Evaluation of *In Vitro* Antioxidant, Antithrombotic And Antihaemolytic Activity of Hydroethanolic Leaf Extract of *Martynia Annua*

Brindha Durairaj[1], Santhi. R[2], Soundaravalli. R[3]
[1]Associate Professor Dept of Biochemistry
[2]Assistant Professor Dept of Biochemistry
[3]Dept of Biochemistry
[1, 2] PSG College of Arts & Science, Coimbatore - 641 014

Abstract- *Plants have been a rich source of medicines as they produce wide array of bioactive compounds. Drugs derived from plants secured importance in recent years because of their unrefuted efficacy as phytomedicines. The principle constituents or active compounds present in these natural plants serves as templates, lead molecules or as intermediates for synthetic drugs. A large number of populations depend on the conventional practitioners, who are depends on medicinal plants to meet their primary health care needs. Since the usage of these herbal medicines has increased regarding their quality, safety and efficacy in industrialized and developing countries. The present study was focus on antioxidant, antithrombotic and antihaemolytic activity of hydroethanolic leaf extract of Martynia annua (L) belongs to the family of Martyniaceae.*

Keywords- *In vitro*, antithrombotic, antihaemolytic, antioxidant, *Martynia annua.*

I. INTRODUCTION

Traditional systems of medicine are popular in developing countries and up to 80% of the population relies on traditional medicines remedies for their primary health care [1]. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. India is the largest producer of medicinal plants and it has the name of "*Botanical Garden of the World*". Medicinal plants contain secondary metabolites (alkaloids, glycosides, steroids or other groups of compounds) which possess pharmaceutical potential such as anticancer, antimalarial, antidiabetic and antidysenteric. In spite of tremendous developments in the field of synthetic medicines during the 20th century, plants still remain as one of the major sources of drugs in modern as well as traditional system of medicine throughout the world [2].

Free radicals contains one or more unpaired electrons due to which they are highly unstable and can cause damage to other molecules by extracting electrons from them in order to attain stability. Free radicals are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function [3]. Free radicals can initiate the oxidation of bio molecules (protein, lipid, amino acids and DNA) which will lead to cell injury and can induce numerous diseases The imbalance between production of reactive oxygen species (ROS) like O_2, H_2O_2, OH^-, ROO^- and the capability of the normal detoxification system in favor of the oxidants lead to oxidative stress, which itself lead to cellular damage caused by the interaction of ROS with cellular constituents. Tissue damage resulting from oxidative stress has been implicated in the pathology of a number of disorder diseases such as (cancer, inflammatory joint disease, cardiovascular diseases, and cataract) and can play a role in neurodegenerative diseases and ageing [4].

Antioxidant refers to a compound that scavenges these free radicals proves to be beneficial for these disorders as they prevent damage against cell proteins, lipids and carbohydrates. Antioxidant activity includes free radical scavenging activity, inhibition of lipid peroxidation, metal ion chelating ability and reducing capacity. Therefore the excessive free radicals scavenging, a dietary antioxidant is required. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation of oxidizing chain reactions.

A blood clot called thrombus is developed in the circulatory system due to the failure of hemostasis which causes vascular blockage and while recovery leads to serious consequences in the arthrothrombotic diseases (myocardial or cerebral infarction) leading to death. Thrombolytic agents that include tissue plasminogen activator (t-PA), Urokinase, Alteplase, Reteplase and enectaplase are commonly used all over the world for the treatment of these diseases and they pose scrows side effects such as bleeding complications, hemorrhagic stroke, allergy and rarely anaphylaxis [6].

Hemolysis leads to lysis of red blood cells by changing the structure of haemoglobin and the damage caused

by reactive oxygen species to the erythrocytes and it might be inhibited by some of the antioxidants especially Vitamin C . The synthetic drugs have many adverse effect but the medicinal plants play an important role in the antihaemoytic activity [7]. In the present study an attempt was made to evaluate the *in vitro* antioxidant, antithromobotic and antiheamolytic effects of hydroethanolic leaf extract *Martyina annua*.

II. MATERIALS AND METHODS

COLLECTION AND EXTRACTION OF PLANT MATERIAL

Martynia annua leaves were collected from the top hill of Yercaud, Salem. Leaves were taken for the investigation of antioxidant, antithrombotic and antihaemolytic properties.

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

The radical scavenging activity of the extract against 1, 1-diphenyl-1-picryl-hydrazyl radical (DPPH) was determined by a slightly modified method of Brand-Williams [8]. The following concentrations of each extract were prepared in methanol at concentration ranging from 0.002 to 1mg/ml. Ascorbic acid was served as standard, and the same concentrations were prepared as the test solution. To 2 mL each of the prepared concentrations in a test tube was added 0.5 mL of 1mM DPPH solution in methanol. The experiments were carried out in triplicates. The test tubes were incubated for 15 minutes at room temperature, and the absorbance read at 517 nm. Rutin was used as a reference.

DPPH radical scavenging activity (%) = [(A0−A1)/(A0)] × 100

where A0 was the absorbance of DPPH radical + methanol;
A1 was the absorbance of DPPH radical + sample extract /standard.

DOT BLOT DPPH METHOD

The screening assay method described by Sivaraj (2011) with slight modification, plant crude ethanol extract dissolved and prepared in ethanol (mg/mL) were plant sample (10 µL) carefully spotted on TLC plate after that kept for dry (5 min). Then, DPPH (0.4 mM) solution was sprayed on TLC plate; Rutin was used as standard.

HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY

A solution of hydrogen peroxide (20mM) was prepared in phosphate buffer saline (pH 7.4), different concentrations of plant extract and standard Rutin solution viz. 100,200,300,400,500µg/ml in methanol (1 ml) where added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

For each concentration, a separate blank sample was used for background subtraction. The percentage inhibition activity was calculated from $[(A_0 – A_1)/A_0]$ x100, where A_0 is the absorbance of the control and A_1 is the absorbance of extract/standard. The antioxidant activity of the extract was expressed as IC_{50}. All the tests were performed in triplicate and the graph was plotted with the average of three observations [9].

NITRIC OXIDE FREE RADICAL SCAVENGING ACTIVITY

About 100, 200, 300, 400 and 500 µg concentration of crude pigment previously dissolved in DMSO, as well as BHA (standard compound) was taken in separate tubes. About 2.0 ml of sodium nitroprusside in phosphate buffer saline was added into each tube. The reaction mixture was incubated at room temperature for 150 minutes. After incubation, 5 ml of Griess reagent was added in the each tube including control. The absorbance was measured at 546 nm on UV- Visible spectrometer using methanol as blank [10].

HYDROXYL RADICAL SCAVENGING ACTIVITY

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell [11]. Stock solutions of EDTA (1mM), $FeCl_3$ (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1mL EDTA , 0.01mL of $FeCl_3$**,**0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration 100,200,300,400,500µg/mL dissolved in distilled water, 0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37^0C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test and control.

SUPEROXIDE RADICAL SCAVENGING ASSAY

The method of Martinez [12] was used to determine superoxide dismutase in superoxide radical scavenging assay. The assay based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Each 3ml reaction mixture contained 0.05M phosphate buffered saline (PBS) (pH 7.8), 13mM methionine, 2µM riboflavin, 100µM EDTA, NBT (75µM) and 1.0ml of test sample solutions 100,200,300,400,500µg/ml. The tubes were kept in front of a fluorescent light for 20 minutes and absorbance was read at 560 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes containing reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was estimated by comparing the absorbance of the control and those of the reaction mixture containing test sample using the equation:

In vitro Nitric Oxide Radical $(NO)^{\cdot}$ Scavenging Assay Nitric oxide generated from sodium nitroprusside (SNP) was measured. The reaction mixture (5.0ml) containing SNP (5 mM) in phosphate buffered saline (pH 7.3), with plant extract at different concentrations, was incubated at 25°C for 180min in front of a visible polychromatic light source. The NO radical thus generated interacted with oxygen to produce the nitric ion (NO_2^-) which was assayed at 30 min intervals by mixing 1 ml of incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylene-dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthyl-ethylene-diamine-dihyhrochloride was measured at 546 nm. Each experiment was carried out in triplicates.

REDUCING POWER

Reducing power of the methanol extract was determined by Oyaizu [13] method with minor modification. Different concentration of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, 6.6 pH) and 2.5 ml of potassium ferric cyanide (1%) and incubated for 30 minutes at 50°C. 2.5 ml of Trichloroacetic acid (10%) was added after incubation and centrifuged at 3000 rpm for 10 minutes. 2.5 ml solution from the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml ferric chloride (0.1%). Absorbance was measured at 700 n m. Ascorbic acid was used as standard.

ANTIHEMOLYTIC ASSAY

Blood was collected from healthy adult human volunteers and collected in sterile Alsevier's solutions and used within 5 hours of collection. The preparation of cell suspension was carried out as described above. In a series of test tubes, take 800 µl of 1 % w/v Triton X-100 and make it up to a volume of 3 ml with phosphate buffer. Similarly 3 ml of distilled water alone served as positive control. Different concentrations of the plant extracts (100-500 µg) were added in a series of tubes in which Triton X-100 was previously incubated. 500 µl of RBC suspension was added to all the tubes mixed gently. Tubes were incubated in a water bath at 37oC for 1 hour and centrifuged at maximum speed for 5 minutes. The supernatant was collected and the absorbance was read at 541 nm against phosphate buffer as blank for calculating the percentage of hemolysis [14].

ANTITHROMBOTIC ACTIVITY

In vitro clot lysis activity of test drug was carried out according to the method of Nicolini [15] with minor modifications. Briefly, venous blood drawn from the healthy volunteers was distributed in different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube –weight of tube alone). To each micro centrifuge tube containing pre-weighed clot, 100 µl of test drug was added separately. As a standard, 100 µl of Streptokinase (SK) and as a non-thrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered I and II. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated with the blood samples of the 5 volunteers.

III. RESULTS AND DISCUSSION

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical scavenging activity was detected and compared with rutin (Figure 1). The activity of DPPH radical scavenging of hydroethanolic extract leaf of *Martynia annua* and rutin was found to have 81.23µg/ml. The IC_{50} values for DPPH scavenging activity for hydroethanolic leaf extract of *Martynia annua* and rutin were found to be 158.64 and 114.3µg/ml respectively.

Dot plot assay is a semi-quantitative and rapid screening method for determining the antioxidant activity of hydroethanolic leaf extract of *Martynia annua*. The radical scavenging effect of hydroethanolic leaf extract of *Martynia annua* were applied as a dot on to a TLC plate and then stained with DPPH solution. The dot plot assay as shown in Plate 1 has revealed the DPPH scavenging activity exhibited by hydroethanolic leaf extract *of Martynia annua* at five different concentrations (100 - 500µg/ml). It was measured by its ability to convert the stable free radical DPPH to a yellow color derivative. The plate 1 shows the results of the rapid screening of hydroethanolic leaf extract of *Martynia annua* against DPPH radicals. Similarly TLC chromatograms of the *Ocimum gratissimum* antioxidant components in the methanol extract revealed DPPH radical solution reported by Okugbo and Oriakhi [16].

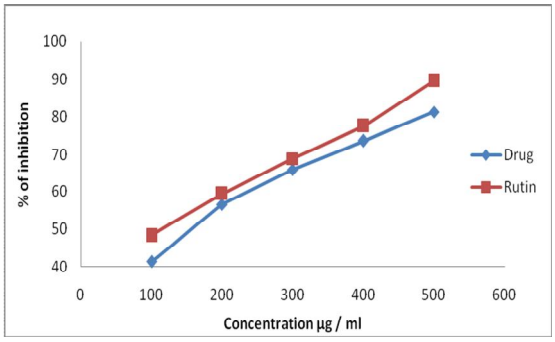

Figure 1: Effect of hydroethanolic leaf extract of *Martynia annua* on percent DPPH scavenging activity

Plate 1: Dot plot assay of hydroethanolic leaf extract of *Martynia annua*

HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY

Hydroxyl radical are the major active oxygen causing lipid peroxidation in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein. From the results the percentage of inhibition of H_2O_2 in different concentration of hydroethanolic leaf extract of *Martynia annua* was found to be 70.89% at 500µg/ml. The IC_{50} values for hydrogen peroxide scavenging activity for hydroethanolic leaf extract of *Martynia annua* and rutin were found to be 277.08µg/ml and 170.92µg/ml respectively (Figure 2). The results of our study are in accordance with the results obtained by Gupta [17].

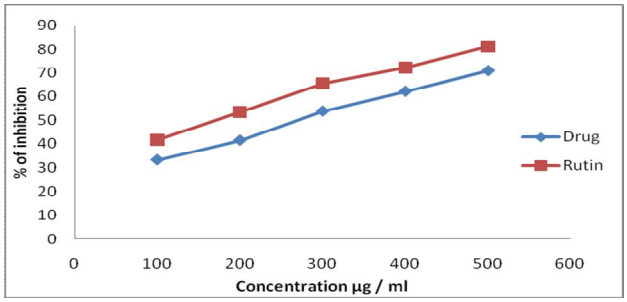

Figure 2: Effect of hydroethanolic leaf extract of *Martynia annua* on H_2O_2

NITRIC OXIDE RADICAL SCAVENGING ASSAY

The nitric oxide assay was preferably adopted method to evaluate the free radical scavenging activity. From the results the percentage of inhibition of nitric oxide in different concentration of hydroethanolic leaf extract of *Martynia annua* was found to be 67.32% at 500µg/ml (Figure 3). The IC_{50} values for Nitric oxide scavenging activity for hydroethanolic leaf extract of *Martynia annua* and the standard (BHA) were found to be 351.68µg/ml and 162.93µg/ml respectively. The results of our study are in accordance with the results obtained by Radhakrishnan [18].

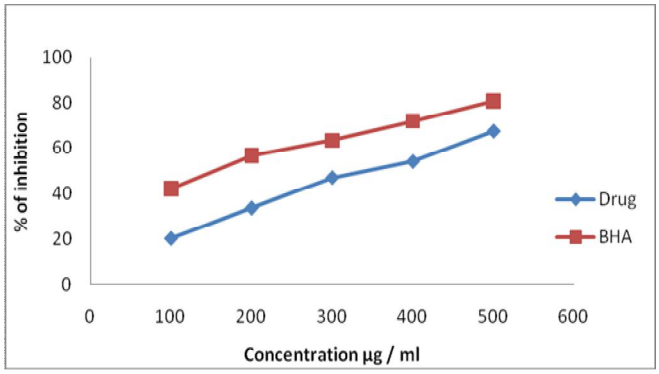

Figure 3: Effect of hydroethanolic leaf extract of *Martynia annua* on Nitric oxide

HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical scavenging effect of different concentration of hydroethanolic leaf extract of *Martynia annua* was found to have 75.77 % at 500µg/ml as shown in figure 4. The IC_{50} values of hydroxyl scavenging activity for hydroethanolic leaf extract of *Martynia annua* and BHA were

found to be 245.04µg/ml and 179.82µg/ml respectively. The results of our study are in accordance with the results obtained by Paulpriya [19].

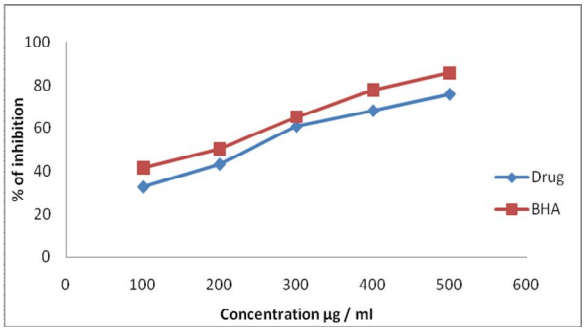

Figure 4: Effect of hydroethanolic leaf extract of *Martynia annua* on hydroxyl scavenging activity

SUPER OXIDE RADICAL SCAVENGING ACTIVITY

The superoxide anion radical is one of the most preferable adopted methods to evaluate the free radical scavenging activity. From the results it may be concluded that hydroethanolic leaf extract of *Martynia annua* was found to posses 64.48 % at 500µg/ml concentration. The IC_{50} values for superoxide scavenging activity for hydroethanolic leaf extract of *Martynia annua* and ascorbic acid were 396.03µg/ ml and 261.8µg/ml respectively (Figure 5). The results of our study are in par with the results obtained by Asadu [20].

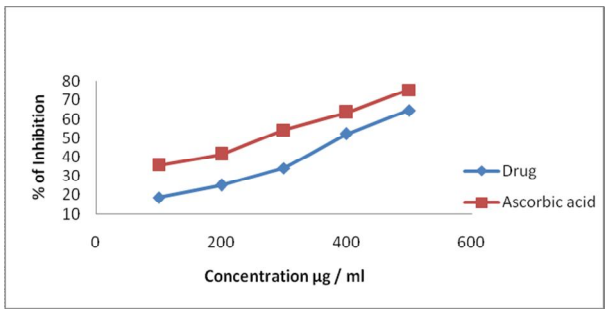

Figure 5: Effect of hydroethanolic leaf extract of *Martynia annua* on Super Oxide

REDUCING POWER ASSAY

The reducing power assay is one of the preferably adopted methods to evaluate the free radical scavenging activity. From the results it can be concluded that the hydroethanolic leaf extract of *Martynia annua* exhibited highest reducing power at 500µg/ml (Figure 6). Thus, the reducing property indicated that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process. The results of our study are on par with the results obtained by Sivaramakrishnan [21].

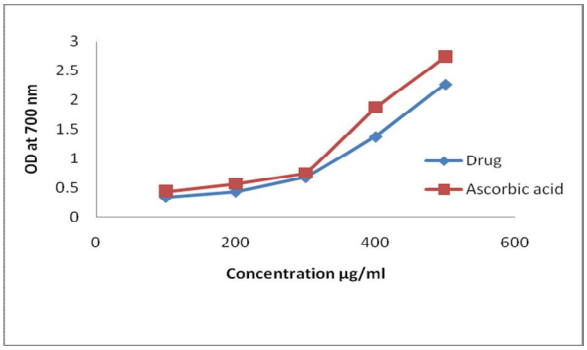

Figure 6: Hydroethanolic leaf extract of *Martynia annua* on Reducing power assay

ANTITHROMBOTIC ACTIVITY OF HYDROETHANOLIC LEAF EXTRACT OF *Martynia annua*

Cardiovascular disease and stroke are caused by blood clot in high-income countries. Thrombolytic agents are used to dissolve clot in patients but their use is associated with lacks specificity. The ability of *Martynia annua* of blood clot is recorded 63.22% and values are compared with standard streptokinase 74.53% as shown in figure 7.

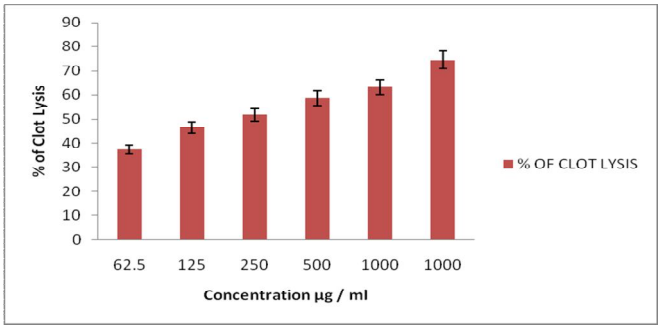

Figure 7: Effect of hydroethanolic leaf extract of Martynia annua on antithrombotic activity

ANTIHEAMOLYTIC ACTIVITY OF HYDROETHANOLIC LEAF EXTRACT OF *Martynia annua*

Oxidative damage and haemolysis caused by reactive oxygen species (ROS) have a major role in the development of diseases such as thalassemia, glucose-6-phosphate dehydrogenase deficiency and sickle cell anaemia. This study demonstrated the capability of the plant extract to stabilize RBC membrane, which is an indication of the ability of plant extract to prevent hemolysis. The maximum inhibition of 65 % was observed at 1000 µg/ml concentration of hydroethanolic extract of *Martynia annua* in concentration dependant manner (Figure 8). The methanolic and aqueous

extracts of Piper Longum seeds was also reported by Manu [22] which show similar results as our study.

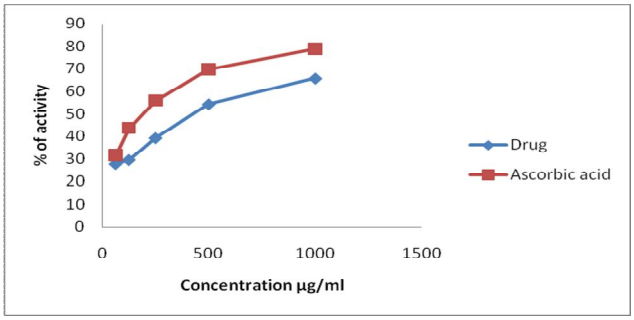

Figure 8: Effect of hydroethanolic leaf extract of *Martynia annua* on antihaemolytic activity

IV. CONCLUSION

Based on the results obtained the present study, it might be concluded that a hydroethanolic leaf extract of *Martynia annua* exhibited high antioxidant and free radical scavenging activities. These *in vitro* assays indicate that this plant extract possess a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stress. The hydroethanolic leaf extract of *Martynia annua* possessed potent antithrombotic and antihaemolytic properties.

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