

Antioxidant and Oxidative DNA Damage Protection Potential of Methanol Extract of Red Tea Stem

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Abstract

This study was undertaken to determine free radical scavenging capacity and oxidative DNA damage protecting activity of methanol extract of red tea stem. The extract was subjected to assess their antioxidant potential using various *in vitro* systems such as DPPH[•], ABTS^{•+}, super oxide and nitric oxide free radicals and it exhibited IC₅₀ values of 68.88 ± 1.1, 12.08 ± 0.65, 404.38 ± 1.6, 93.6 ± 2.7, µg/mL respectively. Red tea extract also showed ferric reducing ability (FRAP) with 2606.85 mmol Fe (II)/g of extract. Furthermore, Methanol extract of red tea stem showed significant DNA damage protecting activity in concentration dependent manner against H₂O₂+UV induced photolysis on pUC19 plasmid DNA. Results of this study showed that the methanol extract of Red Tea stem has strong antioxidant potential along oxidative DNA damage protecting capacity that would be the significant sources of natural antioxidants, which might be helpful in preventing the progress of various oxidative stress generated diseases. Further study is necessary for isolation and characterization of the active antioxidants, which may serve as a potential source of natural antioxidant.

Keywords : Antioxidant, Nitric oxide, Oxidative DNA damage, Red tea stem, Superoxide radicals

Introduction

In living beings, free radicals like superoxide radical (O₂^{•-}) hydroxyl radicals (•OH) and singlet oxygen (¹O₂) are constantly generated as endogenous metabolism process (Porteret al. 1995) as well as by exogenous sources such as ionizing radiations, UV light, pesticides (Briviba and Sies 1994). Fortunately, living beings possess an innate antioxidant enzymes defense mechanism including catalase, superoxidodismutase (SOD), glutathione (GSH) and peroxidase, to protect the functional and structural integrity of the biological molecules such as DNA, proteins, and lipids from detrimental effects of ROS (Ames et al. 1993; Tvazziet al. 2000). However, during oxidative stress, which results from imbalance between formation and neutralization of pro-oxidants, antioxidant defence system become compromised and this oxidative state become sole culprit for the physiological and pathological conditions such as cancer, aging, rheumatoid arthritis, atherosclerosis and neurodegenerative diseases (Florence 1995; Martinez-Cayuela 1995; Schoneich 1999; Young and Woodside 2001; Pardo-Andreu et al. 2006).

The extent of damage caused by free radicals might be mitigated via supplementation with one or more antioxidants. Therefore, in recent years research has been more focused towards to dugout

non-toxic and more effective bioactive phyto-chemicals having antioxidant property, to substitute synthetic preservative antioxidants like butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), whose applications are circumscribed because of their toxic and/or mutagenic concerns (Ito et al. 1983).

The tea plant *Camellia sinensis* (L.) Kuntze (family Theaceae) is grown in about 30 countries worldwide. Research on the effects of tea on human health has been fuelled by the growing need to provide naturally healthy diets that include plant-derived polyphenols. There is already growing evidence that tea polyphenols mitigate the risk of cancer and heart diseases in humans (Vanessa and Williamson 2004). Moreover some studies have reported about anti-allergic (Yamamoto et al. 2004) and antimicrobial properties (Paola et al. 2005) properties of tea. Therefore, tea appears to be an effective chemo-preventive agent for toxic chemicals and carcinogens. The major antioxidant compounds in teas are catechins, then theaflavins, thearubigins, oxyaromatic acids, flavonols, such as kaempferol, myricetin, quercetin; flavones, such as apigenin; derivatives of gallic acid, such as tannins, etc.

Red tea also known as black tea is more oxidized than oolong, green and white teas (Reeves et al. 1987). These all types of tea are prepared from leaves of *Camellia sinensis*. Red tea is

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generally stronger in flavor than the less oxidized teas. However antioxidant property of green tea, as measured by different methods, is usually higher than the antioxidant activity of Red tea or black tea (Cao et al. 1996). But it was also evaluated that theaflavins in black tea and catechins in green tea are equally effective antioxidants (Stewart et al. 2005; Leung et al. 2001). Despite the upsurge of interest in the therapeutic potential of red tea as sources of natural antioxidants, there is no study was carried out by using stem part because of no utility in preparation of tea. However it might contain bioactive compounds and might become the basis for identification and isolation of novel bioactive compounds. Therefore, this study was undertaken to evaluate antioxidant and oxidative DNA damage protecting property of red tea stem.

2. Materials and Methods

2.1 Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2-azino-bis-(3-ethylthiazoline-6-sulphonic acid), ferric chloride, Griess reagent, nitro blue tetrazolium (NBT), xanthine, xanthine oxidase, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), quercetin, sodium nitroprusside (SNP), hydrogen peroxide, were purchased from Sigma-Aldrich (St. Louis, MO, USA). We used pUC19 plasmid DNA obtained from (New England Biolabs, UK). Methanol used for preparation of extract was of highest analytical grade.

2.2 Plant material and extraction

Red tea stem (*Cameliasinensis*) was bought from a special tea store at Hawang market, Kyoungsan city, Republic of Korea in 2013. A total of 200 g of grinded stem of red tea was extracted 3 times for 24 h with the use of 95% ethanol in a laboratory shaker at ambient conditions. After filtration, solvent was evaporated under reduce pressure by using a rotary vacuum-evaporator (Laborota 4000, Heidolph, Germany) at 50°C. Thus after freeze-drying red tea extract (RTSE) was obtained and dissolved in the respective solvent and used for assessment of antioxidant capacity through various assays and oxidative DNA damage protecting potential.

2.3 *In vitro* antioxidant activity

2.3.1 Determination of DPPH[•] radical scavenging activity

DPPH[•] free radical scavenging activity of RTSE was estimated using the slightly modified method of Liyana-Pathirana, and Shahid (2005). 100 μ L of different concentrations (5, 10, 25,

50, 100 and 200 μ g/mL) of RTSE was added in different test tubes. A solution of 0.1 mM DPPH in methanol was prepared and 900 μ l of this solution was added to these tubes, and then shaken vigorously. The tubes were allowed to stand for 20 min at 37°C in dark. The control was prepared as above without any sample. The absorbance of the samples was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radicals by RTSE was calculated by using the following formula:

% radical scavenging activity = [(absorbance of control - absorbance of sample)/absorbance of control] \times 100. Quercetin was used as a standard antioxidant. All the concentrations were used in triplicate and the graph was plotted with the mean values.

2.3.2 Determination of ABTS^{•+} radical scavenging activity

The ABTS^{•+} radical scavenging activity of different concentration of RTSE was determined according to the procedure described by Re et al. (1999) with slight modifications. ABTS^{•+} was dissolved in water to a 7 mM concentration. ABTS^{•+} radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. Prior to assay, the solution was diluted with ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance of 0.700 \pm 0.02 in a 1 cm cuvette at 734 nm. 100 μ L of different concentrations (1.25, 2.5, 5, 10, and 20 μ g/mL) of RTSE was added to 900 μ l ABTS^{•+} free radical solution and incubated for 30 min. OD was taken at exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Radical scavenging activity was expressed as the inhibition percentage of free radicals by the RTSE and was calculated by using the following formula:

% radical scavenging activity = [(absorbance of control - absorbance of sample)/absorbance of control] \times 100. Quercetin was used as a standard antioxidant. All the concentrations were used in triplicate and the graph was plotted with the mean values.

2.3.3 Nitric oxide free radical scavenging activity

At physiological pH, SNP in aqueous solution spontaneously generates nitric oxide (Marcocci et al. 1994), which interacts with oxygen to produce nitric ions that can be determined by using Griess reagent. Scavengers of nitric oxide compete with oxygen to minimize the production of nitric oxide. SNP (10 mM) in 0.1 mM phosphate buffer saline (PBS) was mixed with

different concentrations (25, 50, 100, 200, and 400 $\mu\text{g/mL}$) of RTSE and incubated at 25°C for 150 min. After incubation, Greiss reagent A (2% sulphanilamide in 4% H_3PO_4 and Greiss reagent B (0.2% naphthylethylenediaminedihydrochloride) were added and incubated for 10 min. The OD of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was measured at 540 nm.

Radical scavenging activity was expressed as the inhibition percentage of free radicals by RTSE and was calculated by using the following formula:

%radical scavenging activity = [(absorbance of control - absorbance of sample)/absorbance of control] \times 100. Quercetin was used as a standard antioxidant. All the concentrations were used in triplicate and the graph was plotted with the mean values.

2.3.4 Super oxide free radical scavenging activity

The superoxide anion radical protecting activity of RTSE was estimated according to the method Kuthan *et al.*, (1986) with slight modification. The reaction mixture contained 0.25 mL of 0.8 mM xanthine in 0.1 mM potassium phosphate (pH 7.8), 0.15 mL of 0.5 mM nitro-blue tetrazolium (NBT) in 0.1 mM potassium phosphate (pH 7.8) and 0.09 mL of different concentrations (25, 50, 100, 200 and 400 $\mu\text{g/mL}$) of RTSE. After incubation at 25°C for 15 min, the reaction was started by adding 0.5 U/ml xanthine oxidase and reaction mixtures were kept at 25°C for 30 min and after that, by adding 0.5 mL of 1 N HCl, reaction of samples was stopped. The absorbance was measured at 560 nm.

Radical scavenging activity was expressed as the inhibition percentage of free radicals by RSTE and was calculated by using the following formula:

% radical scavenging activity = [(absorbance of control - absorbance of sample)/absorbance of control] \times 100. Quercetin was used as a standard antioxidant. All the concentrations were used in triplicate and the graph was plotted with the mean values.

2.3.5 Ferric-reducing/antioxidant power (FRAP) assay

The antioxidant capacity of RTSE was estimated according to the method described previously by Pulido *et al.* (2000). The absorbance of the reaction mixture was read at 593 nm. The values are expressed as mmol Fe (II)/g extract.

2.4. *In vitro* DNA damage protection efficiency of RTSE.

DNA damage protective activity of RTSE was checked on

pUC19 plasmid DNA (*E. coli* ER2272, New England Bio labs, UK). Plasmid DNA was oxidized with H_2O_2 +UV treatment in presence of different concentrations of RTSE and checked on 1% agarose according to Russo *et al.* (2000) after slight modifications.

In brief, the experiments were performed in a volume of 10 μL in a microfuge tube containing 186 ng of pUC19 plasmid DNA in 1 \times TE buffer (10 mM Tris - Cl and 1 mM EDTA); pH 8.0, H_2O_2 was added at a final concentration of 33 mM with and without 3 μL of different concentrations of RTSE. The reactions were initiated by UV-C irradiation and continued for 35 seconds with 22500 $\mu\text{W}/\text{cm}^2$ at 280 nm under room temperature on the surface of Trans-illuminator. After irradiation the reaction mixture (10 μL) along with gel loading dye (6X) was placed on 1% agarose gel for electrophoresis in 1 X TBE at 50 V, Untreated pUC19 plasmid DNA was used as a control. Gel was stained with aqueous solution of ethidium bromide and visualized under UV Transilluminator. Quercetin(50 $\mu\text{g/ml}$) was used as positive control.

Gel was scanned on Gel documentation system (Gel Doc- XR, Bio-rad, CA, USA). Bands were quantified by using Gel-Pro Analyzer, Media Cybernetics, USA.

2.5. Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using statistica (Statsoft Inc., Tulsa, USA). Values expressed are means of three replicatedeterminations \pm standard deviation.

3. Results and discussion

3.1. DPPH[•] radical scavenging activity

The DPPH[•] radical is a stable radical with a maximum absorbance at 517 nm that can readily undergo reduction by the antioxidant molecule. The radical scavenging capacity RTSE is showed in Figure. 1 expressed as a percentage reduction of the initial DPPH radical absorption by different concentration. In this assay, RTSE at 100 $\mu\text{g/mL}$ showed 68.84 % DPPH radical scavenging activity in a concentration dependent manner. Consequently, the more rapidly the absorbance decreases, the more potent antioxidant capacity of RTSE observed in terms of hydrogen atom or electron donating ability. At same concentrations quercetin used as a reference compound which showed 81.4% inhibitory effect against DPPH[•] radicals at 100 $\mu\text{g/mL}$ in a dose dependent manner. Therefore, due to presence of bioactive active molecules, RTSE could donate hydrogen

to free radicals, particularly to the lipid peroxides or hydroperoxide radicals that are the major propagators of the chain autoxidation of lipids, and to form non-radical species, resulting in the inhibition of propagating phase of lipid peroxidation (Bamforth et al. 1993).

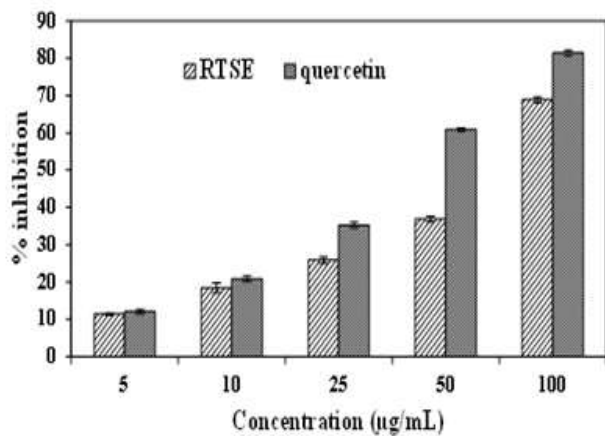


Figure 1. DPPH[•] radical scavenging activity of RTSE and standard compound quercetin.

Values are mean of three replicate determinations (n = 3) ± standard deviation.

3.2. ABTS^{•+} radical scavenging activity

Furthermore, the antioxidant capacity of RTSE was confirmed by ABTS^{•+} radical scavenging activity at different concentrations. As demonstrated in Figure. 2, RTSE showed 78.83 % inhibition of free radicals at 25 µg/mL. RTSE showed a potent scavenging activity toward ABTS radical cations in a concentration dependent manner, showing a direct role in quenching of free radicals as at 25 µg/mL concentration standard compound

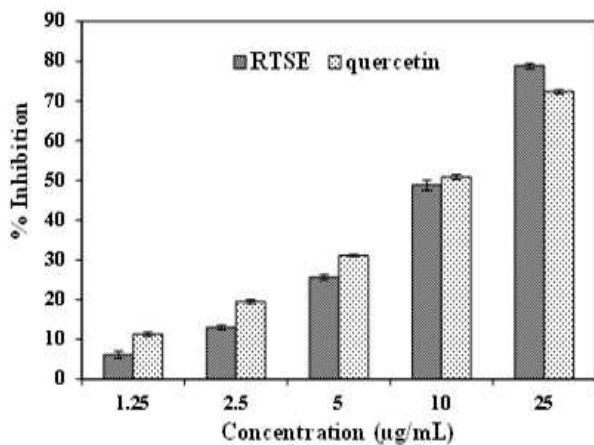


Figure 2. ABTS^{•+} radical scavenging activity of RTSE and standard compound quercetin.

Values are mean of three replicate determinations (n = 3) ± standard deviation. quercetin exhibited 72.53 % inhibition with 12.08 ± 0.65 IC₅₀ value (Table2). There by, RTSE could play a crucial role in scavenging free radicals via a chain breaking reaction, and may survey as potential nutraceutical.

3.3. Nitric oxide free radical scavenging activity

Nitric oxide radical is key molecule that involved in several physiological processes such as neural signal transmission, vasodilatation and regulation of blood pressure (Afanasev 2007). Due to emergence in level of nitric oxide radical during oxidative stress, it becomes the major culprit for numerous carcinomas and inflammatory conditions including arthritis, ulcerative colitis juvenile diabetes, and multiple sclerosis (Rabkin and Klassen 2007). During aerobic metabolism, nitric oxide molecule reacts with oxygen molecule and generates intermediates such as NO₂, N₂O₄ and N₃O₄. These products are highly genotoxic; causing deamination of purines, pyrimidines and denaturation of enzymes such as DNA ligase and DNA alkyltransferase (Lundberg et al. 2008).

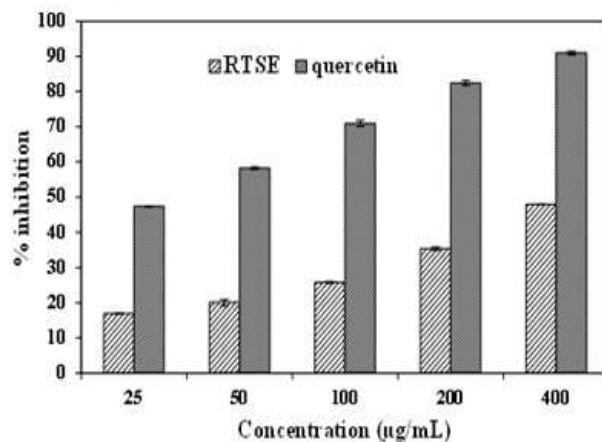


Figure 3. Nitric oxide free radical scavenging activity of RTSE and standard compound quercetin.

Values are mean of three replicate determinations (n = 3) ± standard deviation.

Table 1. FRAP activity of RTSE and standard compound quercetin.

Sample	FRAP mmol Fe(II)/mg extract
RTSE	2606.85 ± 18.7
Quercetin	8326 ± 115.2

Values are means of three replicate determinations (n = 3) ± standard deviation. FRAP-Ferric reducing antioxidant power (Concentration of substance having ferric-TPTZ reducing ability as equivalent to 1 µmol Fe (II)).

In this study, RTSE reduced the levels of nitrite by competing with oxygen to react with nitric oxide radical, a possible protective effect against oxidative damage. As shown in Figure. 3, extract showed most efficient inhibition of nitric oxide radicals with 47.92% at 400 $\mu\text{g/mL}$ in concentration-dependent manner. RTSE showed IC_{50} value of 404.38 ± 1.6 (Table 2). Standard compound, quercetin showed 90.98 % of inhibition at 400 $\mu\text{g/mL}$.

3.4. Super oxide free radical scavenging activity

In living beings superoxide radicals, a highly toxic species, are continuously produced in metabolic and physiological processes. Superoxide free radicals can generate more toxic and reactive free radicals i.e. hydroxyl radicals or singlet oxygen. Biological molecules such as DNA, lipids and proteins are prominent targets of these oxygen species and cause metabolic and cellular disturbances (Martinez-Cayuela, 1995). Superoxide radical protecting activity of RTSE was measured by the NBT which generates superoxide radicals. In Figure. 4, RTSE showed superoxide radical scavenging activity in dose dependent manner with 91.57% inhibition at 400 $\mu\text{g/mL}$. RTSE exhibited 93.67

± 2.7 $\mu\text{g/mL}$ IC_{50} value as compare to standard compound quercetin 4.32 ± 0.8 $\mu\text{g/mL}$ (Table 2).

On the basis of this assay, we can evidence that RTSE contains bioactive compounds which quench superoxide free radicals and can terminate radical chain reaction (Hamid *et al.* 2010).

3.5. Ferric reducing antioxidant power (FRAP) assay

Antioxidants can be explained as reductants, and in-activators of pro-oxidants (Siddhuraju and Becker, 2007). Some previous studies have also reported that the reducing power may serve as a significant indicator of potential antioxidant activity. Anti-oxidative activity has been proposed to be related to reducing power. Therefore, the antioxidant potential of RTSE was estimated for their ability to reduce TPTZ - Fe (III) complex to TPTZ - Fe (II). The ferric reducing ability of the RTSE showed that it has significant FRAP activity with 2606. 85 mmol Fe (II)/g extract (Table 1.) FRAP assay was used by several authors for the assessment of antioxidant activity of various food product samples (Pellegrini *et al.* 2003). Halvorsen *et al.* (2006) suggested most of the secondary metabolites are

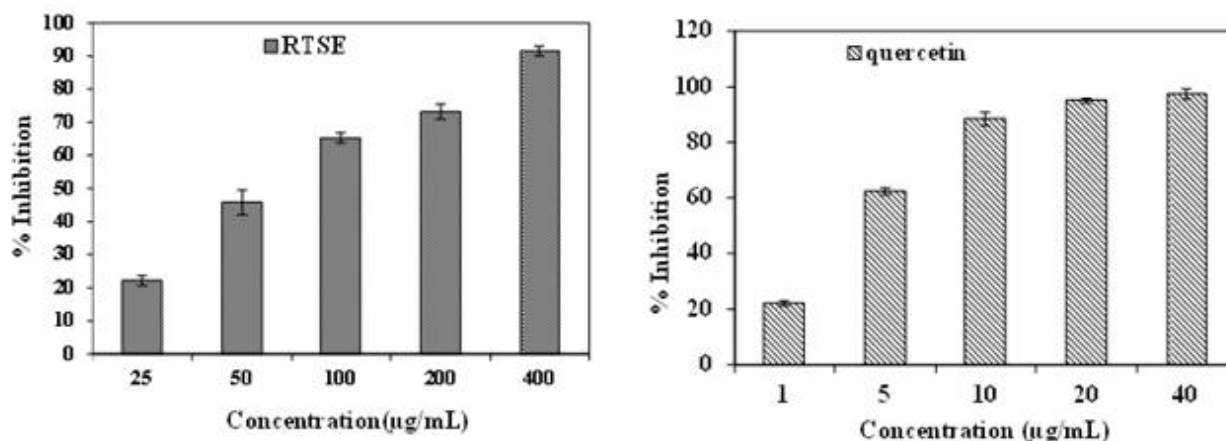


Figure 4. Superoxide free radical scavenging activity of RTSE and standard compound quercetin.

Values are mean of three replicate determinations ($n = 3$) \pm standard deviation.

Table 2. IC_{50} values of DPPH, ABTS, Nitric oxide and Superoxide radicals scavenging potential of RTSE and standard compound quercetin.

Sample	IC_{50} ($\mu\text{g/mL}$)			
	DPPH	ABTS	Nitric oxide	Super oxide
RTSE	68.88 ± 1.1	12.08 ± 0.65	404.38 ± 1.6	93.6 ± 2.7
Quercetin	48.41 ± 0.7	13.62 ± 0.4	29.34 ± 1.3	4.32 ± 0.8

Values are means of three replicate determinations ($n = 3$) \pm standard deviation.

redox-active compounds that will be picked up by the FRAP assay.

3.6. *In vitro* DNA damage protection efficiency of RTSE against hydroxyl radical

Hydroxyl radicals can be produced inside cells by various reactions as UV-induced decomposition of H_2O_2 used in the present study and can damage macromolecules such as lipids, proteins and DNA resulting in the onset of several skin diseases

including skin cancer (Halliwell and Gutteridge 1990). Therefore, in this study, we check the DNA damage protection capacity of RTSE on pUC19 plasmid DNA against hydroxyl radicals generated by UV photolysis of H_2O_2 . Figure. 5 shows electrophoretic pattern of pUC19 plasmid DNA in the presence or absence of different concentration (50, 100, 200, 400 and 800 $\mu\text{g/mL}$) of RTSE. On gel electrophoresis, pUC19 plasmid DNA shows two bands as in lane 1, fast moving prominent band corresponded to native supercoiled circular (Sc) DNA and slow moving, very fade band represents open circular DNA (Oc). After the exposure of UV irradiations in the presence of H_2O_2 , supercoiled DNA converted into prominent band of open circular DNA, due to scission by $\cdot\text{OH}$ free radicals, generated by UV photolysis of H_2O_2 (Lane 2). In biological system, $\cdot\text{OH}$ radicals, generated by the reaction between $O_2^{\cdot-}$ and H_2O_2 are major culprit for oxidative damage (Gutteridge, 1984).

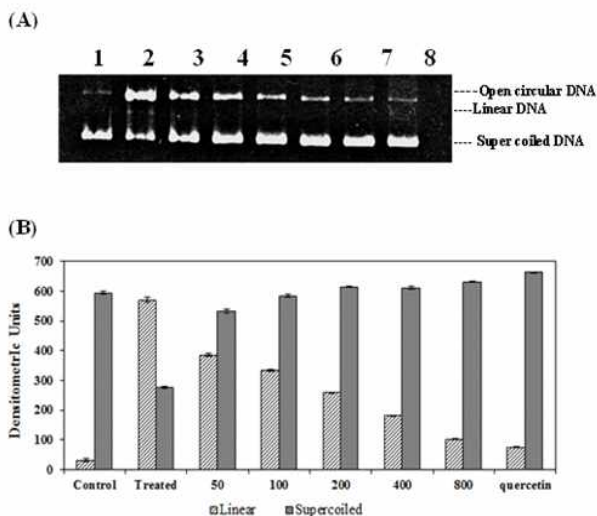


Figure 5. Oxidative DNA damage protecting potential of RTSE.

(A) Electrophoretic pattern of pUC19 DNA UV photolysis of H_2O_2 (33 mM) in the presence and absence of different concentrations of methanol extract of RTSE. Lane 1: (untreated), lane 2: treated (with UV & H_2O_2), lane 3-7: RTSE (50, 100, 200, 400 and 800 $\mu\text{g/mL}$ respectively), and lane 8: Quercetin (50 $\mu\text{g/mL}$) as a positive control.

(B) Densitometry analysis of supercoiled and open circular DNA after the UV-photolysis of H_2O_2 in the presence and absence of different concentration of RTSE (50-800 $\mu\text{g/ml}$).

Values are means of three replicate determinations ($n = 3$) \pm standard deviation.

But It was noted that pre-incubation of RTSE at different concentrations with plasmid DNA pUC19 reduce the extent of DNA damage induced by UV photolysis of H_2O_2 (lane 3-7) and showed significant protection of native super coiled circular (Sc) DNA in dose dependent manner. Quercetin (50 $\mu\text{g/mL}$)

was used as a positive control (lane 8) which reduced the amount of open circular DNA.

Densitometry analysis of supercoiled and linear DNA bands (Fig. 7) revealed that the densitometry units of super coiled circular (Sc) DNA in untreated 694.78 ± 4.12 reduced to 275.53 ± 3.78 after the exposure of UV- H_2O_2 . But densitometry units of super coiled (Sc) DNA were increased in presence of different concentrations (50, 100, 200, 400 and 800 $\mu\text{g/mL}$) of RTSE. At 800 $\mu\text{g/mL}$ RTSE showed protecting activity as densitometry unit of super coiled DNA (Sc) 631.76 ± 2.45 compared with quercetin treated super coiled DNA (662.98 ± 1.59)

This experimental evidence of this present study suggests that methanol extract of Red Tea stem might be good source of bioactive compounds those can to protect oxidative DNA damage from the detrimental effects of solar UV radiation.

Conclusion

In this study of RTSE exhibited good antioxidant and free radical scavenging capacities with different antioxidative values depending on the concentration and the measured antioxidant parameters. Moreover, RTSE showed the oxidative DNA damage protection against H_2O_2 -UV induced photolysis. These experimental evidences proved that RTSE also contain bioactive compounds, as sources of natural antioxidants. It might be a valuable basis for developing food additives or to prevent various oxidative stress induced disease. This study provided the evidence required for the utilization of RTSE for its possible use as a source of cost effective natural antioxidant.

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