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Research Article

IN VITRO EVALUATION OF FREE RADICAL SCAVENGING ACTIVITIES OF *PANAX GINSENG* AND *LAGERSTROEMIA SPECIOSA*: A COMPARATIVE ANALYSIS

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ABSTRACT

Safer antioxidants from plant origin are essential to prevent the progression of free radical mediated disorders. Current research is directed towards finding naturally-occurring antioxidants of plant origin that provided efficacy by additive or synergistic activities. *Panax ginseng* (ginseng) and *Lagerstroemia speciosa* (banaba) are suggested to have bioactive components having health benefits. The present study investigates on the free radical scavenging potential as well as total phenolic and flavanoid contents of aqueous extract of ginseng and banaba. They were analysed for total antioxidant activity by TEAC assay, superoxide, hydroxyl, hydrogen peroxide and nitric oxide radical scavenging activities as well as total phenolic and flavanoid contents. The calculated results with trolox standard curve, the TEAC value explain the antioxidant potential of the GE overhaul BLE. In superoxide and nitric oxide radical scavenging assays, both GE and BLE showed almost similar range of activities when compared to the reference compounds. BLE was found to be less effective in H_2O_2 and hydroxyl scavenging activities compared to GE. The present study provides an evidence that *Panax ginseng* extract even though having comparatively less amount of flavonoid and phenolic contents than leaf extract of *Lagerstroemia speciosa*, shows potential antioxidant and free radical scavenging activity.

Keywords: Ginseng, Banaba, TEAC, Hydroxyl, Phenolic and flavanoid contents

INTRODUCTION

Phytochemicals, especially phenolics are suggested to be the major bioactive compounds having health benefits. Clinical trials and epidemiological studies have established an inverse correlation between the intake of dietary antioxidants and the occurrence of oxidative stress related diseases. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals or prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans[1,2]. Reactive oxygen species including superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$), nitric oxide (NO) exert oxidative stress in the cells of human body rendering each cell to face about 10,000 oxidative hits per second[3,4]. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to development of degenerative diseases. Hence the rationale for the use of antioxidants is well established in prevention and treatment of diseases where oxidative stress plays a major aetiopathological role. Antioxidants may protect the body against ROS toxicity either by preventing the formation of ROS, by bringing interruption in ROS attack, by scavenging the reactive metabolites or by converting them to less reactive molecules[5,6]. The antioxidant capacity gives information about the duration while the activity describes the starting dynamics of antioxidant action[7]. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in prevention of diseases.

Panax ginseng C A MEYER, (Araliaceae) also called Asian Ginseng, is a well known oriental crude drug used in Korea and China, the root of the perennial herb, *Panax ginseng* which contains a series of tetracyclic triterpenoid saponins (ginsenosides) as active ingredients[8,9]. In addition, several investigations strongly support the evidence that ginseng root possess anti-diabetic properties, such as inhibition of intestinal glucose absorption, increase in energy expenditure, improving sensitivity to insulin and stimulation of sugar metabolism[10,11]. It is considered as a tonic cum adaptogenic agent that helps to enhance physical performance (including sexual), promotes vitality and stimulate metabolic function. *Lagerstroemia speciosa* (L.) Pers. (Lythraceae) is called 'banaba' in Tagalog language in Philippines, is another tropical plant found in many parts of South East Asia including Philippines, Vietnam, Malaysia and southern China. Despite its availability in several countries, only few countries are using dried and shredded banaba leaves as a beverage and folk medicine for the treatment, prevention of diabetes and kidney diseases. Hypoglycemic activity of banaba extract was studied in genetically induced diabetic mice and results indicate presence of "insulin- like principle", in leaf extract[12]. Recently, the scientific interest in banaba's potential has resurfaced and has become relatively popular in the form of health promoting tea products in Eastern Asia and the United states.

The objective of the present study was to perform a comparative evaluation on the antioxidant potential and free radical scavenging activity of aqueous extracts of *Panax ginseng* and *Lagerstroemia speciosa*.

MATERIALS AND METHODS

Chemicals

2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid (TROLOX) were obtained from Sigma Aldrich. Gallic acid, butylated hydroxy toluene (BHT), trichloroacetic acid (TCA), deoxy-ribose, ferric chloride, nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), sulfanilamide, naphthylethylene diamine dihydrochloride, quercetin and ascorbic acid were obtained from Merck India Ltd, Mumbai.

Plant Material

Standardized aqueous extract of *Panax ginseng* (Asian Ginseng, GE) (Batch No: GPE80-061108) and leaf extract of *Lagerstroemia speciosa* (Banaba, BLE) (Batch No: BLP01-051608) were procured from Changsha Botaniex Inc, China. Analysis of active components of plant extracts was performed by Changsha Botaniex and the results showed 80% ginsenosides in the GE and 1% corosolic acid fraction in BLE by using high performance liquid chromatography and UV spectrophotomeric analysis.

Trolox equivalent antioxidant capacity (TEAC Assay)

The TEAC assay was determined by adopting the method of Re etal., (1999)[13], using the radical cation $ABTS^{+\bullet}$. The $ABTS^{+\bullet}$ radical cation was pregenerated by mixing 7mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) while incubating for 12–16 h in dark at room temperature until the reaction was complete and the absorbance was stable. The working solution of $ABTS^{+\bullet}$ was obtained by diluting the stock solution in phosphate buffer saline (PBS, pH-7.4) to give an absorption of 0.70 ± 0.02 at λ = 734 nm. About 3ml of ABTS working solution was mixed with 10µl of the trolox standard (50mM) or the test samples (0.05-10 mg/ml)

and the absorbance was measured at every 10 sec for 6 min at 734 nm. Each extract was analysed in triplicate.

The TEAC value was calculated by means of area under curve (AUC_{sample}) and regression coefficient of trolox ($r.c._{trolox}$). The calculation of the area under the curve was performed for one sample dilution which had a final percentage inhibition between 20% and 80%. The antioxidant activity of the plant extracts was expressed as µmol of TE per gram weight of the extract[14].

Superoxide scavenging assay

The activity was evaluated using nitro blue tetrazolium (NBT) reduction method given by Nishikimi et al., (1972)[15]. The reaction mixture consisted of 1ml of NBT solution (156µM) and sample solution at different concentrations. The reaction was started by adding 100µl of phenazine methosulfate solution (60µM, PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 25°C for 5 min and the absorbance at 560mm was measured against blank. Ascorbic acid was used as the standard.

$$\text{Superoxide scavenging activity (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100$$

Where, Abs (control): Absorbance of the control and

Abs (test) : Absorbance of the extracts/standard.

H_2O_2 radical scavenging assay

The ability of the extract to scavenge hydrogen peroxide was determined according to the method given by Ruch et al., (1989)[16]. A solution of hydrogen peroxide (2mmol/l) was prepared in phosphate buffer (pH 7.4). Extracts (1–10 µg/ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound.

$$H_2O_2 \text{ activity (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100$$

Where, Abs (control): Absorbance of the control and

Abs (test) : Absorbance of the extracts/standard.

Hydroxyl radical scavenging assay

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al., (1987)[17]. Stock solutions of EDTA (1mM), $FeCl_3$ (10 mM), ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml of EDTA 0.01 ml of $FeCl_3$, 0.1 ml of H_20_2, 0.36 ml of deoxyribose,1.0 ml of plant extract (1-10µg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1hr. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA to develop the pink chromogen, measured at 532 nm.

$$\text{Hydroxyl scavenging activity (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100$$

Where, Abs (control): Absorbance of the control and

Abs (test) : Absorbance of the extracts/standard.

Nitric oxide scavenging assay

The activity was measured according to the modified method of Sreejayan and Rao, (1997)[18]. To 4ml of the extract having different concentrations (1-50 µg/ml), 1ml of sodium nitroprusside (SNP) solution (5mM) was added and incubated for 2hr at 27°C. An aliquot (2ml) of the incubation solution was removed and diluted with 1.2ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore was read immediately at 550nm and compared with standard, BHT.

$$\text{Nitric oxide scavenging activity (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100$$

Where, Abs (control): Absorbance of the control and

Abs (test) : Absorbance of the extracts/standard.

Total reducing ability

The reducing power of the extracts was determined according to the method of Oyaizu (1986)[19]. Different concentrations of the aqueous extract (1–10µg/ml) in 1.0 ml of deionised water were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min and later 2.5ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3,000rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm.

Total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu reagent according to the method of Singleton et al., 1999)[20]. About 1ml of plant extract was mixed with 5 ml of Folin-ciocalteu reagent (1:10) followed by 4 ml of Na_2CO_3 (0.7M). Subsequently, the mixture was shaken for 2 h at room temperature and absorbance measured at 760nm. All tests were performed in triplicate. The concentration of total phenolic compounds was determined as µg gallic acid equivalents using the following equation obtained from a standard gallic acid graph:

Absorbance = 0.001 x pyrocatechol (µg) + 0.0033

Total flavanoid content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to the known method of Zhishen et al., (1999)[21] using quercetin as a standard. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by 0.03 ml $NaNO_2$ (5%) and incubated for 5 min at 25°C. Later 0.03 ml $AlCl_3$ (10%) was added and further after 5 min, the reaction mixture was treated with 0.2 ml (1mM) NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All tests were performed six times. The flavonoid content was calculated from a quercetin standard curve.

Statistical analysis

Results are shown as the mean ± SE of six measurements. Statistical analysis was performed using one way analysis of variance (ANOVA) with LSD post hoc at $P<0.01$ by SPSS software 15.0. Linear regression analysis was done for calculating IC_{50} values and graphical preparations were done using OriginPro software 7.0.

RESULTS

For TEAC assessment, the standard curve of trolox standard on ABTS was calculated ($R^2 = 0.902$), and the total antioxidant activity for GE was 0.008 µmol of TE /g wt extract and BLE was 0.012 µmol of TE /g wt extract (Fig 1).

Percentage inhibition on superoxide radical generation by both GE and BLE was found increasing in a dose dependent manner, showing the IC_{50} value of 3.18 µg/ml and 6.15µg/ml, respectively, when compared to the IC_{50} value 3.35 µg/ml of ascorbic acid, the standard (Fig 2).

With regard to hydrogen peroxide scavenging activity, IC_{50} value of GE (3.14 µg/mL) was less than BLE (15.14 µg/ml) indicating BLE as a poor scavenger of hydrogen peroxide compared to GE. IC_{50} value of ascorbic acid, the standard was 1.23 µg/ml (Fig 3).

Hydroxyl radical scavenging assay shows the ability of the extracts and standard BHT to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The IC_{50} values (Table 1) of GE, BLE and standard in this assay were 2.15 µg/ml, 7.58 µg/ ml and 2.44 µg/ ml, respectively. The IC_{50} value of the GE was almost same that of standard, while BLE had higher value showing least ability in hydroxyl radical scavenging (Fig 4).

As a function of nitric oxide scavenging activity, the different concentrations of GE showed decrease in percentage inhibition while increase in the concentrations with IC_{50} value 6.09 µg/ml, while that of BLE was 1.39 µg/ml. BHT was used as the reference compound and 0.15 µg/ml of BHT was needed for the 50% inhibition (Fig 5).

Based on the principle that Fe^{3+} was transformed to Fe^{2+} in the presence of the extract with the reference compound, BHT, the reductive ability of phytoextracts were measured. The total reduction ability of both GE and BLE were more or less similar and the absorbance at 700nm exhibited an increase in a dose dependent manner (Fig 6).

Since phenolic compounds may contribute directly to antioxidative action, the total phenolic content measured was 66.83 ± 0.268 and 72.3 ± 0.293 mg/ml gallic acid equivalent per 100 mg plant extract for GE and BLE respectively. The total flavanoid content of ginseng (80% ginsenosides) and banaba (1% corosolic acid) were 120.35 ± 0.03 and 150.5 ± 0.012 mg/ml quercetin equivalent per 100 mg plant extract, respectively.

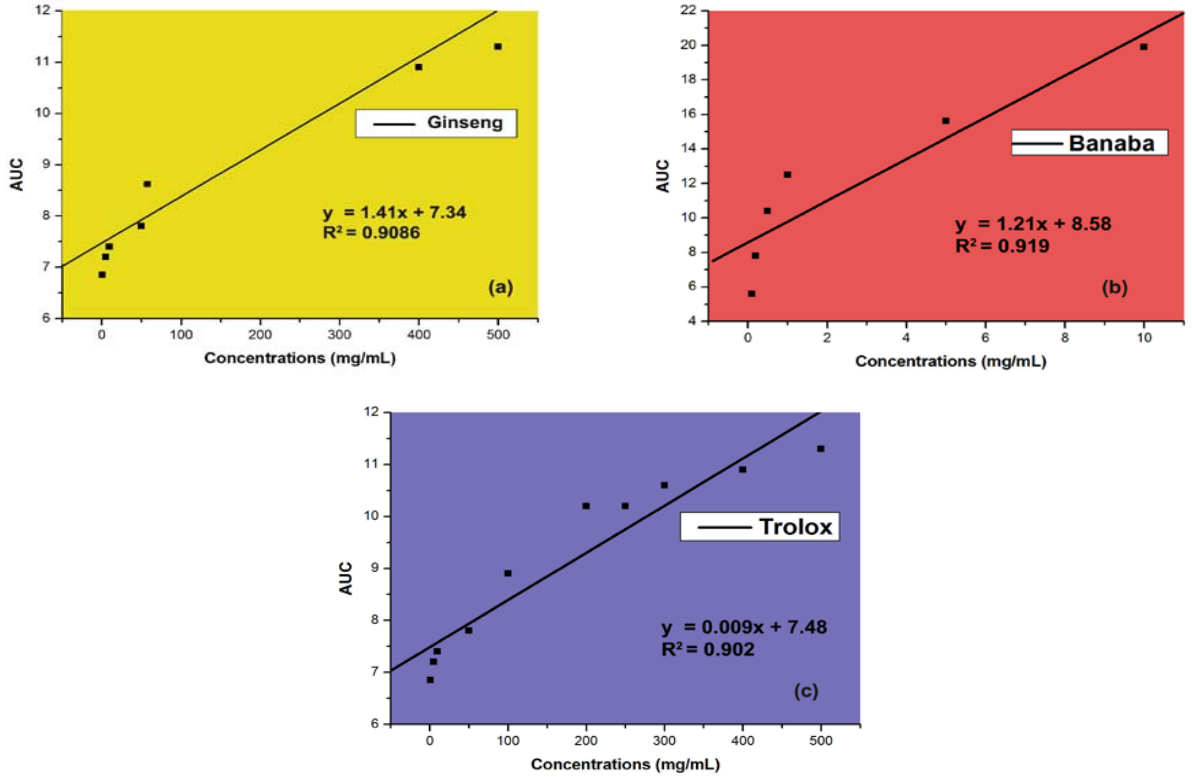

Fig. 1: TEAC assay results showing ABTS radical scavenging activity: Correlation between area under curve (AUC) and different concentrationsof ginseng root (a) and banaba leaf extract (b) as well as the reference compound, trolox (c)

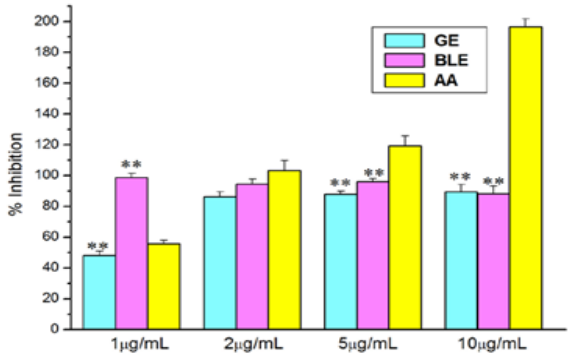

Fig. 2: Superoxide radical scavenging activities of ginseng (GE), banaba extract (BLE) and the standard, ascorbic acid (AA). The data represent the percentage inhibition on superoxide radicals. All data expressed as mean ± SE (n=6). **P<0.01 compared to respective doses of standard

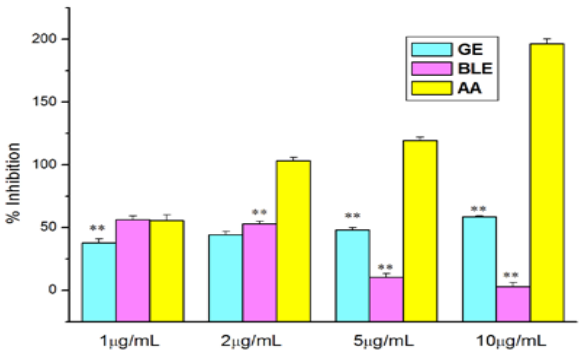

Fig. 3: Hydrogen peroxide scavenging activities of ginseng (GE), banaba extract (BLE) and the standard, ascorbic acid (AA). The data represent the percentage inhibition on hydrogen peroxide. All data expressed as mean ± SE (n=6). **P<0.01 compared to respective doses of standard

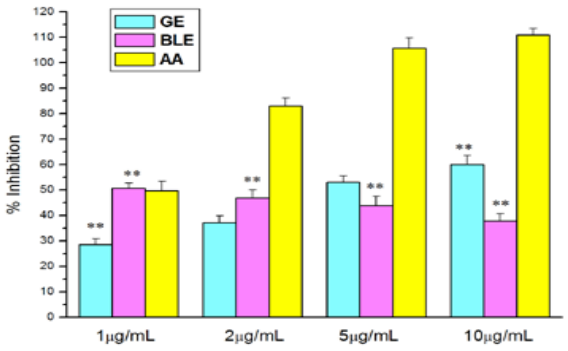

Fig. 4: Hydroxyl radical scavenging activities of ginseng (GE), banaba extract (BLE) and the standard, ascorbic acid (AA). The data represent the percentage inhibition on hydroxyl radicals. All data expressed as mean ± SE (n=6). **P<0.01 compared to respective doses of standard.

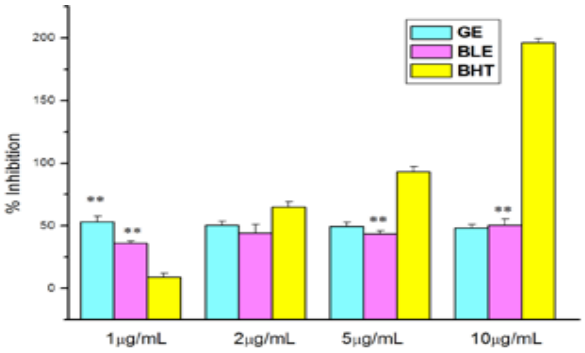

Fig. 5: Nitric oxide radical scavenging activities of ginseng (GE), banaba extract (BLE) and the standard, ascorbic acid (AA). The data represent the percentage inhibition on nitric oxide radicals. All data expressed as mean ± SE (n=6). **P<0.01 compared to respective doses of standard.

DISCUSSION

Dietary component who can either sacrificially scavenge ROS/RNS to stop radical chain reactions, considered as primary chain-breaking antioxidants or free radical scavengers (FRS), or it can inhibit the reactive oxidants from being formed in the first place, considered as secondary or preventive antioxidants[22]. Primary antioxidants, when present in trace levels, the response of antioxidants to different radical or oxidant sources may be different. Therefore, no single assay accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system[23], at least two methods should be employed in order to evaluate the total antioxidant activity[24], due to various oxidative processes. The antioxidant activity of a compound has been attributed to various mechanisms viz. prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging ability.

In the total antioxidant activity, $ABTS^{\bullet\bullet}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and in the presence of the plant extract or trolox, preformed cation radical gets reduced and the remaining radical cation concentration after reaction with antioxidant compound was then quantified[25]. Both the calculated results with trolox standard curve and the TEAC value explain the antioxidant potential of the GE overhaul BLE. The TEAC assay measures the antioxidant capacity of the parent compound plus that of reaction products and often used to rank antioxidants and for the construction of structure-activity relationships[26].

Superoxide anion radicals are produced endogenously by flavoenzymes like xanthine oxidase, which converts hypoxanthine and subsequently to uric acid. The superoxide anion radicals are derived in PMS-NADH-NBT system, where the decrease in absorbance at 560nm with both antioxidants[27] indicates the consumption of superoxide anion in the reaction mixture, thereby exhibiting a dose dependent increase in superoxide scavenging activity. GE with IC_{50} value of 3.18 µg/ml showed strong super oxide radical scavenging activity than BLE; however the values remain below ascorbic acid. H_2O_2 was considered poorly reactive because of its weaker oxidizing and reducing capabilities. Biologically, it acts as a toxicant to the cell by converting itself into hydroxyl radical in the presence of metal ions and superoxide anion and also produces singlet oxygen through reaction with superoxide anion or with hypochlorous acid (HOCl) or chloramines in living systems. Hydrogen peroxide can degrade certain heame proteins, such as heamoglobin, to release Fe ions[28] and therefore the hydroxyl radical scavenging activity of phytoextracts was measured. In this study, BLE in an increasing concentration (1µg -10µg) was less effective in H_2O_2 scavenging activity whereas GE was found more effective.

The effect of GE and BLE on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the hydroxyl radical scavenging assay. The Fenton reaction generates hydroxyl radicals ($OH^{\bullet}$) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component and may cause to DNA fragmentation and DNA strand breakage[29]. The IC_{50} values of GE, BLE and standard in this assay were 2.15µg/ml, 7.58 µg/ ml and 2.44 µg/ ml, respectively. The IC_{50} value of the GE was almost that of standard, while BLE had higher value showing least ability in hydroxyl radical scavenging.

Nitric oxide (NO), being a potent pleiotropic mediator in physiological processes and a diffusible free radical in the pathological conditions, reacts with superoxide anion and form a potentially cytotoxic molecule, the 'peroxynitrite (ONOO-)'. Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant[30]. The main route of damage is the nitration or hydroxyl-lation of aromatic compounds, particularly tyrosine. Under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid and responsible for oxidative damage of proteins[31,32] in living systems. BLE was found to be less scavenging on nitric oxide (IC_{50} value-1.39µg/ml), when compared to dose independent ability of GE (IC_{50} -6.09 µg/ml) and to ascorbic acid, the reference compound. Studies were made on total reduction ability of Fe^{3+} to Fe^{2+} transformation in the presence of both extracts and found increasing in showing reduction ability in a dose dependent manner, with increasing concentrations. Since the reducing capacity of a compound serve as a significant indicator of its potential antioxidant activity, the reducing ability of phytoextracts are measured in this study. The antioxidant activity has been reported to be concomitant with development of reducing power[33].

Phenolic compounds are known powerful chain breaking antioxidants[34], important plant constituents because of their scavenging ability due to their hydroxyl groups and contribute directly to antioxidative action[35]. Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants[37].It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily with a diet rich in fruits and vegetables[36]. In the present study,the total phenolic contents were 66.83 ± 0.268 and 72.3 ± 0.293 mg/ml gallic acid equivalent per 100 mg plant extract for GE and BLE respectively. Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants[37].

In conclusion, the present study provides an evidence that *Panax ginseng* (Asian Ginseng, GE) extract even though having comparatively less amount of flavonoid and phenolic contents than leaf extract of *Lagerstroemia speciosa* (Banaba, BLE), shows potential antioxidant and free radical scavenging activity. These *in vitro* assays demonstrate that plant extracts are important sources of natural antioxidants, which might be useful as preventive agents against oxidative stress and hence currently the evaluation of *in vivo* antioxidant activity of these extracts are in progress. To elucidate

the prime source of antioxidant properties further studies should be carried out with isolate active principles.

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