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Original Article

EVALUATION OF *IN VITRO* ANTIOXIDANT, ANTI-INFLAMMATORY AND THROMBOLYTIC ACTIVITIES OF *SCILLA HYACINTHINA*, AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

Objective: *Scilla hyacinthina* (Roth) J. F. Macbr, an endangered medicinal plant (Liliaceae), was analysed for its *in vitro* antioxidant, anti-inflammatory and thrombolytic potential. Till date, there were no reports about the anti-inflammatory and thrombolytic potential of the plant.

Methods: Leaves and bulbs of *S. hyacinthina* were extracted sequentially with methanol and water. Antioxidant activity of the extracts was studied using DPPH and $ABTS^{\bullet+}$ methods. Anti-inflammatory effect and thrombolytic activity of the plant extracts were determined using protein denaturation (albumin) and clot disruption methods respectively.

Results: Preliminary screening revealed the presence of phenols, saponins, terpenoids, cardiac glycosides, sterols, phlobatannins, alkaloids and flavonoids. The methanolic leaf extracts exhibited significant bioactivities among all analysed extracts. The above extract exhibited considerable dose-dependent antioxidant activity for DPPH and $ABTS^{\bullet+}$, IC_{50} values were 19.79±0.89µg/ml and 21.02±0.72µg/ml resectively. There was a positive correlation between the total phenolic content with DPPH in leaf extract (r^2=0.94). The methanolic leaf extract of the plant also exhibited concentration dependent inhibition of albumin denaturation with maximum inhibition of 60.42%±0.91 and was found to be significant ($p<0.044$) with diclofenac. Methanolic leaf extracts showed maximum clot lytic activity (40%±2.19) and was statistically significant ($p<0.001$), when compared with streptokinase.

Conclusion: The present study promisingly revealed that *S. hyacinthina* may be an effective potential sources of novel antioxidant, anti-inflammatory and thrombolytic drugs. Further research is necessary to identify the bioactive compound(s) responsible for the above mentioned activities.

Keywords: *S. hyacinthina*, Endangered, Antioxidant, Anti-inflammatory, Thrombolytic.

INTRODUCTION

Reactive oxygen species (ROS) are produced during physiological and biochemical processes in the human body. The imbalance between ROS and antioxidant defence mechanism leads to oxidative stress [1]. Oxidative stress causes dysfunction of the tissues and failure of hemostasis, leading to the range of disorders including cancer, arthritis, cardiovascular disease, diabetes, and ageing [2]. In cardiovascular disease, superoxide anion (O_2^-) is an important source of oxidative stress which suppresses the activity of nitric oxide leading to the thrombus formation and activates the inflammatory responses [3]. Due to the inflammation in the blood vessels, the blood flow interrupts, leading to the increase in blood pressure and evolution of cardiovascular disease.

The imbalance between prooxidants and antioxidants play a vital role for the development of atherosclerosis and has prompted the investigation of antioxidants as a possible therapy. Recently, potent antioxidants which are natural phytochemicals have gained attention and exhibited tremendous advantages in human health [4]. The antioxidants may indirectly inhibit platelet aggregation through scavenging of reactive oxygen species, many of which alter platelet function. Oxidative stress and inflammation are intimately linked with the onset of cardiovascular disease and acute coronary syndromes [5].

The drugs which can scavenge ROS, inhibits inflammation and exhibit thrombolytic activities, may be the best choice for the prevention of cardiovascular diseases. Thrombolytic agents such as tissue plasminogen activator (t-PA), Urokinase (UK), Streptokinase (SK) etc. are used all over the world for the treatment of atherothrombotic diseases. In India, though SK and UK are widely used due to lower cost [6, 7], as compared to other thrombolytic drugs, their use is associated with hyper risk of hemorrhage [8], severe anaphylactic reactions and lacks specificity. Because of the above shortcomings of the available thrombolytic drugs, attempts are made to develop drugs of natural origin which are considered as safe and economical.

Scilla hyacinthina is an endangered perennial herb [9] from Liliaceae family with fibrous roots proceeding from the base of a large, tunicate, nearly globular bulb, 4 to 6 inches long and the outer scales are thin and papery, red or orange-brown in colour. This plant is grown in southern part of India and Africa. The previous study of this plant shows that it is used as a cardio tonic for cardio vascular diseases [10]. Therefore, in the present study we aim to investigate the antioxidant potential and anti-inflammatory activity along with the thrombolytic property of *S. hyacinthina.* According to the best of our knowledge, this is the first report on anti-inflammatory and thrombolytic activities of this plant.

MATERIALS AND METHODS

Chemicals and reagents

2,2-diphenyl-1-picryhydrazyl(DPPH), 2,2'-Azinobis-3-ethyl benzotia zoline-6-sulfonicacid ($ABTS^{\bullet+}$), Gallic acid, Quercetin, Egg albumin were purchased from Sigma-Aldrich (St. Louis, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck, Germany. All other chemicals used in the study were of analytical grade.

Sample preparation and extraction procedure

The plant was collected from Bhata village, Sri Potti Sri Ramulu Nellore district, Andhra Pradesh, India and was authenticated by National Ayurveda Dietetics Research Institute, Bangalore with authentication numbers SMPU/NADRI/BNG/2012-13/616 for leaves and SMPU/NADRI/BNG/2012-13/617 for bulbs. The plant material was dried and powered. The dried samples were kept in air tight containers until further use. The powdered samples were extracted sequentially with methanol and water at 1:10 (w/v)

concentrations by using the Soxhlet apparatus. The extract was filtered through Whatman No.1 filter paper and the filtrate collected. The filtrate was concentrated by rotary evaporator, stored at 4°C and used for further studies.

Phytochemical screening

The crude extracts were subjected to the qualitative phytochemical analysis for phenols, saponins, terpenoids, cardiac glycosides, sterols, phlobatannins, alkaloids and flavonoids using standard methods [11].

Determination of total phenolic and flavonoid content

Total phenolic content (TPC) was determined according to standard procedure [12]. Gallic acid was used as a reference for plotting the calibration curve. A volume of 1 ml of plant extracts (100µg/ml) was added to 2 ml of Folin-Ciocalteu reagent (diluted 1:10mix with de-ionized water) and neutralized with 4 ml of (7.5% w/v) sodium carbonate. The reaction mixture was incubated at room temperature for 30 min. The absorbance of the resulting blue colour was measured at 765 nm using double beam UV-VIS spectrophotometer (UV-1800 Shimadzu). TPC was expressed as mg Gallic acid equivalents per gram of sample (mg GAE g^{-1}).

Flavonoid content (FC) was determined as per Arya *et al.* [13] with minor modifications. Quercetin was used as a reference compound. A volume of 1 ml of the plant extract (100 µg /ml) was mixed with 1 ml of 2% aluminium chloride and a drop of acetic acid, and then added two ml of methanol. The absorbance of the reaction was recorded at 415 nm after 30 min. The calibration curve was prepared by quercetin at concentrations of 20 to 100µg ml^{-1}. FC was expressed as mg Quercetin equivalents per gram of the sample (mg QE g^{-1}).

DPPH radical scavenging assay

The free radical scavenging activity of the plant extracts was studied using 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging method with slight modifications [12]. 1 ml of plant extracts of different concentrations (5-25µg/ml) was added to 2 ml of methanolic solution of DPPH (0.025%). The assay mixture was incubated in dark at 37 °C for 30 minutes and the mixture without plant extracts served as control. The decrease in absorbance of each solution was measured at 518 nm using UV-VIS spectrophotometer (UV-1800, Shimadzu). Ascorbic acid was used as standard. The percentage (%) of radical scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = A_C - A_S / A_C \text{ X } 100$$

Whereas= Absorbance of sample, A_C= Absorbance of control

ABTS$^{\bullet}$ assay

The antioxidant capacity of the plant extracts was estimated in terms of the ABTS$^{\bullet}$ radical scavenging activity with slight modifications [12]. Briefly, ABTS$^{\bullet+}$was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium per sulphate and the mixture was allowed to react for 12 h at room temperature in the dark. The resulting solution was then diluted with 5 mM phosphate-buffered saline (pH 7.4). 1 ml of plant extracts of different concentrations (5-25µg/ml) was added 0.5 ml of diluted ABTS$^{\bullet}$ solution, the absorbance was measured at 20 min at 745 nm. The mixture without plant extracts served as control and the ascorbic acid used as standard. The percentage (%) of radical scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = A_C - A_S / A_C \text{ X } 100$$

Where, A_S= Absorbance of sample, A_C= Absorbance of control

The antioxidant activity of each sample in both the assays were expressed in terms of IC_{50} (microgram concentration required to inhibit radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

Anti-inflammatory activity

Anti-inflammatory activity of *S. hyacinthina* extracts was evaluated by protein denaturation method as described by Elias and Rao [14]. Diclofenac sodium was used as standard. The reaction mixture consists of 1 ml of different concentrations of extracts ranging from 20-100 µg/ml and diclofenac sodium and 3 ml of phosphate buffered saline (pH6.4) was mixed with 1 ml of egg albumin solution (1%), the reaction mixture without plant extracts taken as control and incubated at 37 °C for 20 minutes. Denaturation was induced by keeping the reaction mixture at 90 °C in a water bath for two minutes. After cooling the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated by using the following formula:

$$\text{Inhibition (\%)} = (A_t - A_c) / A_c \text{ x } 100$$

Where, A_c= Absorbance of control, A_t= absorbance of test sample

Thrombolytic activity

Experiments for clot lysis were performed according to Prasad *et al.* [15]. 4 ml of venous blood was drawn from healthy human volunteers (n=50) without history of oral contraceptive or anticoagulant therapy (using a protocol approved by the institutional ethics committee of Kempegowda Institute of Medical Sciences, Bangalore with registration No. ECR/216/Inst/ Kar/2013). An earlier consent was taken from healthy human volunteers for collection of blood samples. 500 µl of blood was transferred to each of the previously weighed sterile micro centrifuge and incubated at 37°C for 45 minutes. 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, 100µl of methanolic extract of *S. hyacinthina* was added. Commercially available lyophilised streptokinase vial (1500000 IU) was used as positive control, to which 5 ml sterile distilled water was added and mixed properly. Distilled water was used as negative control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After the 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 performed with the blood samples of the 50 volunteers.

Clot lysis= Actual clot weight – clot weight after addition of extracts X 100/Actual clot weight

Actual clot weight

UV-Visible spectra analysis

The methanolic extract was centrifuged at 5000 rpm for 10 min and filtered. The filtrate was diluted to 1:10 ratio with methanol. The prepared extract having bioactive compounds was analysed by using UV-VIS spectrophotometer (UV-1800 Shimadzu), wave length ranging from 190 nm to 1100 nm [16].

Statistical analysis

All the experiments were carried out in triplicates. Results were expressed as the mean±S.E of three independent experiments (n=3), followed by t-test where $P<0.05$ was considered as statistically significant.

RESULTS

Percentage (%) yield

Methanolic extracts of *S. hyacinthina* gave high percentage of yield (bulbs = 11.45% and leaves = 12.45%) when compared to aqueous extracts (bulbs = 8.3% and leaves = 8.35%).

Phytochemical analysis

The phytochemical analysis of plant extracts revealed the presence phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids (table 1). TPC was found to be maximum (10.33±0.53 mg/GAE/g) in methanolic leaves extracts and FC was found to be highest in aqueous extract (9.32±0.57 mg/QE/g) of leaves (table 2).

Table 1: Phytochemical constituents present in the plant extracts of *S. hyacinthina*

Phytochemicals	Leaves		Bulbs	
	Methanol	Water	Methanol	Water
Alkaloids	+	_	+	_
Saponins	+	+	+	+
Flavonoids	+	+	+	+
Terpenoids	_	+	+	+
Tannins	_	_	+	+
Cardiac glycosides	+	+	+	+
Phenolic compounds	+	_	+	+
Phlobatannins	+	+	_	_
Steroids	+	+	_	_
Anthraquinone	_	_	_	_

+: indicates present; -: indicates absent

Table 2: Total phenolic, flavonoid content and IC_{50} (µg/ml) values of *S. hyacinthina* for DPPH and $ABTS^{\bullet+}$assay

Extract	Total phenol contents (mg GAE/g extract)	Flavonoids (mg QE /g extract)	IC_{50} (µg/ml) for DPPH assay	IC_{50} (µg/ml) for $ABTS^{\bullet+}$assay
Methanolic Leaves	10.33±0.53	6.23±0.58	19.76±0.89	21.02±0.72
Aqueous Leaves	4.48±0.36	9.32±0.57	23.75±0.52	24.56±0.81
Methanolic Bulbs	7.17±0.41	4.42±0.42	22.73±0.73	23.48±0.63
Aqueous Bulbs	3.43±0.42	5.43±0.45	25.44±0.68	25.96±0.79
Ascorbic Acid	-----	-----	11.79±0.83	12.25±0.91

All results are mean±SE of three consecutive experiments.

Antioxidant activity

The plant extracts showed a dose dependent anti-oxidant activity on DPPH and $ABTS^{\bullet+}$ assays and was compared with standard antioxidant, ascorbic acid. IC_{50}values of the different extracts and the correlation between TPC and DPPH of methanolic leaves extracts (r^2= 0.94) were analysed (table 2; fig. 1).

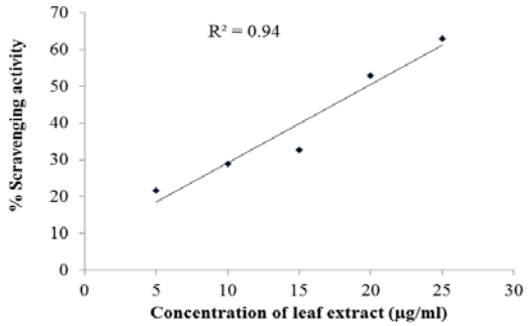

Fig. 1: Correlation between phenolic content and antioxidant activity (DPPH) of *S. hyacinthina* methanolic leaves extracts

Anti-Inflammatory activity

The inhibitory effect of methanolic and aqueous leaf extracts of *S. hyacinthina* on protein denaturation are shown in fig. 2. Both methanol and aqueous extracts of leaves exhibited significant and dose dependent anti-inflammatory activity when compared to diclofenac sodium (20, 40, 60, 80 100 µg/ml). The TPC of methanolic leaves extracts and anti-inflammatory activity showed a positive correlation (r^2= 0.96). The mean difference in percentage (%) inhibition between positive control and methanolic extracts of *S. hyacinthina* was found to be significant (p<0.044).

All results are mean±SE of three consecutive experiments

Thrombolytic activity analysis

The methanolic leaf and bulb extracts of *S. hyacinthina* exhibited 33.55%±2.28 and 40.0%±2.19 clot lysis after incubation at 37 °C for 90 minutes. Whereas SK showed 56.8%±1.03 and distilled water 7.78%±1.29clot lysis (Fig.3).

The mean difference in percentage (%) clot lysis between SK and S. *hyacinthina* methanolic leaves exhibited significance (p<0.001).

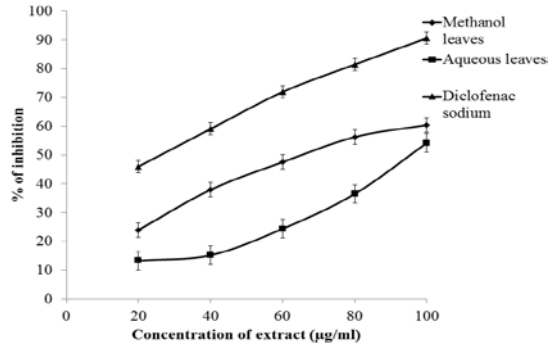

Fig. 2: Anti-inflammatory activity of leaves extracts and diclofenac sodium

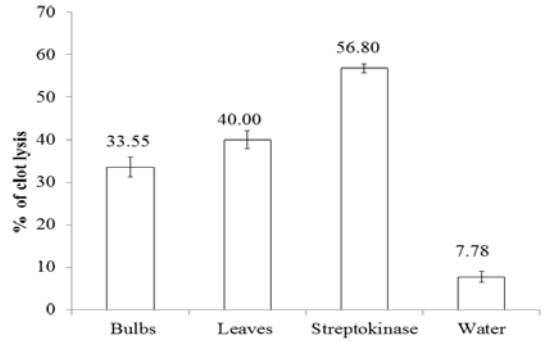

Fig. 3: The percentage (%) clot lysis activity of *S. hyacinthina* extracts and streptokinase. All results are mean±SE of three consecutive experiments.

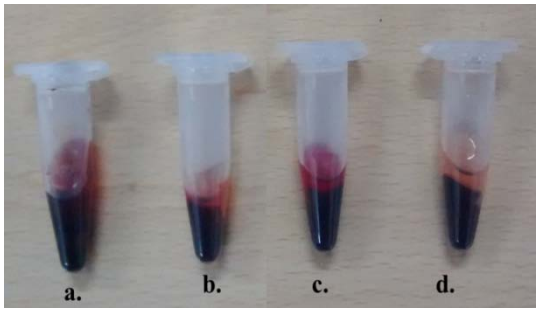

Fig. 4: Clot-lysis of blood samples of normal subjects (positive and negative control)

Where a= *S. hyacinthina* leaves extracts; b= *S. hyacinthina* bulb extracts; c= Streptokinase; d= water

UV-Visible spectra analysis

The UV-visible spectrum of methanol leaf extract showed the absorbance between 190 nm to 350 nm (fig. 5) indicating the presence of phenols and flavonoids.

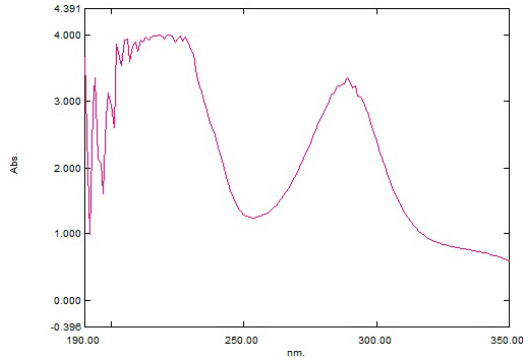

Fig. 5: UV/Visible spectrum of methanolic leaves extract of *S. hyacinthina*

DISCUSSION

In the present study, the methanolic leaf extracts exhibited significant bioactivities among all analysed extracts. Methanolic extracts of *S. hyacinthina* showed 62.89% scavenging activity at 25µg/ml whereas the methanolic extracts of *Urginea indica* demonstrated 98.1% scavenging activity at 30µg/ml [17]. In another study, *S. hyacinthina* extracts significantly ($p<0.01$) counteracted the deleterious effects of DOX by increasing the contents of *in vivo* antioxidant enzymes in rat models [10]. Queiroz *et al.* [18] revealed that methanolic extracts of *Allium sativum* exhibited 82.25% of DPPH radical scavenging activities at 1 mg/ml concentration. A positive correlation ($r^2=0.9$) between radical scavenging activity (DPPH) and the TPC of methanolic leaf extracts of *S. hyacinthina* was observed, the results were in accordance with Saeed *et al.* [19]. The smaller IC_{50} value for DPPH assay corresponds to higher scavenging activity of the plant extracts [20]. Though the DPPH radical scavenging abilities of the extract were less than those of standard ascorbic acid, the extracts in the present study, have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants. A significant difference in inhibition of thermally induced protein denaturation (60.42%) was observed in case of methanolic extracts compared to others. The correlation analysis between TPC and anti-inflammatory activity of methanolic extracts showed a positive correlation ($r^2= 0.9$) confirming that, phenolic compounds might be the factor which contribute to the antioxidant and anti-inflammatory activities of the extract in the present study [21, 22]. The methanolic leaf extracts exhibited significant ($p<0.001$) thrombolytic activity compared to standard drug, streptokinase. Anjum *et al.* [23] demonstrated thrombolytic activity of *Bridelia verrucosa* which showed highest clot lysis of 41.46% whereas in the current study, *S. hyacinthina* showed 40% clot lysis. According to literature, the UV-Visible spectra peaks in the range of 210 – 310 nm are due to the phenolic groups and those in the range of 255 – 280 nm are specific for flavonoids [24]. The present result of UV-Visible spectra of methanolic leaves extracts also confirmed that the extract is rich in phenols and flavonoids.

CONCLUSION

In conclusion, the investigated plant *S. hyacinthina* showed potential antioxidant, anti-inflammatory and thrombolytic activities. *S. hyacinthina* possesses high phenolic content, which might be the contributor to the antioxidant potential leading to the inhibition of thrombus and inflammation. The present study promisingly revealed that *S. hyacinthina* has effective potential sources of novel antioxidant, anti-inflammatory and thrombolytic drugs which may cure cardiovascular diseases. Further studies are necessary for the identification of bioactive compound(s) responsible for the activities.

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CONFLICT OF INTERESTS

Declared None

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