IN VIVO ANTIOXIDANT ACTIVITY OF LIMNOPHILA HETEROPHYLLA AND MICHELIA CHAMPACA

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ABSTRACT

Objective: The present study was aimed at investigating the in-vivo antioxidant activity of the methanol extracts of Limnophila heterophylla and Michelia champaca leaves.

Methods: Methanol extract of both plants were administered to rats separately at three different doses of 125, 250 and 500 mg/kg for 21 d to evaluate oxidative stress parameters such as ferric reducing ability of plasma (FRAP), thiobarbituric acid reactive substance (TBARS) and reduced glutathione (GSH) and to evaluate antioxidant enzyme levels of catalase (CAT) and superoxide dismutase (SOD).

Results: The methanol extracts of both the plants significantly (p<0.05) elevated the ferric reducing ability of plasma (FRAP) on days 7, 14 and 21 of treatment. Significant (p<0.05) decrease of thiobarbituric acid reactive substance (TBARS) levels along with an increase in the superoxide dismutase (SOD) enzyme level in the liver and kidney at three different doses both the plants was observed. Treatment at a dose of 500 mg/kg b.w of both plants caused a significant increase only in the level of CAT in the liver and kidney. However, there was no significant effect of a thiobarbituric acid reactive substance (TBARS), superoxide dismutase (SOD) and catalase (CAT) in the heart and reduced glutathione (GSH) level in liver, heart and kidney at three different doses both the plants.

Conclusion: These outcomes recommend that the leaves of Limnophila heterophylla and Michelia champaca have a potent antioxidant activity which may be responsible for some of its reported pharmacological actions.

Keywords: Limnophila heterophylla, Michelia champaca, FRAP, Catalase, TBARS, GSH

INTRODUCTION

In the development of diseases, free radicals have emerged because the major entity inflicting harm to cells. These cytotoxic metabolites are generated by aerobic metabolism within the cell that successively considerably will increase pathological conditions, resulting in free radical mediated denaturation of protein, enzymatic deactivation, base hydroxylation of nucleic acids, cross-linking or strand cutting, mutation or maybe death [1]. However, the physiological system has a series of defense mechanism including antioxidant enzymes-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), Thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), and other free radical scavengers, β-carotene, vitamin C, vitamin E, α-lipoic acid, curcumin, rutin, BHT and glutathione to protect the cell against cytotoxic ROS (reactive oxygen species) [2]. The antioxidants in a biological system can be either enzymatic or non-enzymatic. The enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD), and glutathione which catalyze neutralization of many types of free radicals [3], while the nonenzymatic antioxidants include vitamin C, selenium, vitamin E, carotenoids, and polyphenols. The enzymatic antioxidants catalyse neutralization of many types of free radicals [3]. However, when the balance between the oxygen species and antioxidants is altered, a state of oxidative stress results, possibly leading to everlasting cellular damage. There is evidence that antioxidants may be useful in preventing the deleterious consequences of oxidative stress, and there is an increasing interest in the protective biochemical function of natural antioxidants contained in vegetables, fruits and medicinal herbs [4]. Generally, plants, herbs, and spice, rich in phenolic compounds like flavonoids, have been demonstrated to have anti-inflammatory, antiallergenic, antiviral, antiaging and anticarcinogenic activities which can be attributed to their antioxidant properties [5]. In this respect, flavonoids and polyphenolic compounds have received the greatest attention [6-8].

Limnophila heterophylla is an aquatic herb, mainly submerged, but with shoots that often emerge above the water surface, rooting at nodes. The plant finds lot of applications in the traditional system of medicine to treat wounds [9]. Different parts of Limnophila heterophylla possess varied pharmacological activities like COX inhibitor [10], antimicrobial [11] and wound healing [12]. The plant encloses terpene, flavanoids, terpinoids and oils [13]. Michelia, known by the scientific name Michelia champaca, is a very tall tree that grows up to 30m tall. Michelia champaca is used ethnomedicinally for the handling of astringent, constipation, dyspepsia, dysmenorrhea, fever, febrile, nausea, stomachic, skin disease, tonic, ulcers and wounds [14]. Earlier pharmacological reports of Michelia champaca had demonstrated its cytotoxic activity [15], anti-inflammatory [16], antihyperglycemic [17], keishmanicidal [18], antibacterial [19], wound healing [20], diuretic [21], antiulcer [22], antifertility [23], antihelmintic [24] and cardioprotective [25] activities. Several phytoconstituents like alkaloids, flavonoids, triterpenoids, saponins, tanins, sterols and steroids have been isolated from different parts Michelia champaca.

Based on these reports, this study was designed to investigate the in vivo antioxidant activity of methanol extracts of leaves of Limnophila heterophylla and Michelia champaca.

MATERIALS AND METHODS

Plant materials

The plants were collected from Tirupati (Andhra Pradesh), India and further, plants were distinguished, affirmed and validated by Dr. Madavchetty, Professor, Botany office, Sri Venkateswara University, Tirupati. A voucher specimen of these plants (Limnophila heterophylla-GIP006/2013-2014 and Michelia champaca-GIP005/2013-2014) have been kept in the GITAM Institute of Pharmacy, GITAM University, Visakhapatnam, Andhra Pradesh, India.
Chemicals and reagents
All chemicals used in the study were of analytical grade and they were procured from Coastal Enterprises Pvt. Ltd., Visakhapatnam, Andhra Pradesh and India.

Extraction
Leaves of Limnophila heterophylla and Michelia champaca were dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. The powder was passed through 40 mesh sieve and extracted with methanol separately in Soxhlet apparatus at 60 °C. The solvent was completely removed by rotary vacuum evaporator and concentrated. The extracts were freeze-dried and stored in a vacuum desiccator for further in vivo antioxidant studies.

Test animals and groups
Wistar albino rats (200–250 gm) of either sex were maintained under standard environmental conditions and had free access to feed and water ad libitum. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee (IAEC). Rats were divided into seven groups of six animals each. Group I served as control and was given the vehicle alone (normal saline). Group II, III and IV received methanol extract of Limnophila heterophylla orichally at 125, 250 and 500 mg/kg body weight, individually. Group V, VI and VII received methanol extract of Michelia champaca orally at 125, 250 and 500 mg/kg body weight, separately. The treatments were given for 21 d and all the animals were sacrificed by decapitation on 22nd day of the experiment. Blood of freshly prepared and warm (37 °C) FRAP reagent (1 ml of 10 heparinised glass tubes at 0, 7, 14 and 21 d of treatment. Briefly, 3 ml samples were collected from the rat retro-orbital venous plexus into 1 ml 20 mmol/l FeCl2 .6H2O, 10 ml of 0.3 mmol/l acetate buffer [pH 3.6]) was mixed with 0.375 ml distilled water and 0.025 ml of test solution. The absorbance of developed colour in the organic layer was measured spectrophotometrically at 560 nm and the concentration of superoxide dismutase (SOD) was expressed as units/mg of protein.

Catalase assay
Catalase activity (CAT) was measured by the method of Aebi [30]. A 0.1 ml of supernatant/serum was added to a cuvette containing 1.9 ml of 50 mmol phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 ml of freshly prepared 30 mmol H2O2. The rate of the decomposition of H2O2 was measured spectrophotometrically at 240 nm. The activity of catalase (CAT) was expressed as units/mg of protein.

Estimation of thiobarbituric acid reactive substance (TBARS)
Lipid peroxidation (LPO) was measured by the method of Liu et al. [31]. Acetic acid 1.5 ml (20% pH 3.5), 1.5 of TBA (0.8%), and 0.2 ml of sodium dodecyl sulfate (8.1%) was added to 0.1 ml of supernatant/serum and was heated at 100 °C for 60 min. The mixture was cooled to room temperature. To this, 5 ml of n-butanol: pyridine mixture and 1 ml of distilled water were added and vortexed vigorously. After centrifugation at 1200g for 10 min, the organic layer was separated and the absorbance was measured at 532 nm using a spectrophotometer. Malondialdehyde (MDA) was an end product of LPO, which reacts with TBA to form pink chromogen–TBA reactive substance. It was calculated using a molar extinction coefficient of 1.56 X 105 M -1 cm -1 and it was expressed as nmol/g tissue.

Statistical analysis
All experimental data were expressed as mean ± standard error of the mean (SEM). This Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnet-test with the SPSS statistical software for comparison to the control group. p<0.05 was considered as statistically significant.

RESULTS

The results of the serum enzymatic levels of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) of individual methanol extract of Limnophila heterophylla and Michelia champaca were mentioned in table 1. At a dose of 250 mg/kg both the extracts exhibited moderate significant (p=0.05, p<0.01 and p=0.001) decrease in the serum level of malondialdehyde (MDA) when compared to control. However, only the higher dose (500 mg/kg) of both the plant methanol extracts produced a significant (p<0.001) increase in the serum level of malondialdehyde (MDA) when compared to control. The extracts of treated rats also evoked a dose-related significant (p<0.05, p<0.01 and p=0.001) increase in the serum levels of catalase (CAT) and superoxide dismutase (SOD) when compared to control. Methanol extracts of both the plants at a dose of 250 mg/kg showed moderate significant (p<0.01) elevation in serum levels of catalase (CAT) and superoxide dismutase (SOD). However, only the higher dose (500 mg/kg) of both the plant methanol extracts caused a significant (p<0.001) increase in the serum levels of catalase (CAT) and superoxide dismutase (SOD) when compared with control. However, there was mild significant (p<0.05) changes of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels were observed in 125 mg/kg of both the plant methanol extracts.
Table 1: Serum enzymatic levels of MDA, SOD and CAT

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nM of TBARS mg⁻¹ of protein)</th>
<th>SOD U/mg protein</th>
<th>CAT U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.46±0.32</td>
<td>46.2±1.28</td>
<td>26.2±0.01</td>
</tr>
<tr>
<td>Group II</td>
<td>0.39±0.01*</td>
<td>48.1±2.62*</td>
<td>26.8±0.18*</td>
</tr>
<tr>
<td>Group III</td>
<td>0.3²±0.12**</td>
<td>53.6±1.37**</td>
<td>28.4±1.12**</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.2¹±1.32***</td>
<td>54.8±1.09***</td>
<td>40.0±1.87***</td>
</tr>
<tr>
<td>Group V</td>
<td>0.4±0.02*</td>
<td>45.2±1.23*</td>
<td>22.1±0.08*</td>
</tr>
<tr>
<td>Group VI</td>
<td>0.3±0.32**</td>
<td>49.7±1.08**</td>
<td>26.3±1.23*</td>
</tr>
<tr>
<td>Group VII</td>
<td>0.3²±0.42***</td>
<td>51.7±1.22***</td>
<td>37.2±1.82***</td>
</tr>
</tbody>
</table>

[p<0.05*, p<0.01** and p<0.001***]

Ferric reducing ability of plasma (FRAP) assay

The ferric reducing ability of plasma (FRAP) level of rats after administration of methanol extracts of Limnophila heterophylla and Michelia champaca over a period of 21 d was presented in fig. 1. In the control group, there was no significant change in ferric reducing ability of plasma (FRAP) value on days 7 (870 nM Fe²⁺/l) and 14 (869 nM Fe²⁺/l) compared with day 0 (864 nM Fe²⁺/l). But, in group II, III and IV on days 7 (947, 1088 and 1101 nM Fe²⁺/l, respectively), 14 (1104, 1202 and 1287 nM Fe²⁺/l, respectively) and 21 (1122, 1309 and 1344 nM Fe²⁺/l, respectively) there was a significant (p<0.05, p<0.01 and p<0.001) increase in ferric reducing ability of plasma (FRAP) value compared with day 0 (869, 870 and 871 nM Fe²⁺/l, respectively). Maximum enrichment of ferric reducing ability of plasma (FRAP) level was found in group IV and VII, which correspond to animals administered with 500 mg/kg body weight of the methanol extract of Limnophila heterophylla and Michelia champaca, individually.

Fig. 1: Changes in rat total antioxidant capacity of the ferric reducing ability of plasma (FRAP) measured by Fe²⁺-equivalent after administration of methanol extract of Limnophila heterophylla and Michelia champaca. Values were mean±s.d (n=6). Group II, III, IV, V, VI and VII (Plant Extracts Treated Rats) Compared to Group I (control rats). p<0.05* and p<0.01**

Estimation of superoxide dismutase (SOD)

The treatment of methanol extract of both plants caused minor significant (p<0.05, p<0.01 and p<0.001) decrease at 125 mg/kg (510 and 498 units/mg of protein), 250 mg/kg (528 and 524 units/mg of protein) and 500 mg/kg (536 and 532 units/mg of protein) bodyweight in the level of superoxide dismutase (SOD) in the heart compared with the control (522 units/mg of protein). However, the level of superoxide dismutase (SOD) in the kidney and liver of the both plants treated rats was not dose related and was found to be significantly increased (p<0.05, p<0.01 and p<0.001) at the three different doses of 125 mg/kg (206, 198 and 428, 419 units/mg of protein, respectively), 250 mg/kg (218, 209 and 455 units/mg of protein, respectively) and 500 mg/kg (220, 218 and 469, 469 units/mg of protein, respectively) compared with the control (52 units/mg of protein, respectively). The results were shown in fig. 2, 3 and 4.

Estimation of catalase (CAT)

The administration of methanol extract of both plants to normal rats for 21 d induced a dose-dependent increase in the level of catalase (CAT) in liver (fig. 2) and kidney (fig. 4), but a decrease in the heart (fig. 3). Methanol extract of both plants were significantly increased at 500 mg/kg body weight dose of the treatment (p<0.001) for liver (344 and 339 units/mg of protein) and kidney (402 and 394 units/mg of protein) compared with the control group of liver and kidney (312 and 305 units/mg of protein, respectively). However, there was a little change in the endogenous antioxidant levels in heart tissue. Similarly, for both the plants at a dose of 125 and 250 mg/kg a significant increase (p<0.05 and p<0.01) in the catalase (CAT) levels were observed for liver (308 and 298 units/mg of protein) and kidney (387 and 374 units/mg of protein) compared with the control group.

Fig. 2: Changes in rat liver thiobarbituric acid reactive substance (TBARS; nmol/g wet weight [wt]), superoxide dismutase (SOD; units/mg protein), catalase (CAT; units/mg protein) and reduced glutathione (GSH; µg/g wet wt) following oral administration of methanol extracts of Limnophila heterophylla and Michelia champaca. Values were mean±SD (n = 6). p<0.05* and p<0.01**, compared with control.
Estimation of thiobarbituric acid reactive substance (TBARS)

The effect of different doses of individual methanol extract of *Limnophila heterophylla* and *Michelia champaca* on the lipid peroxidation and endogenous antioxidants of liver, heart and kidney of rats was shown in fig. 2, 3 and 4 respectively. For groups II, III, and IV, a significant (p<0.05, p<0.01 and p<0.001) decrease in thiobarbituric acid reactive substance (TBARS) concentration in liver (339, 287 and 284 nm/g wet wt tissue, respectively) and kidney (182, 176 and 162 nm/g wet wt tissue, respectively) was observed and compared with the control group (341 and 184 nm/g wet wt tissue in liver and kidney, respectively). Similarly, for groups V, VI and VII a significant (p<0.05, p<0.01 and p<0.001) decrease in thiobarbituric acid reactive substance (TBARS) concentration in liver (329, 292 and 288 nm/g wet wt tissue, respectively) and kidney (176, 179 and 168 nm/g wet wt tissue, respectively) was observed and compared with the control group (341 and 184 nm/g wet wt tissue in liver and kidney, respectively). Also, the experiment study indicated that a negligible change in the level of thiobarbituric acid reactive substance (TBARS) in the heart was observed for all treated groups.

Estimation of reduced glutathione (GSH)

For groups II, III and IV, there was no significant change of reduced glutathione (GSH) level in liver (207, 205 and 206 mg/g wet wt tissue, respectively), heart (285, 287 and 284 µg/g wet wt tissue, respectively) and kidney (91, 87 and 89 µg/g wet wt tissue, respectively) compared with the control group (fig. 2), heart (fig. 3) and kidney (fig. 4) (206, 286 and 88 µg/g wet wt tissue, respectively). Similarly, the methanol extract of *Michelia champaca* showed no significant change in the level of reduced glutathione (GSH) in liver, heart and kidney at a dose of 125, 250 and 500 mg/kg.

DISCUSSION

Excessive production of reactive oxygen species (ROS) plays a very important role within the pathologic process and progression of many diseases together with completely different organs [33]. The principle for the utilization of antioxidants is well established in interference and treatment of chronic diseases wherever aerobic stress plays a serious aetiopathological role, varied population studies support that consumption of natural sources of fruits and vegetables, rich in antioxidant compounds, are related to a lower incidence of aerobic stress evoked diseases [34].

In the present investigation, the ferric reducing ability of plasma (FRAP) test measured total antioxidant capacity determined by non-enzymatic antioxidants. Numerous approaches have been established to assess the total antioxidant capacity of plasma or serum because of the difficulty in measuring each antioxidant component separately in the serum or plasma [35]. One of these is the ferric reducing ability of plasma (FRAP), which measures the reduction of Fe³⁺ to Fe²⁺ in the presence of water-soluble exogenous antioxidants [28]. The significant increase in ferric reducing ability of plasma (FRAP) level after oral administration of methanol extract of both plants (*Limnophila heterophylla* and *Michelia champaca*) indicates the presence of bio-available antioxidants in these plants. As mentioned above, the maximum enhancement was obtained in group IV and VII after 21 d of handling. Also, ROS react with all biological substance; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation (LPO) [36]. Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptor...
It can be concluded that the methanol extracts of CONCLUSION
Extraction process and Andhra Pradesh, India.

...and antioxidative properties of the methanol extracts of... a significant increase in the antioxidant activity of the methanol extracts of... consistent with previous reports...

The free radical scavenging activity of the methanol extracts of... mechanisms, such as inhibition of lipid peroxidation...

...the presence of known bioactive compounds (Bavonoids), which provides maximum conjugation with...

...a dose-dependent manner when compared to control. The present study showed the depletion in the lipid peroxidation as observed by significant decrease in the... the heart.

...providing financial assistance in the form of UGC-MRP fellowship to... and oxidative stress-related ailments (diabetes, Alzheimer's disease, atherosclerosis, arthritis, cancer) of major organs such as liver, kidney and heart.

CONCLUSION
It can be concluded that the methanol extracts of Limnophila heterophylla and Michelia champaca had significant in vivo antioxidant activity. The antioxidant action of methanol extracts of both plants may be attributed to the presence of known bioactive compounds (Bavonoids), which provides maximum conjugation with radical species, thus reducing the number of free radicals available as well as oxidative stress-related ailments (diabetes, Alzheimer's disease, atherosclerosis, arthritis, cancer) of major organs such as liver, kidney and heart.

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AUTHORS CONTRIBUTION
The complete research work was suggested and designed by Raja S. Extraction process and in vivo antioxidant activity was carried out by Ravindranadh K. The manuscript was drafted by Ravindranadh K. The manuscript was edited by Ravindranadh K. Authors read and approved the final manuscript.

CONFLICT OF INTEREST
Declared none

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