *et al.,* 2010).

a) Aqueous Extraction: Dried leaves in ratio of 1:10 were immersed in distilled water, mixed and soaked for 24 hours. Later, mixture was filtered through Whatmann No.4 filter paper to get pure extract.
b) Ethanol Extraction: Dried leaves in ratio of 1:10 were immersed in 95% ethanol for 24 hours at room temperature. Filtered through Whatmann No.4 filter paper, the solvent was evaporated till it became one-fourth of its original.
c) Methanol Extraction: Dried leaves in ratio of 1:10 were immersed in 95% ethanol and kept on shaking at 190-220 rpm for 24 hours at room temperature. After filtration through Whatmann No.4 filter paper and centrifugation at 5000 rpm for 15 minutes, the solvent was evaporated till it became one-fourth of its original.

Phytochemical Screening

Determination of total ascorbic acid contents (ASC)

Ascorbic acid contents were determined according to Kalim *et al.,* 2010. Ascorbic acid was used as standard. Different concentrations ranging 0-10mM were prepared. DTC reagent (0.05% copper sulfate, 0.4% thiourea and 3% 2,4-dinitrophenylhydrazine) was prepared in 9N sulfuric acid. 10% tricholoroacetic acid was added to standard followed by centrifugation. After centrifugation, mixed 1000µLof supernatant with 200µL DTC and incubated at 37°C for 3 hours. 1500µL 65% sulfuric acid was added and absorbance was recorded at 520 nm with a double beam Cecil Aquarius CE 7200UV/Visible spectrophotometer. Same procedure was followed with samples. Ascorbic acid contents were expressed as mg AA/g of plant samples.

Determination of total flavonol content

The content of flavonol was determined by Yermakov *et al*. (1987). Rutin was used as standard and calibration curve was prepared using different rutin concentrations (0.0166, 0.025, 0.05, 0.1, 0.166, 0.2, 0.3, 0.4, 0.5mg/mL). 2mLeach of rutin ethanolic solution and 2% aluminum trichloride were mixed with 6mL 5% sodium acetate and incubated at 20°C for 2.5 hours. Following incubation, absorption was read at 440nm. Flavonol contents in plant extracts were determined in the same way. Flavonol contents were calculated in rutin equivalents (RE) using following formula:

X =C.V/m

Where: X-flavonol content, mg/g plant extract in rutin equivalents;
C-the concentration of rutin solution, established from the calibration curve, mg/mL;
V, m-the volume and the weight of plant extract, mL, g

Determination of total flavonoids contents

Flavonoid contents were determined by aluminum chloride colorimetric method (Chang *et al*., 2002). 0.5mL plant extract (1:10gmL^{-1}) was mixed with 1.5mL methanol, 0.1mL10% aluminum chloride, 0.1mL1M potassium acetate and 2.8mL distilled water and incubated at room temperature for 30 minutes. Following incubation, reading was measured at 415nm. Rutin was used as standard and calibration curve was made by using 12.5µgmL^{-1} to 100 µgmL^{-1}.

Determination of the total amount of phenolic Compounds

Total phenolic contents were determined by Folin-Ciocalteu method (1927). Gallic acid was used as standard and various concentrations were prepared (0.3, 0.105, 0.075, 0.024mg/). 1mL standard was mixed with 5mL Folin-Ciocaleu reagent (1:10 diluted with distilled water) and 4mL (75g/L) 1M sodium carbonate were added and incubated for 30 minutes at 20°C. Absorption was measured at 765nm. Same procedure was followed with 1mL aqueous plant extract (10 g/L) and after 1 hour incubation absorption was measured at 765nm. Phenolic contents were calculated in gallic acid equivalents (GAE) using following formula:

C=c. V/m

Where:
C-Total content of phenolic compounds, mg/g plant extract, in gallic acid equivalents
c-Concentration of gallic acid established from the calibration curve, mg/mL
V-Volume of extract, mL
m-Weight of pure plant methanolic extract, g.

Antioxidant activity determination

DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity

The radical scavenging effect of stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) was measured according to the method of Kriengsak *et al*. (2006). Disappearance of initial purple colour determines the antioxidant capability to scavenge the DPPH free radical. For stock solution, 24 mg DPPH was dissolved in 100 mL methanol and stored at -20°C until needed. 10mL stock solution with 45mL methanol was mixed to obtain working solution with an absorbance of 1.1±0.02 units at 515nm using a double beam Cecil Aquarius CE 7200UV/Visible spectrophotometer. 150 µL sample extracts with 2850 µLDPPH solution kept for 24 hours at room temperature in dark. Meanwhile, the absorbance was taken at 515nm after 15, 30, 60, 120, 1200, and 1440 minutes. Percentage of disappearance of initial purple color determines the ability of extract to scavenge DPPH free radical. Higher the activity, high will be the percentage of initial purple color disappearance. Scavenging activity was calculated as follows:

% of scavenging activity = As-Ai / Ai

Where As is the absorbance of pure DPPH (control), Ai is the absorbance of DPPH in the presence of extracts.

DPPH radical scavenging activity via thin layer chromatography (TLC)

Antioxidant potential of extract was determined by thin layer chromatography using the method of Cavin *et al.,* 1998.Sample/standard was applied on the thin layer chromatography plate with capillary and was air dried; spotting process was repeated on same spot for having suitable amount (10µL). Plate was developed by butanol, acetic acid and distilled water in ratio of 40:50:10. Plates were observed in CAMAG UV Lamp dual wavelength, 254/366 nm to visualize the components showing UV absorbance or fluorescence. Then TLC plates were sprayed with 0.2% of DPPH reagent in methanol and kept for 30 minutes at room temperature. Yellow spots against purple background are indication of positive antioxidant activity. Retention value (*Rf* value) was also determined by given formula:

RF value= (Distance travelled by the component) / (Distance travelled by the solvent)

ABTS (2,2 azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) Assay

For ABTS assay, the procedure followed the method of Arnao *et al.*, 2001 with some modifications. Equal quantities of 7mM ABTS and 2.5mM potassium persulphate were mixed to prepare working solution. It was incubated at room temperature for 12-16 hours in dark before use. Later a value of 0.70±0.02 was established by diluting the intensely-coloured ABTS radical cation with ethanol at 734 nm. 3mL of the $ABTS^{\bullet+}$ solution and 20µL of standard or sample were mixed and absorbance was measured at time intervals of 1-10, 60, 90, 120, 180, 240, 300, 360, 420, 480, 1260 and 1440 minutes at 734nm. Trolox was used as standard. The activity was represented as % inhibition of ABTS radical using the same formula used for DPPH scavenging assay.

Reducing Power assay

Reducing power assay was determined by applying the procedure of Yildrim *et al*., 2001. Different plant concentrations (10-100mg/L) were prepared. 1mL plant extract was mixed with 2.5mL 0.2M phosphate buffer (pH 6.6) and 2.5mL of 1% potassium ferricyanide and incubated at 50°C for 20 minutes. 2.5mL 10% tricholoroacetic acid was added to the mixture after incubation followed by centrifugation at 3000 rpm for 10 minutes. 2.5mL supernatant was mixed with equal quantity of 2.5mL distilled water and 0.5mL 0.1% $FeCl_3$ and measured at 700nm. Ascorbic acid was used as standard and as a blank phosphate buffer was used.

In vitro antioxidant activity using lymphocyte as living model

Normal cellular events are often demonstrated by studying cell cultures. Direct measurement of oxidative changes in cell or tissue cultures makes them model of choice (Halliwell, 2003; Lesuisse *et al.,* 2002). Mostly animal cells are cultured as a monolayer under 95% air, 5% CO_2 (approx. 152mmHg O_2; 1 mmHg=0.133 kPa) in hyperoxic environment (most cells in the human body experience <10mmHg O_2). It is of interest that among blood cells only white blood cells can be cultured. They are primary cells that are explanted directly from donor organisms and express characteristics, which are not seen in cultured cells (Mayne*,* 2003). Lymphocytes are of special interest due to their diversified redox and free radical scavenging systems. They are extensively screened in human population exposed to a variety of toxicants. In this study hydrogen peroxide was used to induce oxidative stress in lymphocytes. Hydrogen peroxide is useful in a sense that it can cross the cell wall easily and stimulate hydroxyl radical formation (Kumar, 2009).

Lymphocyte isolation and preparation for test

Lymphocytes were isolated following density gradient method in heparinized sterilized tube. *Camellia sinensis* aqueous extract was selected although high activity of extracts in ethanol and methanol was observed. But ethanol and methanol may have an effect on the physiological conditions of lymphocytes and in turn on its growth and activity. So to minimize theses artifacts aqueous plant extract of green plant was used. Lymphocytes were processed through pre and post exposure of H_2O_2 for 0 h-4 h, 10 h-72 h and 0 h-72 h. In 0-4 h cell treatment, H_2O_2 was removed by centrifuging the cells for 10 min (1000 r/min), washing once with RPMI medium (without fetal calf serum) and resuspending in complete medium (with fetal calf serum) along with plant extract (10µL/10000 cells of plant extract). In 10-72 h cell treatment, H_2O_2 was followed by resuspending them in complete medium along with plant extract (10µL/10000 cells of plant extract; 50µL/10000 cells of plant extract). In 0-72h-cell treatment, in one group H_2O_2 was left in the culture medium until cell harvest and in another group plant extract (50µL/10000 cells of plant extract) was left in culture medium until cell harvest. This was accomplished by dividing lymphocyte into groups as follow:

Group 1: Only lymphocytes without any treatment acting as negative control

Group 2: 20µL plant treated lymphocytes in fresh media + 100µM H_2O_2 (post-treatment)

Group 3: 100µM H_2O_2 treated lymphocytes as positive control

Group 4:100µM H_2O_2 treated lymphocytes in fresh media + 10µL /10000 cells of plant extract (pre-treatment)

Group 5: 100µM H_2O_2 treated lymphocytes in fresh media + 50µL /10000 cells of plant extract (pre-treatment)

Group 6: lymphocytes + 50µL /10000 cells of plant extract

Visualization of Lymphocytes

After treatments the lymphocytes were centrifuged at 500xg for 10 minutes at 22°C. Supernatant was aspirated and cell pellet was resusupended in 1mL Phosphate buffer saline. 10 µL of cell suspension were mixed with 10µL trypan blue dye. It was observed using Labomed TCM400 Fluorescent microscope. The number of both live cells (cells without TB dye) and dead cells (cells without TB dye) were calculated. Viable cell percentage was determined by using the following formula:

$$\text{Viable cell \% age} = \frac{\text{Total no. of viable cells per mL aliquot}}{\text{Total no. of cells per aliquot}} \times 100$$

Antioxidant activities of lymphocytes

Catalase activity was determined using Catalase assay 96 well kit method (Calbiochem- 219265). In addition, lipid peroxidation (Buege and Aust, 1979), superoxide dismutase activity (Beauchamp and Fridovich, 1971) using nitroblue tetrazolium (NBT) in presence of sensitizing dye (riboflavin) and total protein content (Garcia-Medina *et al.,* 2009) were also measured.

STATISTICAL ANALYSIS

Duplicates were made in each experiment and average of the two measurements was used in subsequent statistical analysis. Values were presented as mean ± standard deviation. One way ANOVA test was used. Significant difference was considered at P   0.05 using Duncan's new multiple range test. Pearson's correlations were also found between phenolic contents and antioxidant activity.

RESULTS

Ascorbic acid contents were calculated as AAmg/g of plant using regression equation of calibration curve. They did not vary in extraction solvents (fig. 1). Highest contents were present in ethanolic extract (29.58±0.1mg/g) while lowest in aqueous extract (28.85±0.5mg/g). Regression equation of calibration curve was used to calculate rutin equivalent (RE) of flavonols and flavonoids. Rutin belongs to flavonoids group that prevents Fe^{2+} binding to H_2O_2, which would otherwise create highly reactive free radicals vicious to the cells (Yang *et al.,* 2002). Flavonoids content ranged from 34.45±0.1mg/g to 17.11±0.1mg/g of extracts. Flavonol contents showed much variations; ranging from 46.11±1.3 mg/g to 3.94±0.0mg/g of extracts. Total phenolic contents were calculated in Gallic acid equivalents (GAE). Antioxidant activity was determined by reducing power assay and radical scavenging methods e.g. ABTS and DPPH radical assays (Zhou *et al.,* 2010). It was also a good scavenger of oxidants as evident by DPPH, ABTS and reducing power assay activity when compared with standard especially in reducing power assay. *In vitro* antioxidant results also showed positive effects in lymphocyte cell culturing. *In vitro* experimental trial showed strong antioxidant repair mechanism of plant against oxidative stress. Results of extraction with solvent methanol showed the highest antioxidant activity.

DISCUSSION

Phytochemical studies

The presence of natural compounds evolved in action to oxidative stress, has widespread the use of traditional herbs and medicinal plants (Sen *et al.,* 2010; Ivanova *et al.,* 2005). The high impact of polyphenols (e.g. flavonols, flavonoids and phenolic compounds) in overall antioxidant activity makes it significant to determine their total amount in medicinal plants (Kratchanova *et al.,* 2010). Despite ascorbic acid, flavonoids and flavonol content vary in extraction solvent (fig. 1). All extracts have high phenolic contents but they behaved differently towards antioxidant assays (fig. 1). Thus, phenolic contents are not the deciding feature on which potential of these plants as antioxidants can be explained. But it could possibly be due to the presence of some other phytochemicals as well as due to their synergistic effects (Awika *et al.,* 2003). In view of extraction solvent, methanol indeed showed high phytochemical contents followed by ethanol and aqueous solvents.

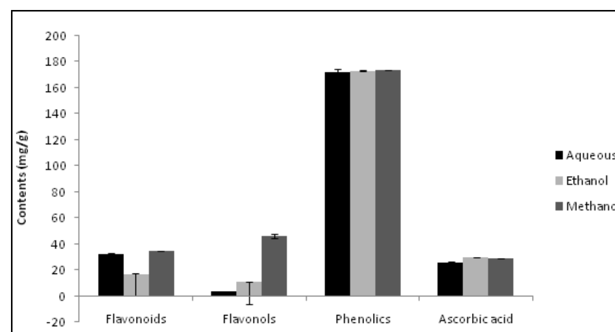

Fig. 1: Phytochemical screening: Flavonoids, flavonol and ascorbic contents, phenolic contents (mg/g dry weight of plant). Mean ± Standard Error.

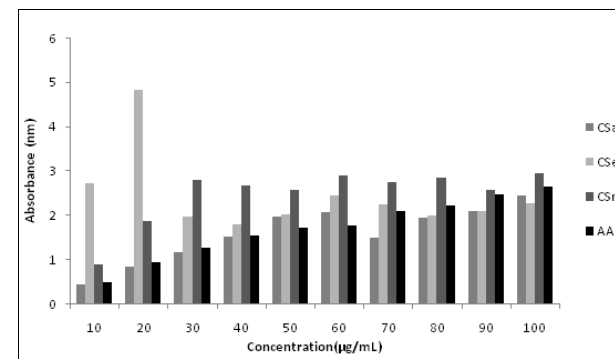

Fig. 2: Reducing power of plant extracts. High absorbance indicates strong antioxidant activity. CSa, *Camellia sinensis* aqueous; CSe, *Camellia sinensis* ethanolic; CSm, *Camellia sinensis* methanol; AA, Ascorbic acid.

***In vitro* antioxidant activity**

In DPPH a hydrogen atom is transferred while in ABTS electron is transferred (Kaviarasan *et al.,* 2007; Sanchez-Moreno, 2002). Reaction with antioxidant compounds denotes hydrogen atom to DPPH and reduces it, which can be visualized by colour change from deep violet to light yellow at 515nm. Tocopherols commonly trolox are very important natural antioxidants present in cereals, legumes, oils and green vegetables. The antioxidant mechanism of tocopherols involves reactions with free radicals (especially the peroxyl radical) resulting in the formation of a relatively stable phenoxy radical or scavenging and quenching of singlet oxygen (Oyaizu, 1986). It was observed that extracts in methanol most effectively scavenged DPPH than extracts in aqueous and ethanol (fig. 2). The %age inhibition of extracts was higher than the both standards e.g. Trolox and Rutin. It declines within an hour and leveled after 22 hours almost. The values ranged in 95% or above should be considered as 100% because even after 24 hours the DPPH radical solution remains yellow irrespective of colorless methanol used as blank.

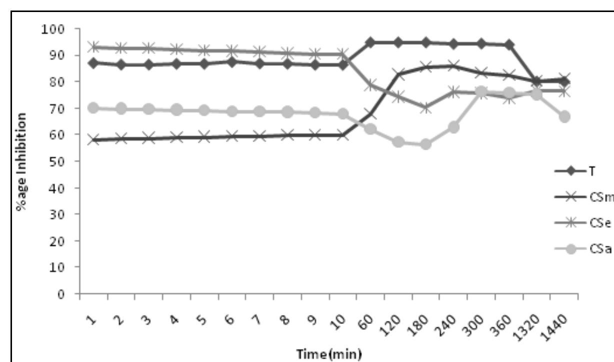

Fig. 3: DPPH absorption inhibition assay. CSa, *Camellia sinensis* aqueous; CSe, *Camellia sinensis* thanolic; CSm, *Camellia sinensis* methanol; AA, Ascorbic acid.

DPPH assay with thin layer chromatography gave instant indication of antioxidant activity of plant extract. Development of yellow spots against purple background indicated the presence of antioxidant activity. The visualization of different colour bands at 366nm point to different phytochemicals present within the plant extracts such as yellow orange color for carotenes, gray color for pheophytin, green for chlorophyll b, blue-green more intense than chlorophyll b for chlorophyll a and yellow for xanthophyll. It gave an indication of bioactive compounds that aids in its antioxidant capacity.

2, 2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was determined using trolox as a standard (fig. 3). Trolox and methanolic extract showed almost the same trend. The scavenging activity increases within an hour that became steady in almost 2hours and moved toward decline in 12 hours. However, ethanolic and aqueous extracts showed aberrant trend; up to 10 minute they were steady but after 10 minutes they started to decline and after 5 hours they again raised eventually again. Generally, maximum activity was from 1 minute to 1 hour and mostly came to stationary phase around three hours. The electron and hydrogen atom donating capacity indicates the presence of reductants, which is associated with reducing capacity of the compound. The presence of reductants in the extract is associated with the development of Perl's Prussian blue coloration. The blue coloration indicates the reduction of Fe^{3+}/ferricyanide complex to ferrous form (Zhou *et al.,* 2010; Nakamura, 2005). In our study, it showed concentration dependency. However, increase in reducing activity is limited to certain concentrations beyond which constancy does not remain. During the assay, the activity increased constantly up to 400-500µg/mL after which the increase was not uniform (data not shown). A steady increase with concentration was observed in standard but same trend was absent in plant extracts. They increase with concentration up to certain limit then showed almost same values but higher than the standard. At 10µg/mL and 20µg/mL ethanolic extracts showed extra ordinary high values. The reason behind this is unknown. Most of the time extracts showed more reducing activity as compared to the standard, ascorbic acid. It suggests that plant extract is a good radical chain reaction terminator that converted reactive oxygen species and free radicals to stable products.

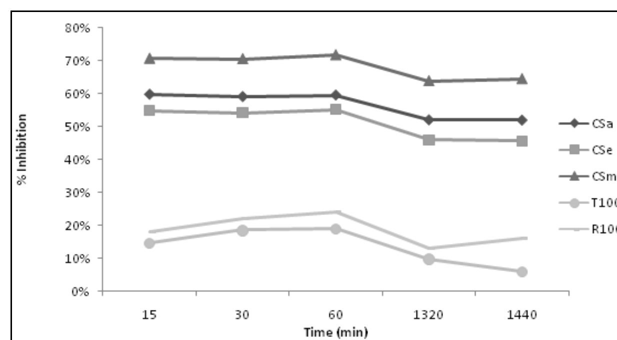

Fig. 4: ABTS absorption inhibition assay. CSa, *Camellia sinensis* aqueous; CSe, *Camellia sinensis* ethanolic; CSm, *Camellia sinensis* methanol; R100, Rutin (100mg/mL); T100, Trolox (100mg/mL).

Higher pressure liquid chromatography (HPLC)

HPLC-UV chromatograms showed presence of unknown bioactive compounds. Numerous peaks at different retention time (tR) were observed out of which 8 major peaks were at the retention time of 0.776, 1.648, 1.872, 2.376, 2.636, 6.6, 10.904, 14.144 minutes with peak areas (area%) 1.4, 3.8, 6.8, 29.9, 48.7, 4, 2.1 and 1.2 respectively (fig. 3A). The two peaks Peak 5 (2.376) and 6 (2.636) closely relates to the peaks of reference compounds. Ascorbic acid and Gallic acid were used as reference compounds. These chromatograms specify the presence of various bioactive compounds concurrently in good concentrations.

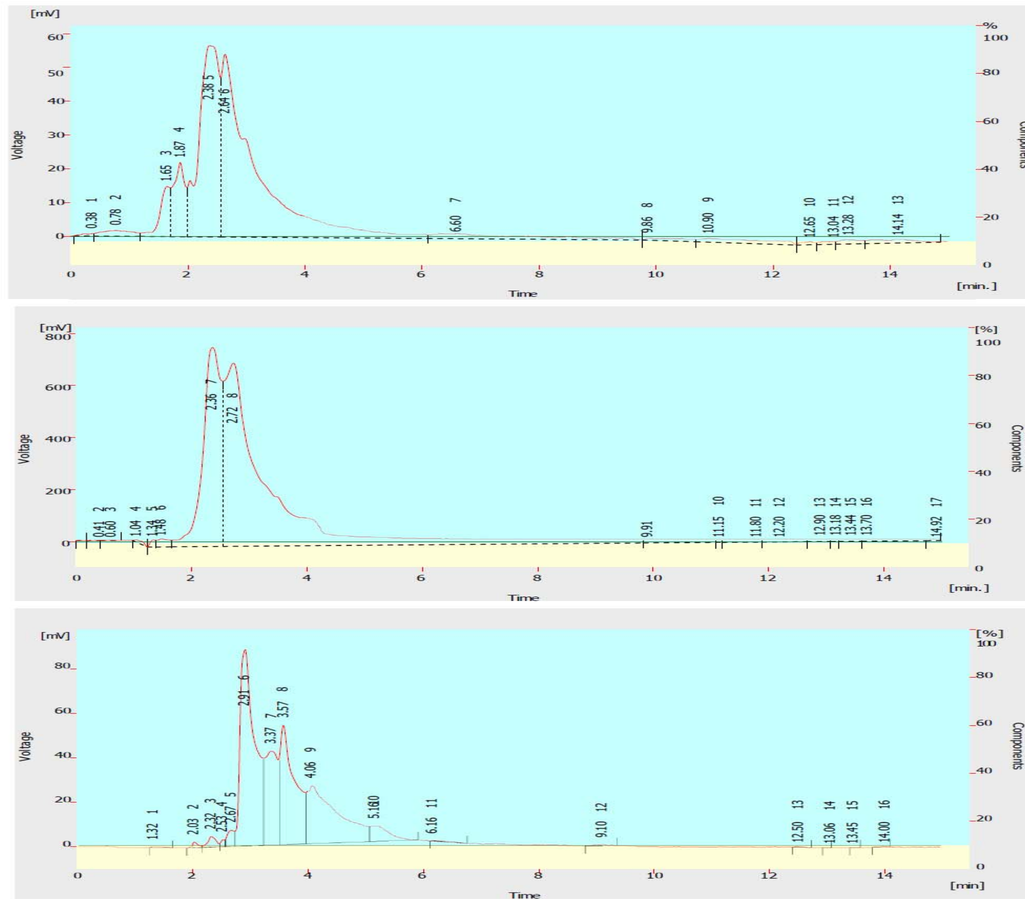

Fig. 5: HPLC chromatogram. A, Plant extract; B, Ascorbic acid used as standard; C, Gallic acid used as standard.

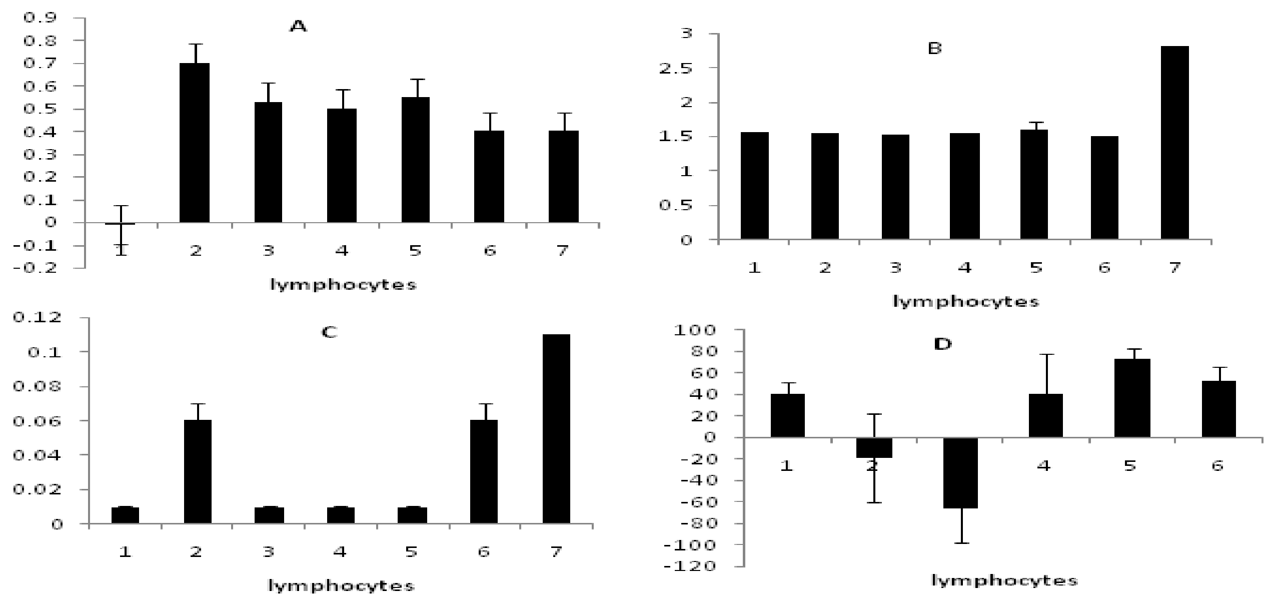

Fig. 6: Antioxidant activity of lymphocytes. 1 (lymphocytes), 2 (plant-treated lymphocytes + H_2O_2), 3 (lymphocytes + H_2O_2), 4 (H_2O_2 + 10µL/10000cells), 5 (H_2O_2 + 50µL/10000cells), 6 (lymphocytes + Plants), 7 (Plant), A: super oxide dismutase (Umg/protein) activity. B: ascorbic acid contents, C: TPC: total protein content (µg/mL), Lipid peroxidation.

Table 1: Catalase activity of lymphocytes

Lymphocyte Groups	CAT activity (nmol/min/mL)	
1 (lymphocytes)	0.34 [c]	0.30 [d]
2 (pre-treated lymphocytes +H_2O_2)	0.22 [b]	-0.33 [a]
3 (post-treated lymphocytes with 10µL/10000cells)	0.130 [a]	-0.17 [b]
4 (post-treated lymphocytes with 50µL/10000cells)	0.40 [d]	0.70 [f]
5 (lymphocytes + H_2O_2)	0.550 [e]	0.22 [c]
6 (lymphocytes + Plants)	0.41 [d]	0.39 [e]

Mean ± Standard Error; Different letters within same column in parenthesis indicate significant difference between treatments using Duncan's multiple range test (P = 0.05)
1 (lymphocytes), 2 (plant-treated lymphocytes + H_2O_2), 3 (lymphocytes + H_2O_2), 4 (H_2O_2 + 10µL/10000cells), 5 (H_2O_2 + 50µL/ 10000 cells), 6 (lymphocytes + Plants)

Table 2: Correlation between phytochemical and antioxidant assays

	Flavonol	Phenolics	Ascorbic acid	Reducing power	ABTS	DPPH
Flavonoids	.438	-.109	-.593	.856	-.111	.818
Flavonols		.846	.464	.840	.845	.875
Phenolics			.865	.421	1.000**	.483
Ascorbic Acid				.942	.333	-.022
Reducing power					.419	.998*
ABTS						.481

**Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

In vitro lymphocyte activity

In the organism, active oxygen molecules are converted into non-toxic compounds by first line of defence, the antioxidative enzymes (SOD, CAT, and GSH) (Ames *et al.,* 1993). Lymphocytes were isolated following density gradient method in heparinized sterilized tube. Viable cell percentage was 73.43% estimated by trypan blue dye method. SOD activity (fig. 7A) showed that when lymphocytes were treated with plant extract prior to exposure to hydrogen peroxide, the activity is enhanced as compared to plant treatment after exposure to hydrogen peroxide (G4, G5). It is also noteworthy that when plant concentration was increased as in G5, the activity also improved as compared to G4 where less plant concentration was used. Ascorbic acid contents (fig. 7B) did not vary among different groups while total protein contents (fig. 6C) were high only in lymphocytes treated with plant extract prior to exposure to hydrogen peroxide (G2) and without exposure to hydrogen peroxide (G6 and G7). This showed that exposure to hydrogen peroxide deteriorates the protein content of lymphocytes and plant extract prevent the protein damage. Catalase (oxidoreductase) is a omnipresent antioxidant enzyme present in most aerobic cells. Upon complete reaction, purple color variations were observed in 96 well microplate. The samples were grouped into with and with no dilutions. Diluted (10µL sample: 10µL sample buffer) samples for all groups were observed. Formaldehyde was used as standard. The catalase activity results were described in table 1. G 6 (lymphocytes + plant extract) had the highest catalase activity as compared to the control (G 1 + G5) both in diluted (0.34 nmol/min/mL) and with no diluted sample (0.30 nmol/min/mL) except in with dilution (0.41 nmol/min/mL). G1 also showed increased activity in diluted samples (0.34 nmol/min/mL) as compared to with no dilution (0.30 nmol/min/mL). Post-treated lymphocytes showed decreased catalase activity as compared to both positive (G5) and negative (G1) control. Pretreatment with 10µL/10000cells (G3) showed increased activity in diluted sample (0.13 nmol/min/mL) as compared to in sample with no dilution (-0.17 nmol/min/mL). However post treatment with 50 µL/10000cells (G4) showed increased activity in sample with no dilution (0.70 nmol/min/mL) than in diluted sample (0.40 nmol/min/mL). It was observed that catalase activity was lower in diluted samples as compared to not diluted samples. These results were also analyzed with Duncan's and Tukey's test. Homogenize tests through Duncan's test showed that the significant difference between lymphocyte groups (with dilution) was highest for group 5. In lymphocyte groups (with no dilution) the significant difference was highest in post-treated lymphocytes with 50µL/10000 cells. Accumulation of free radicals reduces the activities of SOD, GSH and CAT (Santhakumari *et al.,* 2003). O^{2-} radicals are disrupted only by SOD, which is present in all cells especially in erythrocytes. CAT stop the OH^- radicals production by preventing the formation of hydrogen peroxide or by SOD reaction (Urso and Clarkson, 2003; Li *et al.,* 2007).

Correlations findings

Assessment of antioxidant activity using different assays comprehensively evaluates potential of natural products as antioxidant. One assay is specific for only one property; the correlation between the different assay procedures certifies the reliability of different methods (El-Toumy *et al.,* 2011). Some authors (Loganayaki *et al.,* 2010, Patel *et al.,* 2010, Djeridane *et al.,* 2006) had reported a linear correlation between phenolic contents and their antioxidant capacity. However, others (Javanmardi *et al.,* 2003, Kahkonen *et al.,* 1999) observed poor or no linear correlation. In present study significant correlation between phenolics and antioxidant activity was observed however correlation was observed between assay of reducing power and DPPH (Table 2).

Table 3: Correlation between antioxidant assays

Antioxidant Assays	Total protein content	SOD activity	Ascorbic acid
Lipid peroxidation	-0.154	-0.319	-0.128
Total protein content		0.139	0.775*
SOD activity			-0.080

*Correlation is significant at the 0.05 level (2-tailed)

CONCLUSION

Experimental plant is potential source of natural antioxidants. *In-vitro* experimental trial showed strong antioxidant mechanism of plant against oxidative stress.

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