ANALYSIS OF THE METHANOLIC EXTRACT OF THE SHOOT OF *PLECTRANTHUS HADIENSIS*Darsan B. Menon¹, Sasikumar J.M¹, Gopalakrishnan V.K^{2*}¹Department of Biotechnology, Karpagam University, Coimbatore, Tamilnadu, India²Department of Biochemistry and Bioinformatics, Karpagam University, Coimbatore, Tamilnadu, India

*Corresponding Author Email: vkgopalakrishnan@gmail.com

Article Received on: 11/03/13 Revised on: 09/04/13 Approved for publication: 01/05/13

DOI: 10.7897/2230-8407.04549

IRJP is an official publication of Moksha Publishing House. Website: www.mokshaph.com

© All rights reserved.

ABSTRACT

The herbal renaissance has been taking place throughout the world over the last few years. Over the period, herbal medicines have moved from an empirical basis to its present as an increasingly scientifically based system of healing, being referred as phytotherapy. Although the traditional medicinal systems adopt a holistic approach while using herbs or their combinations, knowledge of individual constituents facilitates in developing quality control methods, extraction procedures, understanding of pharmacological activity and pharmacokinetics. In tune with this effort, our current study focuses on the phytochemistry of the methanolic extract of the shoot part of *Plectranthus hadiensis*, a plant used in Ayurvedic preparations as well as other traditional systems of medicine. The preliminary phytochemical studies had revealed the presence of terpenes in the extract. TLC, HPTLC, GC-MS and NMR studies confirmed the presence of mono-, sesqui- and diterpenes in the extract. The terpene-rich methanolic extract when tested for its cytotoxicity using brine shrimp lethality assay showed an LC₅₀ of 145µg/mL. The presence of terpenes thus supported the use of this plant in various medicinal preparations.

Key words: *Plectranthus hadiensis*, GC-MS, NMR, Brine shrimp assay.

INTRODUCTION

Knowledge of medicinal plants used in traditional system of medicines has always guided the search for new therapeutic molecules. These herbs used in traditional medicinal systems are often cheap, locally available and easily consumable, either raw or as multi-herbal preparations¹. Nowadays, traditional medicinal practices form an integral part of complementary or alternative medicine. Ayurveda, one of the major traditional forms of medical practice in India, has produced many useful leads in developing medications for chronic ailments. *Plectranthus hadiensis* (Hribra) is one such herb that is widely used in Ayurvedic preparations. This plant is used in various Ayurvedic formulations for treating inflammation and related ailments². Our previous studies have shown potential anti-oxidant, anti-inflammatory and anti-proliferative activity of the extract^{3,4}. Several studies have shown the presence of terpenes in solvent extracts and essential oil extracts obtained from the plant^{5,6}. Our present study focuses on the chromatographic profiling of the terpenes present in the methanolic extract of this plant using TLC, HPTLC, GC-MS and NMR, and also checking the cytotoxicity of the extract using brine shrimp lethality assay.

MATERIALS AND METHODS

The shoot part of the plant was collected from Kerala, India and authenticated by Dr. Kunhikannan, Scientist E, IFGTB, Coimbatore, India (Voucher specimen no: IFGTB-27/07/11-001). The sample was shade dried, pulverized and extracted with methanol using soxhlet apparatus. The crude extract was concentrated using rotary flash evaporator (Buchi type). This extract was used for the chromatographic profiling and different concentrations of the extract were used for assessing the cytotoxicity.

TLC

Thin layer chromatography was performed to generate a chromatographic profile of the methanolic extract. 20µL of the extract was applied on the plate and developed to a distance of 8cm. The solvent systems, Ethyl acetate:

Chloroform: Methanol (4:3:3) and Methanol: Chloroform (9:11) were selected after standardization. After development, the plate was allowed to dry in air and sprayed with Sulphuric acid to detect the terpene-rich compounds⁷.

HPTLC

A densitometric HPTLC analysis was performed for the development of characteristic fingerprinting profile. The *Plectranthus hadiensis* methanolic extract (PV) of shoot was dissolved with HPLC grade methanol (100mg/0.5mL). The solution was centrifuged at 3000rpm for 5minutes. Then, 2µL of the sample solution was loaded as 5mm bands on the 3 x 10 Silica gel 60F₂₅₄ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample loaded plates were kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (for Terpenes) and the plate was developed in the respective mobile phase up to 90mm. The developed plates were dried by hot air to evaporate solvents from the plates. The plates were kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm. The plates were then sprayed with LB reagent to detect the presence of steroids and terpenes⁷.

GC-MS

The extract was analyzed using a Thermo GC-Trace Ultra Ver: 5.0, Thermo MS DSQ II (Column: 30mts, ID: 0.25mm, Film: 0.25µm) with He as carrier gas. Flow rate was fixed at 1mL/min. The oven temperature was programmed from 140°C-260°C with a total run time of 47mins⁷.

NMR

The ¹H and ¹³C NMR spectrum of the methanolic extract was recorded on a Bruker type AV300 at 300MHZ and the chemical shifts were recorded as δ values. The solvent used was CDCl₃^{8,9}. The result graph was compared with the reference chart and possible functional groups present were determined.

Figure 1: TLC chromatogram of methanolic extract (Sol sys- EA: Chlrfm: Meth, 4: 3: 3)

Figure 2: TLC chromatogram of methanolic extract (Sol sys- Chlrfm: Meth, 9: 11)

Figure 3: HPTLC chromatogram of the methanolic extract

Figure 4: Gas chromatography chromatogram of the methanolic extract

Figure 5: Peak fragmentation of GC-MS spectrum (RT - 15.44)

Figure 6: Peak fragmentation of GC-MS spectrum (RT - 24.49)

Figure 7: Peak fragmentation of GC-MS spectrum (RT - 36.46)

Figure 8: Peak fragmentation of GC-MS spectrum (RT - 41.87)

Figure 9: ^1H -NMR spectrum of the methanolic extract Figure 10: ^{13}C -NMR spectrum of the methanolic extract Figure 11: Brine shrimp lethality assay of methanolic extract of *Plectranthus hadiensis* against *A. salina*

Brine shrimp lethality assay

Dried *Artemia* cysts (1gm = 1.5-2 lakh cysts per liter) were taken and incubated in a hatcher in artificial sea water at 28-30°C with continuous aeration under light regime. Approximately 12 h after hatching, the live nauplii were collected with a pipette and transferred into small vials. Each test consisted of exposing groups of 10 *Artemia* nauplii aged 12 h to various concentrations of the extract (50, 100, 250, 500 and 1000 µg/mL) which was dried and redissolved in artificial sea water. Artificial sea water was taken as control. The lethality was determined after 24 h (nauplii in instar II/III) of exposure¹⁰. The numbers of survivors were counted and percentage of death was calculated. Larvae were considered dead if they did not exhibit any internal or external movement towards light (phototropic movement) 30 seconds. The larvae did not receive food, to ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not to starvation; we compared the dead larvae in each treatment to the dead larvae in the control. In any case hatched brine shrimp nauplii can survive for up to 48 h without food because they still feed on their yolk-sac^{11,12}.

The percentage of mortality (% M) was calculated using the formula:

$$\% M = \frac{\text{Total number of larvae used in each concentration} - \text{total number of larvae alive in each concentration}}{\text{Total number of larvae used in each concentration}} \times 100$$

RESULTS

TLC

Ethyl acetate: Chloroform: Methanol (4: 3: 3) and Methanol: Chloroform (9: 11) were used as solvent systems which showed good resolution. Five bands were visualized. The bands became more prominently visible after the plate was sprayed with sulphuric acid. Bluish-green colored bands were observed in both the solvent systems indicating the presence of terpene-rich compounds. This TLC chromatogram can be used as a reference standard for further separation and isolation of compounds from the plant (Figures 1 and 2).

HPTLC

The HPTLC chromatogram of the methanolic extract confirmed the presence of terpenoids after they were sprayed with LB reagent after plate development. The various bands with shades of blue reveal the presence of terpenes in the extract (Figure 3). The selection of the mobile phase was based on the adsorbent material used as stationary phase and physical and chemical properties of the analyte used.

GC-MS

The GC-MS analysis of the shoot extract of *Plectranthus hadiensis* was carried out to identify the nature of the components present. The GC-MS chromatogram showed the presence of four major components at retention times 15.44, 24.49, 36.46 and 41.87 minutes (Figure 4). The respective fragmentation patterns are shown in the figures 5, 6, 7 and 8

respectively. The mass spectrum of the compound with retention time 15.44 gave two major peaks (m/z) at 105.0 and 132.9. The element combinations for these peaks show probability for $C_9H_{10}O$. The above molecular formula indicates the possibility of an aromatic ring containing compound that could be a terpene. The compound with retention time 24.49 gave three major peaks (m/z) at 76.9, 103.0 and 163.9. The combinations show probability for $C_{10}H_{12}O_2$, indicating the possibility of styrene or phenolic – OH group attached compound. The compound with retention time 36.46 gave two major peaks (m/z) at 130.7 and 163.9 again corresponding to a compound with formula $C_{10}H_{12}O_2$, which corresponds to either an aromatic phenol group or eugenol or styrene. The last compound with retention time 41.87 gave single major peak (m/z) at 148.8. The combinations for the major and minor peaks show probability for $C_{12}H_{14}O_4$, indicative of the possibility of a diethyl ester compound. Therefore, it may be concluded that the compound with these characteristic retention times and fragmentation patterns could be categorized into the class of terpenes.

NMR

The ^1H -NMR spectrum of the extract exhibits signals between 2 and 3 indicative of a methyl group and presence of an aromatic ring. The signals between 3 and 4 indicate the presence of a CH_2 group. The quartet signals between 6 and 7 indicate the presence of CH group as well. Consistent with the ^1H -NMR spectrum, the ^{13}C -NMR spectra exhibited signals at δ 76-77 (indicating presence of C-O), 121-125 (indicating presence of C=C), 132-137 (indicating presence of aromatic rings) and 149-156 (again indicating presence of aromatic rings) (Figures 9 and 10). These signals confirmed the presence of compounds with aromatic ring, indicating towards the occurrence of terpene-rich compounds in the extract.

Brine shrimp assay

In the Brine Shrimp lethality assay with varying concentration of the extract ranging from 50-1000 $\mu g/mL$, the LC_{50} was found to be 145 $\mu g/mL$ (Figure 11). The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity (10). For the past 30 years, the *Artemia* nauplii have been used to detect general toxicity, in teratology screens and in ecotoxicology¹³.

DISCUSSION

Various chromatographic techniques are used nowadays for separation and isolation of compounds from crude plant extracts. Thin layer chromatography and Gas chromatography are very effective and have been used for getting consistent separation of compounds from crude extracts. Thin layer chromatography was used to separate the individual chemical constituents of the methanolic extract of the shoot of the plant. Analysis of phytochemicals present in this plant has been reported by us and was found to be rich in terpenes. Essential oil extracted from the leaves and root of this plant have been reported to be rich in mono-, sesqui- and diterpenes^{5,6}. Thus the TLC specific for separating terpene-rich compounds were used and good separation of the compounds were observed. HPTLC is an enhanced form of TLC with relative independence in sample application, chromatogram development and detection. In the present study, the chromatogram after development and spraying

with the LB reagent showed the presence of steroids and terpenes in the methanolic extract, thus confirming their presence similar to the result obtained in TLC. GC separates volatile and semivolatile compounds with great resolution and MS provides structural information on most compounds such that they can be exactly identified. The methanolic extract of the plant, which was found to be rich in terpenes when injected, was separated into its various components. These components were identified using the mass spectrum generated and concluded to be aromatic terpenoid compounds. NMR is one of the most powerful tools available for determining the structure of organic compounds. This technique relies on the ability of atomic nuclei to behave like a small magnet and align themselves with an external field. The NMR spectrum of the methanolic extract revealed the presence of carbon atoms having double bonds with other carbon atom and also single bond with oxygen. The presences of methyl and methylene groups were also confirmed along with aromatic rings which showed that the extract was rich in terpenes¹⁴.

From a pharmacological point of view, a good relationship has been found with the brine shrimp assay to detect cytotoxic and anti-tumoral compounds in terrestrial plant extracts. The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts¹³. The present study thus employed brine shrimp bioassay as a reliable method for the assessment of bioactivity of plant extract and lends support for their use in pharmacology. The activity at lower concentration suggests the presence of compounds which can be used as potential source for cytotoxic or anti-proliferative drugs.

CONCLUSION

Earlier studies performed using the methanolic extract of shoot of *Plectranthus hadiensis* had revealed its bioactive potential viz., antioxidant, anti-inflammatory and anti-proliferative activity. The present study aimed at identifying the components responsible for its activities. The methanolic extract showed the presence of terpene-rich compounds which was confirmed using TLC, HPTLC, GC-MS and NMR studies. The brine shrimp assay showed the cytotoxic potential of the extract which substantiates our earlier studies of anti-proliferative activity of the extract. Further isolation and characterization of the individual components are necessary to corroborate the use of this plant in the traditional systems of medicine.

ACKNOWLEDGEMENT

We, the authors are thankful to Chancellor, Chief Executive Officer, Vice-Chancellor and Registrar of Karpagam University for providing facilities and encouragement.

REFERENCES

1. Amin KA, Nagy MA. Effect of Carnitine and herbal mixture extract on obesity induced by high fat diet in rats. Diabetology and metabolic syndrome. 2009; 1: 17-31. <http://dx.doi.org/10.1186/1758-5996-1-17> PMID:19835614 PMCID:2772188
2. Sivarajan VV, Balachandran I. Botanical notes on the identity of certain herbs used in Ayurvedic medicines in Kerala. III. *Hribera* and *Amragandha*. Ancient Science of Life. 1986; 5: 250-254. PMID:22557534 PMCID:3331476
3. Menon DB, Sasikumar JM, Latha K. Anti inflammatory and cytotoxic activity of methanolic extract of *Plectranthus hadiensis* stem. Pharmacologyonline. 2011; 3: 275-282.
4. Menon DB, Sasikumar JM, Latha K. Phytochemical analysis and antioxidant activity of methanolic extract of *Plectranthus hadiensis* (Forssk) Schweinf. ex. Spreng. Aerial parts. Indian J Nat prods resour. 2012; 3: 359-365.

5. Mehrotra R, Vishwakarma RA, Thakur RS. Abietane diterpenoids from *Coleus zeylanicus*. *Phytochemistry*.1989; 28: 3135-3137. [http://dx.doi.org/10.1016/0031-9422\(89\)80293-6](http://dx.doi.org/10.1016/0031-9422(89)80293-6)
6. Lukhoba CW, Simmonds MSJ, Paton AJ. *Plectranthus*: A review of ethnobotanical uses. *J. Ethnopharmacol.* 2006; 103: 1-24. <http://dx.doi.org/10.1016/j.jep.2005.09.011> PMID:16289602
7. Harborne JB. *Phytochemical methods to modern techniques of plant analysis*. Chapman & Hall, London, 1984. <http://dx.doi.org/10.1007/978-94-009-5570-7> PMCID:1193218
8. Goto H, Usuki T. H-NMR analysis of terpene trilactones (TTLs) in *Ginkgo biloba*: green female leaves contain the most TTLs. *Phytochem anal.* 2012;23:84-87.<http://dx.doi.org/10.1002/pca.1328> PMID:22144104
9. Mills NS. Complete assignment of proton chemical shifts in terpenes: An experiment combining 2D NMR spectroscopy with molecular modeling. *J of Chem Educ.* 1996; 73: 1190. <http://dx.doi.org/10.1021/ed073p1190>
10. McLaughlin JL, Chang CJ, Smith DL. Bench-top bioassays for the discovery of bioactive natural products: an update. *Studies in Natural Products Chemistry*.1991; 9: 383-409.
11. Lewis GE. Testing the toxicity of extracts of Southern African plants using brine shrimp (*Artemia salina*) *S Afr J Sci.*1995; 91: 382-384.
12. Pelka M, Danzl C, Distler W, Petschelt A. A new screening test toxicity testing of dental materials. *J Dent.* 2000; 28: 341-345. [http://dx.doi.org/10.1016/S0300-5712\(00\)00007-5](http://dx.doi.org/10.1016/S0300-5712(00)00007-5)
13. Carballo JL, Hernandez-Inda ZL, Perez P, Garcia-Gravalos MD. A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnology.* 2002; 2: 17- 21. <http://dx.doi.org/10.1186/1472-6750-2-17> PMID:12270067 PMCID:130034
14. Silverstein RM, Webster FX, Kiemle DJ. *Proton NMR spectrometry. Spectrometric identification of organic compounds*. USA: John Wiley and Sons, Inc.; 2005. p. 127-204

Cite this article as:

Darsan B. Menon, Sasikumar J.M, Gopalakrishnan V.K. Analysis of the methanolic extract of the shoot of *Plectranthus hadiensis*. *Int. Res. J. Pharm.* 2013; 4(5):233-237

Source of support: Nil, Conflict of interest: None Declared