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

SAUMYA S M & MAHABOOB BASHA P*
Department of Zoology, Bangalore University, Bangalore - 560 056, India Email: pmbashabub@rediffmail.com

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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 flavonoid 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 over haul 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 H2O2 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 lipoperoxidation and scavenger free radicals or prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans\(^5\). Reactive oxygen species including superoxide (O\(2^-\)), hydrogen peroxide (H\(2\)O\(_2\)), 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 aetiological 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\(^8\). 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\(^9\)\(^10\). 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 banana extract was studied in genetically induced diabetic mice and results indicate presence of ‘insulin- like principle’, in leaf extract\(^9\). Recently, the scientific interest in banaba’s potential has resurfac ed 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’-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxonic acid (Trolox) were obtained from Sigma Aldrich. Gallic acid, butylated hydroxy toluene (BHT), trichloracetic 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 spectrophotometric analysis.

Trolox equivalent antioxidant capacity (TEAC Assay)

The TEAC assay was determined by adapting the method of Re et al., (1999)\(^11\) using the radical cation ABTS\(^+\). The ABTS\(^+\) radical cation was regenerated 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\(^+\) 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 A = 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 (AU(AUCsample)) 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 1 ml of NBT solution (15 μg/mL) and sample solution at different concentrations. The reaction was started by adding 100 μL of phenazine methosulfate solution (60 μM, PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 25°C for 5 min and the absorbance at 560 nm was measured against blank. Ascobic acid was used as the standard.

\[
\text{Superoxide scavenging activity} (%) = \frac{\text{Abs(test)} - \text{Abs(control)}}{\text{Abs(control)}} \times 100
\]

Where, Abs [control]: Absorbance of the control and
Abs (test) : Absorbance of the extracts/standard.

**H₂O₂ 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 (2 mmol/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.

\[
\text{H₂O₂ activity} (%) = \frac{\text{Abs(control)} - \text{Abs(sample)}}{\text{Abs(control)}} \times 100
\]

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 (1 mM), FeCl₃ (10 mM), ascorbic acid (1 mM), H₂O₂ (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml of EDTA 0.01 ml of FeCl₃, 0.1 ml of H₂O₂, 0.36 ml of deoxyribose, 1.0 ml of plant extract (1–10 μg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 hr. About 1 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.

\[
\text{Hydroxyl scavenging activity} (%) = \frac{\text{Abs(control)} - \text{Abs(sample)}}{\text{Abs(control)}} \times 100
\]

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 4 ml of the extract having different concentrations (1–50 μg/mL), 1 ml of sodium nitroprusside (SNP) solution (5 mM) was added and incubated for 2 hr at 27°C. An aliquot (2 ml) of the incubation solution was removed and diluted with 2.2 ml of Griess reagent (1% sulfanilamide in 5% H₂PO₄ and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore was read immediately at 550 nm and compared with standard, BHT.

\[
\text{Nitric oxide scavenging activity} (%) = \frac{\text{Abs(control)} - \text{Abs(sample)}}{\text{Abs(control)}} \times 100
\]

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 (1–10 μg/mL) in 1.0 ml of denoised water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min and later 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with FeCl₃ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm.

**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 1 ml of plant extract was mixed with 5 ml of Folin–Ciocalteu reagent (1:10) followed by 4 ml of Na₂CO₃ (0.7 M). Subsequently, the mixture was shaken for 2 h at room temperature and absorbance measured at 760 nm. 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:

\[
\text{Absorbance} = 0.001 \times \text{pyrocatechol} [\mu g] + 0.0033
\]

**Total flavonoid content**

The total flavonoid content was determined with aluminum chloride (AlCl₃) according to the known method of Zhishen et al., (1999) using quercetin as a standard. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by 0.03 ml NaNO₂ (5%) and incubated for 5 min at 25°C. Later 0.03 ml AlCl₃ (10%) was added and further after 5 min, the reaction mixture was treated with 0.2 ml (1 mM) 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 were 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 IC₅₀ values and graphical preparations were done using OriginPro software 7.0.

**RESULTS**

For TEAC assay, the standard curve of trolox standard on ABTS was calculated (R² = 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 IC₅₀ value of 3.18 μg/mL and 6.15 μg/mL, respectively, when compared to the IC₅₀ value 3.35 μg/mL of ascorbic acid, the standard (Fig 2).

With regard to hydrogen peroxide scavenging activity, IC₅₀ 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. IC₅₀ 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 Fe³⁺-EDTA-ascorbic acid and H₂O₂ reaction mixture. The IC₅₀ values (Table 1) of GE, BLE and standard in this assay were 2.15 μg/mL, 7.59 μg/mL and 2.44 μg/mL, respectively. The IC₅₀ value of the GE was almost same that of standard, while BLE had higher value showing less 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 flavonoid 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.

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**Fig. 1:** TEAC assay results showing ABTS radical scavenging activity: Correlation between area under curve (AUC) and different concentrations of 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
The effect of GE and BLE on the inhibition of free radical-mediated deoxyribonucleic acid (DNA) damage was assessed by means of the hydroxyl radical scavenging assay. The Fenton reaction generates hydroxyl radicals (•OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component and may cause to DNA fragmentation and DNA strand breakage. The IC_{50} values 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 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. The main route of damage is the nitration or hydroxyl--amination 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 in living systems. BLE was found to be less scavenging activity compared to a dose independent ability of GE (IC_{50} 6.09 µg/ml) and to ascorbic acid, the reference compound. Studies 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 phytostantrates are measured in this study. The antioxidant activity has been reported to be concomitant with development of reducing power.

Phenolic compounds are known powerful chain breaking antioxidants, important plant constituents because of their scavenging ability due to their hydroxyl groups and contribute directly to antioxidative activity. Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human, when ingested up to 1g daily with a diet rich in fruits and vegetables. In the present study, the total phenolic contents were 66.83 ± 0.268 and 72.3 ± 0.293 mg/g/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.

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.
REFERENCES


