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FREE RADICAL SCAVENGING ACTIVITY OF *PREMNA INTEGRIFOLIA* LINN.

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ABSTRACT

Purpose: The present paper was aimed to study an *in-vitro* and *in-vivo* antioxidant activities of hydro alcoholic extracts (HAE) of root bark of *P. integrifolia*. **Methods:** The hydro alcoholic extract was subjected to various *in-vitro* assays such as DPPH, nitric oxide scavenging activity, reducing power assay, superoxide anion radical scavenging and iron chelation assays. For *in-vivo* analysis sixty Wistar rats were taken and they were divided into six groups; the first group served as control, the second group was fed with high fat diet, the other three groups were fed with high fat diet along with various concentrations of HAE and the last group was treated with atorvastatin for 30 days. Analysis of lipid peroxides, superoxide dismutase (SOD) and catalase activities were carried out. **Results:** The IC_{50} values of 142.93, 293.9, 1000, 810.67, 69.03µg/ml for DPPH, nitric oxide, reducing power assay, superoxide anion radical scavenging and iron chelation assays respectively were found in the present study. The lipid peroxides and conjugated dienes were increased and SOD, catalase activities were decreased in blood, liver, heart and kidney of atherosclerosis induced rats when compared to control. The activities were reversed to near normalcy on treatment with different doses of HAE. **Conclusion:** HAE extract was showed an increased activity by 3.21 fold and 3.09 fold in nitric oxide and DPPH scavenging activity respectively. The activity was directly proportional to the concentration of the HAE treatment. The *in-vitro* free radical scavenging

activity is correlated well with the *in-vivo* anti oxidant activities in high fat diet induced atherosclerosis.

KEY WORDS: Nitric oxide; DPPH; SOD; Lipid peroxides; *Premna integrifolia.*

1.0 INTRODUCTION

Reactive oxygen species (ROS) are continuously formed in cells as consequence of oxidative biochemical reactions. It is increasingly being realized that many pathophysiology of today's diseases are due to the oxidative stress that results from the imbalance between formation and neutralization of pro-oxidants. Under these conditions the endogenous antioxidants may be unable to counteract ROS formation; therefore formed ROS may cause cellular damage. Oxidative stress is initiated by free radicals, which seek for stability through electron paring with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause damage to proteins and DNA along with lipid peroxidation. These changes contribute to the development of various diseases such as cancer, atherosclerosis, cardiovascular diseases, ageing, inflammatory diseases etc.[1] All human cells protect themselves against free radical damage by antioxidants and antioxidant enzymes[2]. Sometimes these protective mechanisms disrupted by various pathological processes and exogenous antioxidant supplements are vital to combat oxidative damage. Recently, much attention has been directed towards the development of ethno medicines with strong antioxidant properties but low cytotoxicities.

Premna integrifolia L. (syn: *Premna serratifolia*), belongs to Verbenaceae family and have a prominent role in ASU systems of medicine. The plant is known for its cardio-tonic[3], anti-inflammatory[4], anti-diabetic[5], anti-arthritic[6] and anti-obesity[7] activities. Many medicinal plants have become major candidates of research for identifying their rich chemical constituents and possess antioxidant potential which in turn protects cellular damage induced by ROS. There has been a remarkable increase in claim of herbal drugs due to their wide biological activities and have higher safety margin and low costs than the synthetic drugs. In case of allopathic medicine, complete cure is not attained without side effects. Moreover they are often associated with serious side effects. Polyphenolic compounds are widely distributed in plants are known to possess tremendous antioxidant activities. These compounds are capable of scavenging free radicals and provide antioxidant defense[8]. The present study is to evaluate the free radical scavenging activity of *P. integrifolia* by *in vitro* and the same was compared with high fat diet induced atherosclerosis in rats.

2.0 MATERIALS AND METHODS

2.1. Preparation of plant extract from root bark

The root bark of *P. integrifolia* was procured from Registered Pharmacy and authenticated by Shri. C. Arunachalam, Research Officer (Botany), Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute, Chennai and the voucher specimen (00641/2014) was deposited in the Botany Department. The coarse powder of root bark in the ratio of hydro alcohol (60:40, v/v) 1:10 (w/v) for 72 hrs with intermittent shaking at room temperature. The extract was filtered through Whatman No.1 filter paper; the filtrate was evaporated to dryness and stored in an air-tight container.

2.2 Determination of *in vitro* free radical scavenging activity of *P. integrifolia*

Free radicals such as nitric oxide[9], superoxide anion radical scavenging activity[10], total free radical scavenging activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) method[11], iron chelating activity[12], and reducing power assay[13] by standard procedures using UV-Visible Spectrophotometer (Perkin-Elmer λ EZ 201)

2.3. Animals

Wistar rats of 6-8 weeks of age were purchased from King's Institute of Preventive Medicine, Guindy, Chennai-600032, India. Experiment was conducted in 60 (30 males + 30 females), 10 rats (5M + 5F) in each group. The rats were maintained in institutional animal house facility with 12h light/dark cycles. Temperature was maintained at 25±3°C and feeding schedule consisted of rat pellet diet and water *ad libitum*. HAE and vehicle (0.5% SCMC) were administered for 30 days by intra gastric route using appropriate graduated syringe. The proposal was duly approved by Institutional Animal Ethical Committee as per CPCSEA guidelines (Registration No. IAEC/CSMDRIAS/10/2014).

2.4. Atherosclerosis model

Rats were fed with atherogenic diet consisting of 2.0g of cholesterol, 8.0g of saturated fat and 0.1g of calcium were mixed thoroughly with 90g of powdered standard commercial pellet diet. The rats were fed with high fat diet along with weekly challenge of oral vitamin D_3 (3,00,000 IU) through per oral route[14]. Feed was prepared daily and intake was recorded.

2.5. Preparation of Hemolysate and Biochemical analysis

At the end of experimental period (i.e.31st day) 4mL of blood sample was collected in an EDTA-K_2 tube from retro-orbital plexus under mild ether anesthesia. Plasma was separated

using cooling centrifuge (Remi C-24) at 3000 rpm for 10min at 4°C and used for the estimation of lipid peroxides[15] and conjugated dienes[16]. The bottom portion of red blood cells were washed with ice cold phosphate buffered saline (0.1 M; pH 7.4) in a high speed cooling centrifuge at 4°C for 10 minutes at 3000 rpm. This procedure was repeated till the red cell pellet changed to pale red color. The pellet obtained was suitably diluted with phosphate buffered saline and used for the biochemical estimations such as SOD[17], catalase[18] and total protein[19]. Tissues such as liver, heart and kidney were removed and homogenized using Teflon homogenizer and biochemical analysis were carried out using UV-Visible Spectrophotometer (Perkin Elmer Lambda EZ).

2.6. Statistical analysis

Statistical analysis was carried out using graph pad prism software, version 5. All the values were expressed as mean ± SD (n=10). Analysis of variance (ANOVA) was used for multiple comparison of treatment groups with the vehicle control and disease control followed by Dunnett's test. $p<0.05$ was considered to be statistically significant.

3.0 RESULTS

An *in vitro* free radical scavenging and anti oxidant activity of HAE is shown in Table.1. DPPH (IC_{50}: 142.93µg) and nitric oxide (IC_{50}: 293.9µg) scavenging activity was found higher than that of standards (IC_{50}: 442.3µg; 943.94µg) respectively. Whereas for HAE, the superoxide anion radical (IC_{50}: 810.67µg), reducing power assay (IC_{50}: 1000µg) and iron chelating activity (IC_{50}: 69.03µg) was observed. There was no hydrogen peroxide activity was found for HAE of the present study.

The levels of conjugated dienes (plasma) and lipid peroxides in plasma, liver and kidney of control and HAE treated groups are depicted in Fig.1. The level of conjugated dienes were significantly increased in group II ($p<0.0001$), III ($p<0.001$), IV ($p<0.01$) and VI ($p<0.05$) when compared to group I. The level was significantly decreased in group III ($p<0.05$), IV ($p<0.01$), V ($p<0.001$) and VI ($p<0.01$) when compared to group II. The levels of lipid peroxides were significantly increased in plasma of group II ($p<0.001$); III ($p<0.01$) & VI ($p<0.01$), whereas in liver of group II & V ($p<0.05$) and in kidney of group II ($p<0.0001$); III ($p<0.001$); IV ($p<0.01$) & VI ($p<0.05$) when compared to control. No significant change was noticed in V when compared to group I. The levels were significantly decreased in plasma of group IV ($p<0.05$) & V ($p<0.01$) whereas in kidney of group III ($p<0.01$); IV ($p<0.001$); V ($p<0.0001$) & VI ($p<0.001$) when compared to group II.

The activity of SOD in hemolysate, liver, heart and kidney of control and HAE treated groups are depicted in Fig.2. The activity was significantly decreased in hemolysate of group II ($p<0.001$); III ($p<0.01$); IV ($p<0.05$) & VI ($p<0.01$); liver ($p<0.01$), kidney and heart ($p<0.05$) of group II when compared to group I. The activity was significantly increased in group IV ($p<0.05$) & V ($p<0.05$) when compared to group II.

The activity of catalase in hemolysate, liver, heart and kidney of control and HAE treated groups are depicted in Fig.3. The activity was significantly decreased in hemolysate of group II, III, IV, VI ($p<0.001$) & V ($p<0.01$) in liver of group II ($p<0.01$), heart of group II ($p<0.01$), III ($p<0.05$) and kidney of group II ($p<0.05$) when compared to group I. The activity was significantly increased in group V ($p<0.05$) of liver and heart when compared to group II.

Table 1: *In vitro* Anti-oxidant Activity of HAE.

S. No.	Antioxidant Assay	HAE (µg)	Standard (µg)	Activity
1.	Nitric Oxide scavenging activity	293.90	943.94	Increased by 3.21 fold
2.	DPPH scavenging activity	142.93	442.30	Increased by 3.09 fold
3.	Superoxide anion radical scavenging activity	810.67	617.14	Decreased by 1.31fold
4.	Reducing power	1000.00	800.00	Decreased by 1.25 fold
5.	Fe^{2+} chelating activity	69.03	14.62	Decreased by 4.72 fold

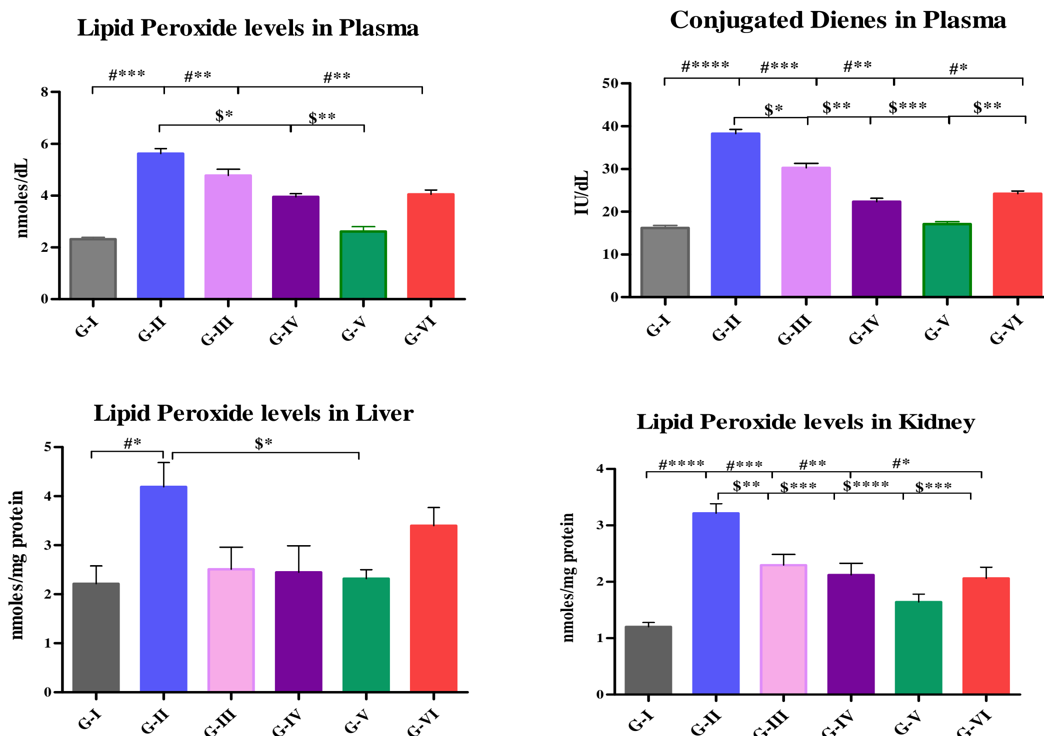

Fig. 1: Estimation of LPO and Conjugated dienes in Plasma.

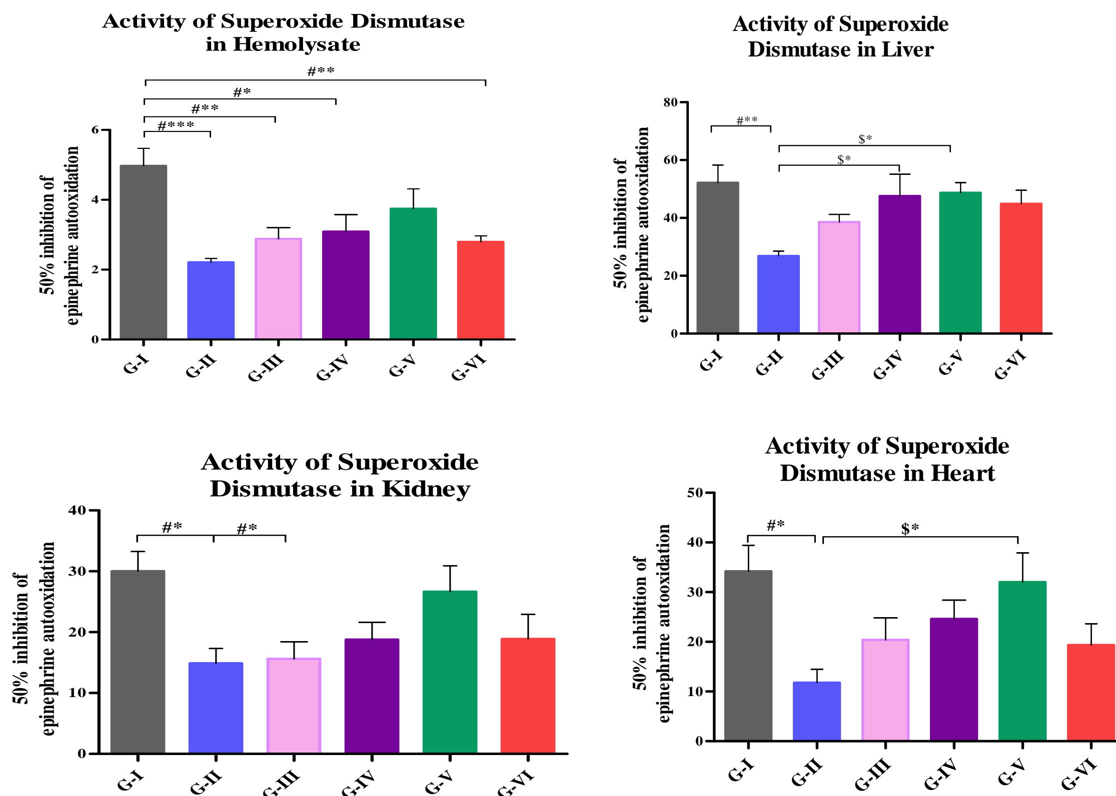

Fig. 2: Assay of SOD in Hemolysate, liver, Heart and Kidney.

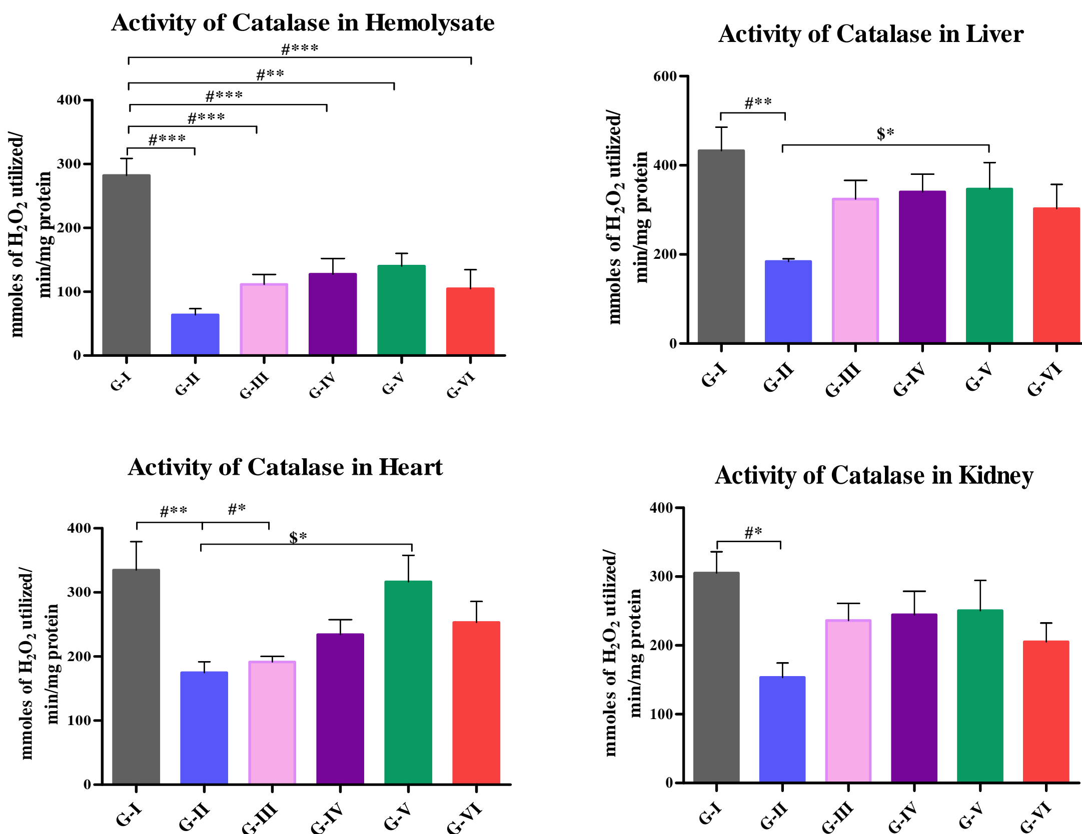

Fig. 3: Assay of Catalase Hemolysate, liver, Heart and Kidney.

4.0 DISCUSSION

The free radical scavenging activity of natural compounds can be evaluated through their ability to quench the synthetic free radicals, in which the absorbance of the reaction mixture is taken in visible range to know whether the compound is having antioxidant activity. DPPH method is simple, accurate and cost effective for the evaluation of total antioxidant capacity in plants and this assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical. DPPH is relatively stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule[20]. DPPH radicals react with suitable reducing agent as a result of which electron become paired off forming the corresponding hydrazine[21, 22]. From the present results, it is postulated that the HAE may have hydrogen donors, thus scavenge the free radical DPPH. Based on the data obtained from this study, IC50 value for DPPH radical scavenging was increased by 3.09 fold for HAE when compared to standard quercetin. Whereas the nitric oxide scavenging activity of the HAE was increased by 3.21 fold when compared to standard ascorbic acid. Hence the present study showed HAE had higher antioxidant capacity than standard quercetin. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. The decomposition of hydrogen peroxide by root bark extract of *P. integrifolia* results from its antioxidant and free radical scavenging activity. Flowers of *P. serratifolia* L. had a higher nitric oxide scavenging activity was reported[23]. Hence the present study showed that the HAE of root bark of *P. integrifolia* L. showed a significant anti-oxidant and free radical scavenging activity.

Lipid peroxidation is a free radical mediated process and it is involved in the formation of lipid radicals. A re-arrangement of the unsaturated lipids that results in the variety of degraded products like alkanes, malondialdehyde, conjugated dienes and lipid hydro peroxides and eventually causes destruction of membrane lipids[24]. Oxidative stress resulting from increased production of ROS, play a key role in the pathogenesis of several diseases. Increased lipid peroxidation and decreased cellular antioxidant defense mechanisms are susceptible to oxidative stress[25]. The imbalance between pro-oxidants and antioxidant levels in high fat diet fed group were noticed in the present study this may be due to the oxidation of LDL cholesterol. In addition, the increased ROS and MDA a product of lipid peroxidation was elevated in the atherosclerotic rats and decreased in HAE treated groups. Taken together with above results, the imbalance between the level of oxidative stress and antioxidants could

be seen in high fat diet fed group. HAE could prevent the excessive formation of lipid peroxides which indicated that its therapeutic and preventive nature. Treatment with HAE inhibited atherogenic diet induced MDA accumulation in kidney this might be due to the anti-lipid peroxidative property of extract. Atorvastatin also prevented the elevated level of malondialdehyde in some extent. The ethanolic extract of leaves of *P. serratifolia* reported to have higher concentrations of flavonoids and alkaloids which possess antioxidant properties by scavenging the free radicals[21].

The red blood corpuscles (RBC) are intrinsically prone to oxidative stress because they are exposed to high oxygen tension and have a characteristic structural composition with polyunsaturated fatty acids in membrane, besides the presence of hemoglobin-bound iron[26]. However, membranes and cytoplasmic compartments of RBC have an efficient antioxidant mechanism that maintains their integrity. Oxygen free radicals have been implicated in the development of hyperlipedimic atherosclerosis[27]. SOD and catalase helps in detoxification of oxygen free radical and hydrogen peroxide[28]. Among these, SOD converts the highly toxic superoxide to less toxic hydrogen peroxide and O_2 by the enzyme catalase[30] and SOD involves in the first line of defense to protect the cells from the injurious effects of superoxide. It is clearly depicted that the HAE treated rats showed decreased lipid peroxide levels and increased activities of SOD and catalase when compared to high fat diet induced rats. Hence HAE of *P. integrifolia* protects liver from the free radical damage.

The activities of SOD and catalase in kidney were lowered but not a very significant reduction in high fat diet fed rats than normal control rats. High fat diet is accompanied by increased hepatic, heart, and renal tissues oxidative stress, which is characterized by reduction in the antioxidant enzyme activities and glutathione levels, that correlate with the increase in MDA levels in most tissue[30]. In conclusion, the root bark of *P. integrifolia* is extensively used in the Ayurvedic systems of medicine for treating various ailments. As with the other plant parts, the root bark have been shown antioxidant activities but the active principles have not so far identified. Further research is needed to identify the biomolecules responsible for its antioxidant activity.

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