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Evaluation of Anti-arthritic, HRBC Membrane Stabilization and Antioxidant Properties of the Edible *Phlogacanthus thyrsiformis* (Hardow) Mabb Flower Extracts and Their Correlation Studies

Poppy Das[1], M. Himaja[1*], V. Vinodhini[1] and S. Manimegalai[2]

[1]Department of Chemistry, School of Advanced Sciences, VIT University, Vellore 632 014, Tamil Nadu, India
[2]Department of Biotechnology, School of Bio Science and Technology, VIT University, Vellore 632 014, Tamil Nadu, India

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ABSTRACT The present experiments were designed to scientifically prove the anti-arthritic and anti-inflammatory properties of traditionally used *P. thyrsiformis* flowers. The flower extracts were subjected to qualitative and quantitative screening. Gas chromatography mass spectrometry technique was employed and eight molecules were identified from the ethyl acetate extract using the NIST library. The extracts were also evaluated for *in vitro* antioxidant, anti-arthritic and anti-inflammatory activities. Potent antioxidant activity was observed for the ethyl acetate extract in both antioxidant assay methods with an IC_{50} value of 63.36 µg/mL and 64.60 µg/mL, respectively. The ethyl acetate extract also exhibited significant activity in both anti-arthritic and anti-inflammatory activities. Correlation studies revealed that the presence of phenol, flavonoid, terpenoid and ß-sitosterol in the ethyl acetate extract played a crucial role in its impressive activity. This study provided experimental evidence towards the traditional use of *P. thyrsiformis* flowers against arthritis and inflammation.

INTRODUCTION

Phlogacanthus thyrsiformis (Hardow) Mabb. is a common seasonal plant belonging to the Acanthaceae family. It grows in northeastern India in sub-tropical Himalayas in the upper Gangetic plain in the states of Bihar and Bengal (Sharmistha and Jogen 2012). It is characterized by brick red velvety flowers in the early spring season and has cylindrical leaves (Khare 2007). The flowers are used in various recipes and consumed in northeastern India. The roots, leaves and flowers are traditionally used in the treatment of fever, inflammation, jaundice, cold and cough, pox and many other ailments (Ningombam and Singh 2014; Sarbadhikari et al. 2015). Though the plant is well established as a folklore medicine in northeastern India, no phytoconstituents have been identified or reported from this flower and its activities have not been established on a scientific level. The widespread use of *P. thyrsiformis* as a traditional medicine against inflammatory ailments generated an ardent interest to unravel its inhibitory activity and identify the different phytoconstituents present in it. Thus, an attempt was made to study the antioxidant, anti-inflammatory and anti-arthritic activity of the plant.

In this study the researchers have tried to explore the various medicinal properties of edible *P. thyrsiformis* flower extract on a scientific level and justify them by correlating its activities to the phytoconstituents present. Qualitative and quantitative phytochemical screening was done for the edible flower extract to identify as well as quantify the phytoconstituents present in the flower. Gas chromatography mass spectrometry analysis was carried out and the chromatograms were screened through NIST library to identify the phytoconstituents from the extracts. Antioxidants have been investigated for the prevention of many diseases and the prevalence of phenols and polyphenols in plant extracts contributes to antioxidant activity. Hence, *in vitro* antioxidant activity for the extracts was estimated by two approaches, the 2, 2-diphenyl-1-picrylhydrazyl assay and hydrogen peroxide scavenging assay. Anti-arthritic activity of the extracts was measured by egg albu-

Address for correspondence:
Dr. (Mrs). M. Himaja
Professor
Department of Chemistry,
School of Advanced Sciences,
VIT University, Vellore 632 014, India
Mobile: 9944796228,
E-mail- drmhimaja@gmail.com

min denaturation method and anti-inflammatory activity of the flower extracts was carried out by human red blood cell membrane stabilization method. An attempt was made to establish a correlation between the phytoconstituents and the activities of the extracts, which was never reported for this plant earlier. The phenol content was directly correlated with both 2, 2-diphenyl-1-picrylhydrazyl assay and hydrogen peroxide scavenging activities.

METHODOLOGY

Preparation of Extract

The plants were collected from Tinsukia district in Assam, India. It was authenticated in Tinsukia college with a reference no TC/IDENT/CERTIFICATE/14/2648. The flowers were washed, dried and powdered. Extraction was carried out in a soxhlet extractor using water, ethanol and ethyl acetate as solvents for 24 hours. The extracts were concentrated and screened for qualitative phytochemical analysis. The results are tabulated in Table 1.

Quantitative Phytochemical Screening

The total phenol content of each extract was assessed by Folins Ciocalteau method (Anamaria et al. 2014). The results were stated in terms of mg/g gallic acid equivalent. Total flavonoid content was determined using the technique reported by Ordonez et al. (2006) and Gursoy et al. (2009). The results were stated as quercetin equivalent (mg/g). Total terpenoid content was achieved by the method described by Gayathri et al. (2014). The results were stated as linalool equivalent (mg/g). The total phytosterol content of the extracts was obtained by Libermann-Burchard colorimetric method (Feng et al. 2014) and the contents were stated as cholesterol equivalent. The results were expressed as mg cholesterol/100 g.

GC-MS Analysis

GC-MS analysis of the ethyl acetate crude extract was performed to detect the phytoconstituents present. The relative percentage of the phytoconstituents was expressed in terms of percentage by peak area normalization. Identification of compounds was based on the retention time and computer matching of the mass spectra with the standards.

Antioxidant Activity

The extracts were evaluated for antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay (Gulcin et al. 2006; Gulcin et al. 2010). The IC_{50} value of the extracts and the standard were calculated by obtaining a linear regression graph. The DPPH scavenging activity was calculated based on the following formula:

DPPH scavenging activity (%) = $[(A_0 - A_1) / A_0] X 100$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the plant extracts.

The hydrogen peroxide scavenging activity of the extracts was estimated using the procedure reported by Ruch et al. (1989). The IC_{50} value for the samples and the standard were measured from a linear regression graph. The antioxidant activity was calculated using the following formula:

H_2O_2 scavenging activity (%) = $[(A_0 - A_1) / A_0] X 100$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the plant extracts.

Anti-arthritic Activity

The water, ethanol and ethyl acetate extracts were evaluated for anti-arthritic activity using egg albumin denaturation method (Singh et al. 2011; Ashok Kumar et al. 2013). The anti-denaturation activity was calculated using the following formula:

% Inhibition = 100 x Absorbance of Test/ 1-Absorbance of Control

Membrane Stabilization Activity

The anti-inflammatory activity of the extracts was tested by HRBC membrane stabilization method (Azeem et al. 2010; Sakat et al. 2010; Leelaprakash and Dass 2011; Kar et al. 2012). The percentage of HRBC membrane stabilization was measured using the following formula:

Percentage of stabilization =

Absorbance of control - Absorbance of test/ Absorbance of control x 100

Statistical Analysis

All tests were performed in triplicates for each individual sample. Linear regression analysis was performed and the correlation coeffi-

cient r^2 was quoted. All results were expressed as mean ± S.D. One-way ANOVA was used to determine the significance. $P < 0.001$ was considered statistically significant.

RESULTS

Qualitative Phytochemical Screening

Phenols, flavonoids, phytosterols and terpenoids were observed in all the three extracts, which indicated that these phytoconstituents were extracted efficiently by all the three solvents. The presence of carbohydrates was observed in aqueous extract and ethanol extracts, which revealed that carbohydrates were not extracted by ethyl acetate. Saponins were detected only in aqueous extract, which indicated that saponins were extracted only by water and not by the other two solvents. The results are tabulated in Table 1.

Table 1: Qualitative phytochemical screening results

Phytoconstituents	*PTA*	*PTEA*	*PTE*
Alkaloids	-	-	-
Carbohydrates	++	-	++
Glycosides	-	-	-
Phytosterols	+	+	+
Phenols	+	+++	++
Tannins	-	-	-
Terpenoids	+	+++	+
Saponins	++	-	-
Flavonoids	+	+++	++

+++ = present in good quantity, ++ = present in medium quantity, + = present in low quantity, - = absent. PTA = Aqueous extract, PTEA = Ethyl acetate extract and PTE = Ethanol extract
Source: Authors

Quantitative Phytochemical Screening

Ethyl acetate extract was observed to have maximum phenol content of 107.16±1.27 mg/g followed by ethanol extract (89.12±1.63 mg/g) and aqueous extract (41.59±0.99 mg/g). The linear regression equation was employed to obtain the total phenolic content.

Aqueous extract presented the least flavonoid content of 72.53±1.32 mg/g preceded by ethanol extract (116.21±1.04 mg/g) and ethyl acetate extract (171.35±1.68 mg/g), respectively. The regression curve was used to obtain the flavonoid content.

The terpenoid content in aqueous extract was found to be the least that is, 45.61±1.70 mg/g. The terpenoid content in ethanol extract was 59.64± 1.44 mg/g, which was not very higher than aqueous extract. Ethyl acetate extract was detected to have the maximum content of 103.47±2.03 mg/g.

All the three extracts were observed to contain comparable contents of phytosterol. The total phytosterol content in aqueous, ethanol and ethyl acetate extract were 49.48±1.15 mg/g, 59.69± 1.58 mg/g and 74.40±0.91 mg/g, respectively. These outcomes suggested that the ethyl acetate extract contained maximum levels of all major phytoconstituents whereas the aqueous extract contained the least levels. The quantitative screening results are tabulated in Table 2.

GC-MS Analysis of Ethyl Acetate Extract

The ethyl acetate extract was examined using GC-MS, leading to the identification of 8 compounds. The compounds were identified using online NIST library search database. The chemical name, retention time and peak percentage value of the eight identified compounds are presented. The chemical constituents identified were N-hexadecanoic acid (RT - 17.81, Peak % - 15.16%), 3-fluorobenzoic acid (RT - 25.9, Peak % - 11.34%), 2-thiopheneacetic acid (RT – 19.46,

Table 2: Quantitative analysis and IC_{50} values

						IC_{50} *Values (µg/ mL)*	
Extract	*% Yield*	*TPC(mg/g)*	*TFC(mg/g)*	*TTC(mg/g)*	*TSC(mg/g)*	*DPPH*	H_2O_2
PTEA	11.64	107.16±1.27	171.35±1.68	103.47±2.03	64.40±0.91	63.36	64.60
PTE	31.04	89.12±1.63	116.21±1.04	59.64±1.44	59.69±1.58	72.94	76.98
PTA	27.68	45.61±1.70	72.53±1.32	41.59±0.99	49.48±1.15	88.83	88.71
Ascorbic acid	-	-	-	-	-	50.59	55.36

Note: All the values expressed as mean± standard deviation. PTA = Aqueous extract, PTEA = Ethyl acetate extract and PTE = Ethanol extract.
Source: Authors

Peak % - 10.83%), ß-sitosterol (RT – 28.64, Peak % - 9.78%), 5,8,11,14,17 Ecosapentanoic acid (RT – 27.32, Peak % - 6.87%), Methyl 9,12-Heptadecadienoate (RT – 19.41, Peak % - 6.84%), Ethyl-9,12,15-octadecatrienoate (RT – 19.67, Peak % - 4.59%) and Ethyl-9.Cis.,11.Trans-Octadecadienoate (RT – 19.62, Peak % - 3.69%).

Antioxidant Activity

All the extracts presented a gradual decrease in the absorbance with increasing concentration, which indicated that all the extracts could reduce the DPPH into a stable molecule. The three extracts demonstrated substantial activity when compared to standard. The IC_{50} value of ascorbic acid was established to be 50.59 µg/mL. The IC_{50} value of ethyl acetate, ethanol and aqueous extract was found to be 63.36 µg/mL, 72.94 µg/mL and 88.83 µg/mL, respectively. The IC_{50} values of the extracts are tabulated in Table 2. The antioxidant activity in terms of percentage inhibition and the IC_{50} values supported the potent antioxidant nature of the extracts compared to standard. The correlation between phenolic content and antioxidant activity using DPPH assay was established by a correlation graph ($r^2 = 0.98$).

The results of H_2O_2 assay demonstrated a similar result as the DPPH method. A gradual decrease in the absorbance was observed for all extracts with increasing concentration. The IC_{50} values for all the extracts and the standard were generated from the linear regression equation. The IC_{50} value obtained for ascorbic acid was 55.36 µg/mL. The IC_{50} value of ethyl acetate, ethanol and aqueous extract was 64.60 µg/mL, 76.98 µg/mL and 88.71 µg/mL, respectively. The IC_{50} values of the extracts are tabulated in Table 2. The correlation between phenolic content and antioxidant activity using H_2O_2 assay was established by a correlation graph ($r^2 = 0.92$).

The antioxidant activity of the extracts can be correlated with the total phenol and polyphenol content. The correlation between phenolic content and antioxidant activity was established individually for DPPH and H_2O_2 assay using a correlation graph. Ethyl acetate extract was observed to have maximum phenol and polyphenol content, hence it exhibited potent antioxidant activity followed by ethanol and aqueous extract, respectively.

Anti-arthritic Activity

Different extracts of *P. thyrsiformis* flower were tested for anti-denaturation activity and the results were compared with Diclofenac sodium (standard). Ethyl acetate extract exhibited potent results in comparison to ethanol extract and aqueous extract. However, when overall results were compared all the extracts exhibited significant results in a dose dependent manner. In some literatures anti-denaturation activity is related to polyphenolic and terpenoidal compounds. The presence of the two phytoconstituents in fairly good quantity in ethyl acetate extract, justifies its potent activity. The reduction in polyphenolic and terpenoidal content in ethanol extract and further in aqueous extract also justifies the decrease in activity in ethanol extract and further in aqueous extract. The anti-denaturation activity results are represented in Table 3.

Table 3: Anti-arthritic activity

S. No.	*Sample*	*Concentration (µg/ml)*	*Inhibition (%)*
1	*Diclofenac Sodium*	50	28.3
		100	66.18
		200	91.32
2	*Aqueous Extract*	50	11.21
		100	29.3
		200	53.37
3	*Ethanol Extract*	50	25.87
		100	43.83
		200	71.69
4	*Ethyl Acetate Extract*	50	33.35
		100	54.52
		200	84.46

Membrane Stabilization Activity

The membrane stabilization activity of *P. thyrsiformis* flower extracts were evaluated by HRBC membrane stabilization method. The rupturing of the RBC membrane was initiated by inducing heat. The inhibition of the rupturing of RBC cell membrane was measured for each extract and compared to standard Diclofenac sodium. The activity of all the three extracts were observed to be less than the standard. The activity gradually increased with increasing concentration for all extracts. Among the three extracts, ethyl acetate and ethanol extract exhibited potent activity than aqueous extract. The GC MS analysis revealed the occurrence of ß-sito-

sterol in ethyl acetate extract in a considerable amount. ß-sitosterol is reported to possess membrane stabilization activity, and hence its presence might lead to the better activity of the extract. The results are illustrated in Table 4.

Table 4: HRBC membrane stabilization activity

S. No.	*Sample*	*Concentration (µg/ml)*	*Inhibition (%)*
1	*Diclofenac Sodium*	50	51.48
		100	72.79
		200	88.05
2	*Aqueous Extract*	50	12.20
		100	36.54
		200	54.59
3	*Ethanol Extract*	50	24.52
		100	48.05
		200	66.21
4	*Ethyl Acetate Extract*	50	38.58
		100	55.74
		200	73.70

DISCUSSION

In this present work, the aqueous, ethanol and ethyl acetate extracts of the edible *Phlogacanthus thyrsiformis* (Hardow) Mabb. flowers were subjected to qualitative and quantitative phytochemical screening. The results exhibited the presence of phytosterols, phenols, terpenoids and flavonoids in all three extracts, carbohydrates in aqueous and ethanol extracts and saponins only in aqueous extract. Based on the qualitative phytochemical screening phenols, flavonoids, terpenoids and phytosterols were identified to be the major phytoconstituents present in all three extracts. Ethyl acetate extract was observed to contain a high concentration of all the four phytoconstituents whereas aqueous extract was observed to contain least concentration. The extracts were subjected to GC-MS analysis and using NIST library eight phytoconstituents were identified and reported for the first time in the ethyl acetate extract. Among the eight compounds, ß-sitosterol is known to possess membrane stabilization property. Further the extracts were evaluated for antioxidant, anti-arthritic and membrane stabilization activities. The increase or decrease in the concentration of terpenoid and flavonoid content greatly affected the activity of the extracts.

All the extracts demonstrated potent antioxidant activity when compared against the standard Ascorbic acid. The extracts exhibited good activity when tested for anti-denaturation and membrane stabilization activity, where Diclofenac sodium was taken as the standard. However, ethyl acetate extract demonstrated the best activity compared to ethanol and aqueous extract. The presence of different phytoconstituents was correlated with the biological activities. The potent antioxidant activity of the extracts can be accredited to the presence of phenols and flavonoids (Liu et al. 2009; Rengasamy et al. 2010). Based on literatures, phenols, flavonoids, terpenoids and phytosterols have been identified to possess anti-arthritic (Amer et al. 2013; Cui-Ping et al. 2014; Furtado et al. 2016; Zhao et al. 2016) and membrane stabilization activity individually. The phytosterol, ß-sitosterol has been reported to possess membrane stabilization activity, and hence its presence in ethyl acetate extract justifies its impressive activity (Ji et al. 2007; Shailaja and Anita 2011; Nícolas et al. 2015). Thus, the potent anti-denaturation and membrane stabilization activity of ethyl acetate extract can be accredited to the presence of terpenoids, flavonoids and the phytosterol that is ß-sitosterol in the extract.

The correlation between the occurrence of phenols and flavonoids and antioxidant activity was established by plotting a correlation graph. An evident correlation between the phenolic content and antioxidant activity was obtained for H_2O_2 assay ($r^2 = 0.929$) and DPPH assay ($r^2 = 0.98$). An evident correlation was also obtained between flavonoid content and antioxidant activity that is, for H_2O_2 assay ($r^2 = 0.997$) and DPPH assay ($r^2 = 0.956$). A very good positive correlation was also established between anti-denaturation activity and phenol ($r^2 = 0.992$), flavonoid ($r^2 = 0.945$), phytosterol ($r^2 = 0.998$) and terpenoid ($r^2 = 0.804$) content. The use of flavonoids and terpenoids against anti-arthritic activity is already reported, which is further supported by the correlation study. An effective positive correlation was observed between membrane stabilization activity and phenol ($r^2 = 0.947$), flavonoid ($r^2 = 0.992$), phytosterol ($r^2 = 0.965$) and terpenoid ($r^2 = 0.906$) content. The presence of ß-sitosterol (an anti-inflammatory agent) in the flower extract justifies the correlation between sterol content and membrane stabilization activity.

CONCLUSION

Phlogacanthus thyrsiformis (Hardow) Mabb. flowers, generally used in different recipes in northeast India, are rich in phenols, fla-

vonoids, terpenoids and sterols. Eight compounds were identified from the flower extract using NIST library search database. The ethyl acetate extract exhibited potent antioxidant activity compared to ethanol and aqueous extract. It also efficiently inhibited protein denaturation in egg albumin and stabilized HRBC membrane indicating effective anti-denaturation and membrane stabilization activity. This supports the traditional use of *Phlogacanthus thyrsiformis* (Hardow) Mabb. flowers against inflammatory and arthritic ailments. A strong positive correlation was established between the antioxidant, anti-denaturation and membrane stabilization activities and the phytoconstituents present. The potency of *Phlogacanthus thyrsiformis* (Hardow) Mabb. flower extracts as antioxidant, anti-denaturation and membrane stabilizing agent can be considered as the basis for further *in-vivo* testing and isolation of phytoconstituents.

RECOMMENDATIONS

The present study provides a platform for further *in vivo* studies for the development of alternate medicines. This study supports the traditional use of P. thyrsiformis flowers against arthritis and inflammation.

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