In vitro determination of anti-oxidant activities of *Garuga pinnata* Roxb

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Abstract: Medicinal plants are alternative source for the anti-oxidants which don’t possess side effects and more potent than synthetic drugs. The present investigation was carried out to evaluate anti-oxidant properties of *Garuga pinnata* leaves, stem bark and fruit which are extracted with methanol. The anti-oxidant properties of these plant extracts of various concentrations (50, 150 and 250 µg/ml) was determined by using various methods like DPPH radical scavenging, hydroxyl radical scavenging, superoxide anion, hydrogen peroxide and nitrous oxide the results are compared with known standards. All the plant extracts exhibited notable inhibition of free radicals generation at all concentrations tested. The highest scavenging activity was shown by stem bark extract followed by leaf and fruit. A systemic investigation was also carried out to evaluate to identify the total phenolics and flavanoid to explore relation with anti-oxidant properties.

Keywords: anti-oxidant; flavanoids; medicinal plants; Phenolic compounds; scavenging activity.

Introduction

Reactive oxygen species (ROS) are free radicals such as superoxide anion radical, hydrogen peroxide, hydroxyl radical etc., are generated from mitochondrial respiratory chain of living organisms and have been implicated in various metabolic disorders which includes cancer, diabetic mellitus, haemorrhagic shock, acute hypertension and atherosclerosis (Hemnani and Parihar 1998). Many synthetic antioxidants are available which can effectively prevent rancidity of food materials (Newkirk et al.1993). However, the efficacy of these compounds in animal studies and clinical trials has been unsatisfactory due to dose restrictive toxicity. In view of that, there is a shift towards identifying natural compounds which do not have undesired effects (Jovanovic and Simic 2000). Owing to the bio integrity and ease in isolation, the naturally derived antioxidants are preferred more in comparison to synthesized compounds (Ghanta et al. 2007).

It has been estimated that approximately two-thirds of anticancer drugs approved worldwide up to year 2010 were derived from plant sources (Vickers 2002).

*Garuga pinnata*, belongs to family *Burseraceae* commonly called as kakad is Chhattisgarh, and kondavepa in Andhra Pradesh, India and seen to bloom throughout India and most commonly grows in forests. This specific Indian medicinal plant reaches to a medium height, growing as a deciduous tree. The stem bark of this plant appears thick, soft, greyish or brown from the outside and red when stem is peeled off. The leaves are imparipinnate and are arranged at alternate intervals, varying from 15 to 45 centimetres in length. The presence of traditional medicinal properties, *Garuga pinnata*, made a good contribution for the development of alternative medicine in medicinal plant therapy (Kathad et al. 2010; Mohammad et al. 2008; Jain et al. 1991).
In contrast, to medicinal properties of this plant, a systemic investigation was undertaken to evaluate anti-oxidant effect of *Garuga pinnata* leaves, stem bark and fruit which were extracted with methanol.

**Materials and methods**

**Plant material**

Leaves, fruits and stem bark of *Garuga pinnata* were collected from Rampet village, Warangal district, Andhra Pradesh, India. Plant has been authenticated by Professor V.S Raju, Taxonomist, Department of Botany, Kakatiya University warangal and parts of this plant were maintained in the Department of Biotechnology, Chaitanya Postgraduate college.

**Chemicals**

Sulphanilamide, Folin- Ciocalteu reagent, Sodium nitroprusside, Nitro blue tetrazolium (NBT), tannic acid were procured from SRL Mumbai. Catechin, 2,2-diphenyl-1-picrylhydrazil (DPPH) was purchased from Sigma-Aldrich Chemical Co. All the other chemicals and reagents purchased were of research grade.

**Extraction procedure**

Leaves, fruits and stem bark were chopped in to smaller fragments, shade dried and grinded in homogenizer in to coarse powder. The 100 g of each powdered material is extracted with methanol and concentrated under rotavapour at 40-50 °C. Further on, the extracts of leaves, fruits and stem bark were abbreviated as GPL, GPF and GPSB for better understanding.

**Reagents preparation**

Preparation of Nash Reagent: 75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and distilled water was added to total volume of 1 L.

Preparation of Griess Reagent: 1% sulphanilamide, 2% Phosphoric acid and 0.1% N-1-napthylethlyenediamine di hydrochloride in distilled H$_2$O.

Preparation of Ferrous EDTA: 0.13% ferrous ammonium sulfate and 0.26% EDTA in distilled H$_2$O.

**Determination of total phenol content**

The amount of total phenolics in extract was determined with Folin–Ciocalteu reagent by following described method with slight modifications (Singleton and Rossi 1965). Separately, 1 ml of each methanolic extract of GPL, GPF and GPSB of different concentrations (50,100 and 250 µg/ml) and standard solution of tannic acid (10, 15, 25 µg/ml) was added separately to 100 ml volumetric flask separately, that contained about 60 ml distilled water and followed by the addition of 5 ml of Folin–Ciocalteu reagent. The content was mixed thoroughly and kept constant for about 10 min. To this, add 15 ml Na$_2$CO$_3$ (20 %) and make up to 100 ml using distilled water. The mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm using a UV-visible spectrophotometer.

**Total flavonoid assay**

Flavonoid content was measured by aluminium chloride colorimetric assay with slight modification (Kartashova and Sudos 1997). 1 ml of each GPL, GPF and GPSB methanolic extracts with different concentrations (50,100 and 250 µg/ml) and standard solution of catechin (10, 15, 25 µg/ml) was added separately to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture 0.3 ml of 5% NaNO$_2$ was added, followed by the addition of 0.3 ml of 10% AlCl$_3$ after 5 min. After incubation period of 6 min 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm.

**Anti-oxidant assay**

**DPPH radical scavenging activity**

Free radical scavenging capacity of GPL, GPF and GPSB methanolic extract was determined by using DPPH as described elsewhere (Naznin and Hasan Nur 2009). DPPH radical scavenging activity was done by serial dilution
by taking diluted methanol (1:20) as standard. 10 ml of various diluted methanolic extracts of various concentrations (50,100 and 250 µg/ml) were added to 1 ml DPPH solution (0.004%) and incubated for 10 min at room temperature. Absorbance of test and reference standard, ascorbic acid was measured at 517 nm. The amount of DPPH scavenging was calculated by using the above formula: % DPPH radical scavenging = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100

**Hydroxyl radical activity**

Hydroxyl radical activity was measured by the method described elsewhere (Klein et al. 1981). 1 ml of each GPL, GPF and GPSB methanolic extracts of various concentrations (50,100 and 250 µg/ml) were placed in tubes and evaporated to dryness. 1 ml of ferrous-EDTA (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of 0.018% EDTA, 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) and 0.5 ml of freshly prepared 0.22% ascorbic acid were added to each tube. The tubes were capped tightly and heated in a water bath at 80-90°C for 15 min. The reaction was terminated by adding 1 ml of ice cold TCA (17.5% w/v). Latter 3 ml of Nash reagent was added to each tube and left at room temperature for 15 min for color development. The intensity of color formed was measured at 412 nm against the reagent blank. The percentage inhibition was compared with standard and test compounds.

**Nitric oxide scavenging activity**

Nitric oxide scavenging activity was determined by the method described by (Govindarajan et al. 2003). Briefly, 5 mM sodium nitroprusside was prepared in phosphate buffered saline and mixed with different concentrations of GPL, GPF and GPSB methanolic plant extracts (50,100 and 250 µg/ml) followed by incubation at 25°C for 30 min. A control without the extracts but with equivalent amounts of methanol was taken. After 30 min, 1.5 ml of incubated solution was removed and diluted with 1.5 ml of Griess reagent. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequence coupling with N-1-naphthylethylenediamine dihydrochloride was measured at 546 nm and percentage scavenging activity was measured with reference standard.

**Superoxide radical scavenging activity**

The super oxide radical scavenging activity was measured by the method described by (Nishikimi et al. 1972; Yen and Duh 1994). 1 ml of each GPL, GPF and GPSB methanolic extracts of various concentrations (50,100 and 250 µg/ml) were mixed with 1 ml of nitro blue tetrazolium (NBT) solution (156 mM NBT in phosphate buffer of pH 7.4) and 1 ml NADH in phosphate buffer of pH 7.4. The reaction was initiated by adding 100 µl of phenazine methosulfate (PMS) solution (60 mM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm against blank sample and compared with standards. Decreased absorbance of reaction mixture indicated increased superoxide anion scavenging activity. The percentage of inhibition of superoxide anion generation was calculated using the following formula:

% inhibition = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100

**Scavenging of hydrogen peroxide**

Scavenging of hydrogen peroxide was measured by the method described elsewhere (Ilhami et al. 2005). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 1 ml of each GPL, GPF and GPSB methanolic plant extract of different concentrations (50,100 and 250 µg/ml) were added to 0.6 ml of 40 mM hydrogen peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of plant extract and standard compounds was calculated using the following formula:

% scavenged [H₂O₂] = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100
Statistical analysis

The data from the experiments are presented as mean_S.E.M (n=3). Student’s t-test was used for statistical analyses (SAS software 9.0). Values were considered statistically significant when \( p<0.001 \).

Results

Total phenol content

Total phenolic content of \( G. \ pinnata \) stem bark is significantly increased with increase in the concentration of plant extract. The percentage yield of phenolic content was found to be 48, 58, 71 and 56, 63, 81 and 52, 60, 76 at 50, 150, 250 µg/ml of GPL, GPSB, GPF respectively. The highest yield was noticed with stem bark and leaf extracts and are comparable with reference standard tannic acid 58.9, 74.5, 88.9 at 10, 15, 25 µg/ml respectively (Figure 1).

Total flavonoid assay

The percentage yield of total flavonoid content is found to be 39, 52, 69 and 58, 69, 75 and 45, 53, 62 (75%) 76 at 50, 150, 250 µg/ml of GPL, GPSB, GPF respectively. The highest yield was noticed with stem bark and leaf extracts and are comparable with reference standard catechin 60.8, 73.4, 86.9 at 10, 15, 25 µg/ml respectively (Figure 2).

Antioxidant assay

DPPH radical scavenging activity

The anti-oxidant activity of methanol extracts of the \( G. \ pinnata \) stem bark was investigated by DPPH scavenging assay. This method was proved to be effective when methanolic extract compared to the standard antioxidant, ascorbic acid. All the plant extracts exhibited significant percentage of inhibition (Table 1).

Table 1: Scavenging activity of various plant extracts of \( G. \ pinnata \).

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<tr>
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<th>DPPH</th>
<th>Hydroxyl</th>
<th>Nitric Oxide</th>
<th>Super Oxide</th>
<th>Hydrogen Peroxide</th>
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<td>50 µg/ml</td>
<td>150</td>
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<td>GPL</td>
<td>0.5±</td>
<td>0.37±</td>
<td>0.4 ±</td>
<td>0.72±</td>
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Values are mean±S.E.M. n=3

*significance is set at \( p<0.001 \) (Student’s t-test).

Abb. GPLG. \( pinnata \) leaf, GPSB- \( G. \ pinnata \) stem bark, GPF- \( G. \ pinnata \) fruit.
Hydroxyl radical activity

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. The present study reveals that the hydroxyl radical scavenging activity significantly found at 250 µg/ml with GPL and GPSB extracts respectively (Table 1).

Nitric oxide scavenging activity

Nitric oxide (NO) is a potential pleiotropic mediator of various physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. The percentage inhibition of nitric oxide generation by all tested concentrations of GPL, GPS and GPSB were found significant at 250 µg/ml concentrations and are comparable with reference standard ascorbic acid (Table 1).

Super oxide radical scavenging assay

The superoxide anion radical scavenging activity of plant extract was assayed by the PMS-NADH method. As shown in Table 1, the percentage inhibition of superoxide radical generation by GPL, GPS, GPSB were compared to standard reference compound ascorbic acid. The percentage inhibition of superoxide generation was found as high found at 250 µg/ml concentration with GPSB which was shown equality with the value obtained by the standard ascorbic acid at 25 µg /ml.

Scavenging activity of hydrogen peroxide

Scavenging of hydrogen peroxide (H₂O₂) is another method used to determine the antioxidant properties. In the present study, H₂O₂ scavenging activity of GPL, GPS, GPSB at all tested concentrations (50, 150, 250 µg/ml) noticed significant results in comparison to ascorbic acid 10, 15 and 25 µg/ml as reference standard. All extracts obtained inhibition percentage of H₂O₂ and are very much comparable with reference standard ascorbic acid (Table 1).

Discussion

Enormous generation of free radicals, leads to oxidative stress that entangle several disorders in living systems and other side the use synthetic anti-oxidants was restricted to minimize hazardous effects which are associated with them. Thus, an attention was made to search for the drugs which are derived naturally from medicinal plants (Shahidi 1997). In this pertinent, we investigated few fractions of G. pinnata leaves, stem bark and fruits to evaluate the anti-oxidant properties. The present studies recorded, high amounts of poly phenols in the stem bark rather than in leaves and fruits. Thus, the stem bark established a good relationship with anti-oxidant properties. It is known fact that phenolic compounds and flavanoids are effective hydrogen donors which inhibits the lipid oxidation and chelating metal ions, making them good anti-oxidants and involve in protecting us from serious diseases such as strokes, heart attack and even cancer, etc, (Shahidi 1997) . Generally, these natural anti-oxidants strengthen the endogenous defenses against reactive oxygen species (ROS) and restore optimal balance by neutralizing the reactive species. However, the anti-oxidant activities of leaf and fruit extracts are also comparable. With the below reference mentioned it is evident that the leaves extracted with methanol noticed, significant anti-oxidant activity, than extracted with ethanol (Kathad et al. 2010). This is might because of difference in polarity of the solvents used. When the anti-oxidant activity of all extracts which are tested and compared with ascorbic acid is, the stem bark exhibited good concentration dependent inhibition of DPPH activity which is directly proportional reduction of DPPH radical in stoichometric manner (Sanchez Moreno 2002).

The reaction of superoxide and hydrogen peroxide generates the production of hydroxyl radical which involves in depolymerisation of hyaluronic acid (polysaccharide) and it may result in premature connective tissue destruction and also involves is the fragmentation of DNA strand (Kaneko et al. 1996). The plant extracts which were tested possessed scavenging activity. In general, H₂O₂ is not that much reactive but sometimes it can be toxic and cause cell damages as it may give rise to hydroxyl radical in cells. So therefore, it is important to remove H₂O₂ for better protection. Additionally, nitric oxide is a diffusible free radical which plays a significant role in signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. These natural anti-oxidants are effective hydrogen donors which inhibits the lipid oxidation and chelating metal ions, making them good anti-oxidants and involve in protecting us from serious diseases such as strokes, heart attack and even cancer, etc, (Shahidi 1997) . Generally, these natural anti-oxidants strengthen the endogenous defenses against reactive oxygen species (ROS) and restore optimal balance by neutralizing the reactive species. However, the anti-oxidant activities of leaf and fruit extracts are also comparable. With the below reference mentioned it is evident that the leaves extracted with methanol noticed, significant anti-oxidant activity, than extracted with ethanol (Kathad et al. 2010). This is might because of difference in polarity of the solvents used. When the anti-oxidant activity of all extracts which are tested and compared with ascorbic acid is, the stem bark exhibited good concentration dependent inhibition of DPPH activity which is directly proportional reduction of DPPH radical in stoichometric manner (Sanchez Moreno 2002).

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various roles as an effector molecule in diverse biological systems (Hagerman 1998). The scavenging activity of methanolic leaf, stem bark and fruit were found as concentration-dependent. Among, these extracts stem bark exhibited greater inhibitory percentage rather than others, in comparison to the known standards.

The superoxide radical formation occurs either by enzymatic or non-enzymatic process leads to the generation of more amounts of ROS in the cell and involve in several metabolic and physiological processes (Halliwell and Gutteridge 1985; Gqlcin et al. 2004; Blaszczyk et al. 1994; Bedard et al 2001). The present studies on superoxide radical, revels that at all tested concentrations of various extracts noticed significant scavenging effect (P < .001).

Conclusion

The results of in vitro analysis of G pinnata leaf, stem bark and fruit extracts posses significant free radical scavenging activity might be attributed to its various phyto constituents such as flavanoids and phenolic compounds. The present studies are the direct relationship for the use of this plant material in traditional medicine practice. Thus, in continuation to present work, further studies will be taken up with in vitro cellular systems.

References


Sanchez Moreno, C. 2002. Methods used to evaluate the free radical scavenging activity in foods and biological systems, Food Science and Technology International, 8(3): 121-137.


