

ACTIVE PHENOLIC CONSTITUENTS AND BRINE SHRIMP LETHALITY ASSAY OF HYDROALCOHOLIC EXTRACT OF *PLECTRANTHUS AMBOINICUS*

DAYANA JANAKIRAMAN¹, PARAMESWARI CS^{2*}

¹Post Graduate and Research Department of Biochemistry, Bharathi Women's College, Chennai, Tamil Nadu, India. ²Principal, Government Arts College for Women, Ramanadapuram, Tamil Nadu, India. Email: cspresearch12@gmail.com

Received: 08 September 2016, Revised and Accepted: 25 October 2016

ABSTRACT

Objective: *Plectranthus amboinicus* (PA) leaves contain pharmaceutically important phytoconstituents that attribute to greater medicinal values. The purpose of this study was to investigate the active principles present in the potent hydroalcoholic extract of PA (HAPA) and brine shrimp lethality studies to understand the toxicity profile of PA, to unravel many potential applications in the field of science.

Methods: An approach toward high performance liquid chromatography (HPLC) screening of (HAPA) and selective partitioning using various solvents such as hexane, chloroform, ethyl acetate, and butanol were performed to identify the active principles present. Acute oral toxicity or brine shrimp lethality studies on *Artemia salina* L. using active fraction of PA were also attempted.

Results: The HPLC profile of ethyl acetate fraction of hydroalcoholic extract showed flavonoids such as Rutin, quercetin, luteolin and gallic acid, which was found to be considerably different from hydroalcoholic extract and this fraction also showed good acute oral toxicity effect and the LC50 value obtained was 198.630 µg/ml.

Conclusion: These results attribute to the pharmacological importance of the plant. Selective isolation of individual phytochemical constituents and subjecting it to biological testing will definitely give promising results for the medicinal field.

Keywords: High performance liquid chromatography, *Plectranthus amboinicus*, *Artemia salina* L., Brine shrimp, Phenolic constituents.

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INTRODUCTION

Herbal immunosuppression: Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity. Many plants used in traditional medicine have immunomodulating activities, where activation of T and B lymphocytes, macrophages, and apoptosis of immune effector cells play critical roles [1,2]. Herbal drugs have gained importance and popularity because of their safety, efficacy, and cost-effectiveness.

Plectranthus amboinicus (Lour) (PA) Spreng, commonly called as country borage or Indian borage is a native plant of Taiwan. It is a large succulent aromatic perennial herb, shrubby below, hispidly villous, or tomentose [3,4]. Juice of leaves mixed with sugar acts as a powerful aromatic carminative, given in colic and dyspepsia [5]. The antibacterial [6], antifungal, anti-inflammatory [7], antidiabetic, antioxidant, antinociceptive, antihelminthic, and hepatoprotective nature of PA were studied and reported by many scientists worldwide. PA is considered as a useful medicinal plant because it contains lots of bioactive compounds which actively play a role for immune response.

Medicinal plants are gaining importance due to their positive effects with no harmful results. Thus, to enhance the health status, with a deep insight of phytochemical constituents present in them, an attempt toward studying active phenolic constituents of hydroalcoholic extract of PA (HAPA) and acute oral toxicity bioassay on *Artemia salina* L. were performed.

METHODS

Collection and preparation of plant extract

Fresh leaves of PA were collected from a local garden in Chennai during the month of August. The leaves were authenticated by Prof. Dr. P Jayaraman, Director, Plant Anatomy Research Centre (PARC),

Chennai, India. A voucher specimen (No: PARC/2013/2063) was deposited in the institution.

About 1 kg of powdered dry leaves was incubated with 70% hydroalcohol for 48 hrs in a mechanical shaker. The extract was concentrated using a rotary evaporator, and the distilled solvent was collected. The crude extract was now reincubated with Hexane, chloroform, ethyl acetate and butanol successively in a separating funnel for 1 hr and the solvent phase formed as a separate layer was removed, respectively, and analyzed qualitatively for flavonoids (Shinoda and Lead acetate tests) [8,9].

High-performance liquid chromatography (HPLC)

The HAPA which possessed good anti-inflammatory, antioxidant capacity and notable phenolic content as reported in our earlier studies [10,11] was subjected to HPLC analysis for quantitative interpretation of compounds present in it. Later, the ethyl acetate fraction of HAPA (EAHAPA), which answered positively for flavonoids, was subjected to HPLC analysis for quantitative interpretation of compounds present in it with the same set of parameters.

C18 column of MERCK make was used for HPLC run. Mixture of alcohol, water and hydrochloric acid (50:20:8) was used as extraction solvent. Mixture of methanol, water and phosphoric acid was prepared in the ratio 100:100:1 and adjustments were made if necessary and used as mobile phase. Sample flow rate was at 1 ml/min. The injection volume was 20 µl and the eluate was monitored at 270 nm.

The HAPA and EAHAPA were injected under these conditions along with the authentic samples of rutin, quercetin, luteolin, kaempferol, and gallic acid (prepared as 1 mg/ml, respectively). The chromatograms obtained were recorded and areas of major peaks were measured. The percentages of each phenolic and flavonoid samples were calculated

using authentic standards. The purity of each identified peak was determined by comparison of the retention times and ultraviolet spectra at upslope and downslope inflexion points from available literature sources.

Acute oral toxicity studies using *A. salina* L.

Lethal toxicity assay an emerging new assay for determining oral toxicity is done based on the method of Meyer *et al.* and Solis *et al.* [12,13]. It is also done to assess cytotoxicity [13,14].

About 10 shrimps were transferred to each test tube and artificial sea water was added to make 5 mL. Samples were prepared at different concentrations and diluted appropriately and transferred to each tube. The nauplii can be counted macroscopically in capillary against a lighted background. The test tubes were maintained under illumination. Survivors were counted; with the aid of the $\times 3$ magnifying glass for every 3 hrs up to 24 hrs and the percentage death at each dose were determined. 24 hrs count was more useful.

The percentage of mortality in each concentration was determined, and LC50 values were obtained from the best fit line plotted with concentration versus percentage lethality using Microsoft Excel 2007 using linear regression analysis.

RESULTS

The HPLC analysis of the hydroalcoholic extract revealed the presence of various flavonoid compounds including quercetin, vitexin, rutin, luteolin, hispidulin (Fig. 1 and Table 1) and phenolic constituents including hydroxy benzoic acid, vanillic acid, gallic acid, coumaric acid, syringic acid, caffeic acid, and cinnamic acid (Fig. 2 and Table 2).

Further purification of hydroalcoholic extract using different solvents such as hexane, chloroform, ethyl acetate, and butanol based on polarity were done by successive extraction using separating funnel. Among which ethyl acetate fraction alone showed a significant presence of flavonoid compounds and was analyzed in HPLC column with the same set of parameters.

The HPLC profile of EAHAPA showed flavonoids such as rutin, quercetin, luteolin, and gallic acid (Fig. 3 and Table 3). The compounds were quantified and were expressed in mg/g units.

The results were also compared with the standard graphs of rutin, luteolin, gallic acid, galangin, etc., (Figs. 4 and 5).

Acute oral toxicity assay

Acute oral toxicity assay of EAHAPA was done with concentration ranging from 50,100,200,400 and 800 $\mu\text{g/ml}$, respectively.

Fig. 6 shows the graph of lethal toxicity assay for dosage fixation study plotted for concentration in $\mu\text{g/ml}$ of EAHAPA (X-axis) against % mortality (Y-axis) of brine shrimps and linear regression analysis done showing equation ($y=0.073x+35.50$) and $R^2=0.791$ value obtained for it. The LC50 value thus obtained is 198.630 $\mu\text{g/ml}$.

DISCUSSION

HPLC

The HPLC chromatograms of crude leaf extracts of PA indicated the presence of phenolics like caffeic acid, coumaric acid, rutin, quercetin and gallic acid, which were present in the range of 0.01-1.41 mg/g in ethyl acetate and 0.03-1.93 mg/g in the acetone extract [15]. Antibacterial compounds - 1, 2-Benzene diol 4-(1, 1 dimethyl ethyl), phytol, squalene and eudesma-4(14), 11-diene have been isolated from the acetone extract by gas chromatography-mass spectrometry (GC-MS) analysis done by Jasmine and Selvi, 2013 [16].

The chemical entities identified from plants vary according to geographical location, time and season of collection. Chemical entities including carvacrol, caryophyllene, thymol, eugenol, patchoulane,

chacicol, and flavonoids have been reported to be isolated from *Coleus aromaticus*. Monoterpenes and sesquiterpenes have been reported

Table 1: Flavonoid compounds present in HAPA

Flavonoids	Retention time (minutes)	mg/g
Quercetin	3.37	5.43
Vitexin	4.13	0.102
Rutin	8.17	1.264
Luteolin	10.19	1.05
Hispidulin	20.98	0.21

HAPA: Hydroalcoholic extract of *Plectranthus amboinicus*

Table 2: Phenolic compounds present in HAPA

Phenols	Retention time (minutes)	mg/g
Vallinic acid	2.78	2.12
Hydroxy Benzoic acid	3.76	1.44
Gallic acid	4.04	0.93
Coumaric acid	4.68	1.68
Syringic acid	5.91	1.22
Caffeic acid	6.91	2.26
Cinnamic acid	11.68	0.28

HAPA: Hydroalcoholic extract of *Plectranthus amboinicus*

Table 3: Flavonoid compounds present in EAHAPA

Flavonoids	Retention time (minutes)	mg/g
Quercetin	3.19	0.536
Gallic acid	4.08	0.327
Rutin	7.47	0.059
Luteolin	10.18	0.34

EAHAPA: Ethyl acetate fraction of hydroalcoholic extract of *Plectranthus amboinicus*

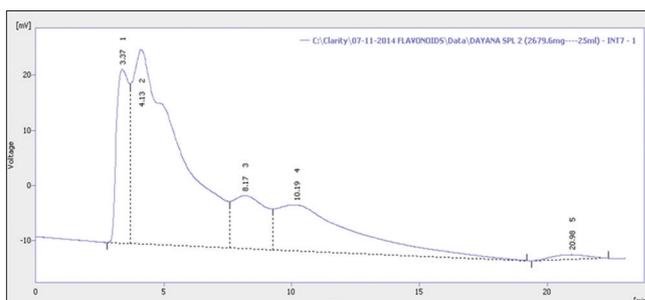


Fig. 1: High performance liquid chromatography chromatogram showing flavonoids of hydroalcoholic extract of *Plectranthus amboinicus*

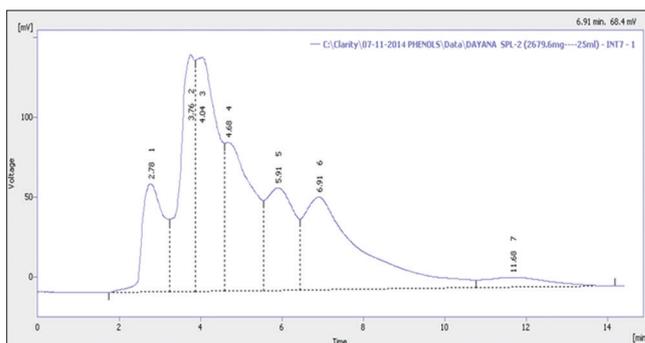


Fig. 2: High performance liquid chromatography chromatogram showing phenolic compounds of hydroalcoholic extract of *Plectranthus amboinicus*

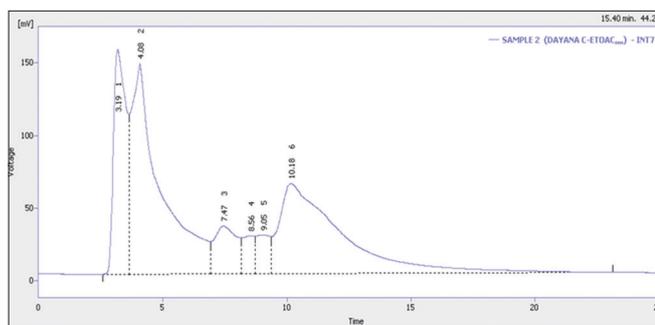


Fig. 3: High performance liquid chromatography chromatogram of ethyl acetate fraction of hydroalcoholic extract of *Plectranthus amboinicus*

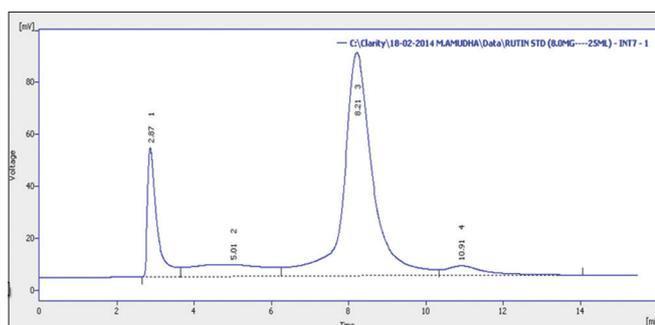


Fig. 4: High performance liquid chromatography chromatogram of standards rutin and luteolin

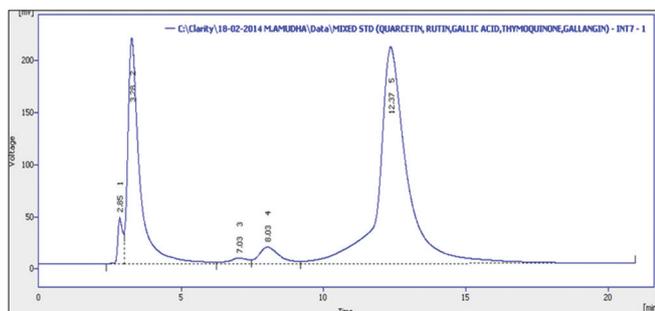


Fig. 5: High performance liquid chromatography chromatogram of standards quercetin, galangin, gallic acid and thymoquinone

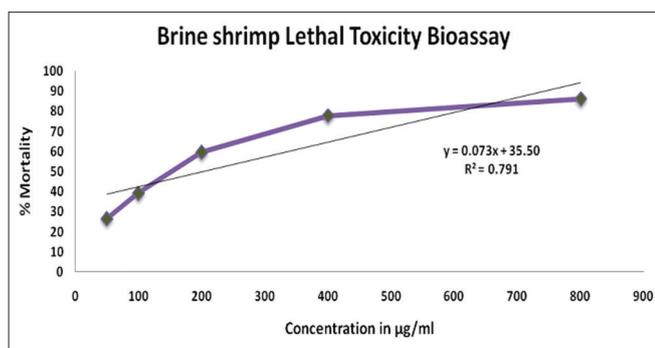


Fig. 6: Lethal toxicity assay of brine shrimp and linear regression analysis plot showing the R^2 value and the equation of the line

from *C. aromaticus* which had antimicrobial activity including limonene, linalool, myrcene and thymol also, alpha-amorphene, beta cubebene, and phenolics [17].

GC-MS characterization of essential oils from roots, stem and leaves of two *Plectranthus* genus (*Plectranthus barbatus* and *Plectranthus caninus*) cultivated in northern Italy were done by steam distillation and chemical structures were characterized. The leaves showed the second highest yield (64.34 and 26.65 mg/kg) followed by roots and stems. 128 and 121 structures were identified respectively [18].

Leaves of *C. aromaticus* contain flavones salvigenin, 6-methoxygenkwanin, quercetin, chrysoeriol, luteolin and apigenin, the flavanone eriodyctol and the flavanol taxifolin, triterpenic acids; oleanolic acid, 2,3-dihydroxyoleanolic acid, crategolic acid, ursolic acid, pomolic acid, euscaphic acid, tormentic acid, and 2,3,19,23-tetrahydroxyursolic acid as reported by Sahaykhare *et al.* [19]. The major compounds including carvocrol, thymol, Cis-caryophyllene, t-caryophyllene, and p-cymene were reported by GC-MS analysis of PA essential oil [20].

Acute oral toxicity assay

The percentage of mortality increases with the increase in concentration of test samples. Brine shrimp lethality bioassay (BST) is an efficient, rapid and inexpensive assay for testing the bioactivity of plant extracts. It is an excellent choice for elementary toxicity investigations based on the ability to kill laboratory-cultured *Artemia nauplii* [21]. A positive correlation between the lethality to brine shrimp and the corresponding oral lethal dose in mice of medicinal plants has also been demonstrated [22].

Similar results were observed in *Elaeis guineensis* methanolic extract [23] after 24 hrs was 3.87 mg/ml. Sahqal *et al.*, 2010, [24] also studied for brine shrimp lethality on *Swietenia mahagoni* (Linn.) Jacq. seed methanolic extract and analyzed moderate cytotoxicity at high concentration with LC50 value for the extract was 0.68 mg/ml.

CONCLUSION

This study has been found useful, where a variety of active compounds have been found in HAPA. The presence of various bioactive compounds such as vanillic acid, hydroxy benzoic acid, Gallic acid, Coumaric acid, syringic acid, caffeic acid and cinnamic acid, and flavonoids including quercetin, vitexin, rutin, luteolin and hispidulin, essential oils, terpenoids, sugars, aromatic aldehydes justifies the use of the plant for various ailments by traditional practitioners. The analysis time is an important factor in analytical work and the run time should be reduced to a minimum to optimize equipment use and reduce solvent consumption [25].

It could be concluded that PA plant is of phytopharmaceutical importance. However, isolation of individual phytochemical constituents and subjecting it to biological testing will definitely land down in promising results.

In this study, the brine shrimp lethality bioassay was performed to assess the preliminary toxicity of PA and dosage of 200 mg/ml ($\times 1000$) was planned for further *in vivo* animal studies. Even though the brine shrimp test does not provide adequate information regarding the mechanism of action of the plant extracts it is useful to assess the toxicity from the LC50 value obtained and indicates the cytotoxic nature of the plants and warrants further investigation [26].

ACKNOWLEDGMENTS

The authors thank for the financial support provided by DST_INSPIRE fellowship (AORC scheme) and Department of Biochemistry, Bharathi Women's College for the lab facilities.

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