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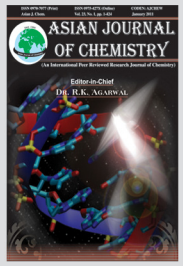

Isolation, Characterization and Antibacterial Evaluation on Long Chain Fatty Acids From *Limnophila polystachya* Benth.

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Limnophila polystachya Benth. is a herb belongs to the family *Scrophulariaceae*; widely used in traditional Indian medicine for various debilitating diseases such as pestilent fever, dysentery, elephantiasis, dyspepsia and also exhibits significant antibacterial, antifungal and antineoplastic activities. Preliminary photochemical screening on this plant showed that the presence of alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids. The ethanolic extract of *Limnophila polystachya* was subjected to thin layer and column chromatography using petroleum ether:ethyl acetate in the ratio of (3.75:1.25) as solvent system for the isolation of bioactive phytoconstituents obtained after fractional crystallization using silica gel (60-120) mesh. The colourless crystalline compounds further purified using chromatographic methods and elucidated with spectral techniques such as UV, IR, ^{1}H NMR, ^{13}C NMR, elemental analysis and LCMS. The amorphous crystalline compound was elucidated as CH_3-$(CH_2)_{30}$-COOH (Do-triacontanoic acid, Syn: lacceroic acid). Antibacterial activity was assessed on isolated crystals against gram negative and positive bacterial pathogenic organism. The elucidated crystals showed that significant activity against the pathogenic organisms at the diameter 600 and 1000 µg/mL at significant zone of inhibition. Lacceroic acid has been exhibited antibacterial activity against different pathogenic organism.

Key Words: *Limnophila polystachya*, NMR, IR, UV, LC-MS.

INTRODUCTION

Limnophila polystachya Benth. belongs to the family *Scrophulariaceae* is an herb and rarely trees, mostly autotrophic and less often hemi parasitic. Stipules are absent; leaves are alternate, opposite, whorled or basally opposite and apically alternate (ZipcodeZoo.com). The plant species widely used in traditional Indian medicine in the treatment of pestilent fever, dysentery, elephantiasis, dyspepsia, antipyretic, expectorant, lactogogue and also exhibits significant antibacterial, antifungal and antineoplastic activities[1]. The *Limnophila polystachya* has been reported for its antitumor promoting agent, antimutagenic[2], antimycobacterial[3] and antioxidant activity[3,4]. The plant genus of species *Limnophila polystachya* has not been reported any phytochemical investigation and further logistic pharmacological actions further its no more revealed that any work in this clinical investigation. In the past studies the photochemical studies reported that *Limnophila indica* resulted the isolation of 5-hydroxy-6,8-dimethoxy-3',4'-methylenedioxyflavone[5] and 5,8-dihydroxy-6,7,4'-trimethoxyflavone[6]. The essential oil has been isolated from *Limnophila rugosa*[7]. Two novel flavonoids have been reported on *Limnophila indica*[8]. Long chain fatty acid isolated from *Limnophila indica* possess immense antimicrobial activity against some gram positive and gram negative organisms[9]. The present study described the isolation and structural characterization of bioactive constituent of the elucidation of the crystals from the ethanolic extract of *Limnophila polystachyea* (EELP) and evaluated its antibacterial activity on different pathogenic organism.

EXPERIMENTAL

Limnophila polystachya Benth. (*Scrophulariaceae*) was collected from Samayapuram, Tiruchirappalli District in Tamil Nadu in the month of September-November 2008 and identified by the taxonomists in Botanical Survey of India, Coimbatore Tamil Nadu, India. The voucher specimen Number is BSI/SC/5/23/08-09/Tech.275. After authentication, fresh plant was collected in bulk washed, shade dried and pulverized in a mechanical grinder to obtain coarse powder.

Pharmacological activity of *Limnophila* species: Flavones and the fatty acid derivative isolated from *Limnophila geoffrayi* exhibits antimycobacterial, antioxidant properties and mutagenic activities. The inhibition activity was found against *Mycobacterium tuberculosis*. Significant antioxidant activity was found against the radical scavenging ability of DPPH

model. Mutagenic activity was found in the isolated fatty acid derivatives by well diffusion method[3].

Extraction and isolation: The powdered plant (900 g) was successively extracted with ethanol for 48 h in a Soxhlet extractor. Following extraction, the liquid extracts were concentrated under vacuum to yield 9.5 g of dry extracts. The dried extract was subjected to preliminary photochemical screening of the different solvents to know the nature of phytoconstituents present in ethanolic extract of *Limnophila polystachya*. The ethanolic extract of *Limnophila polystachya* upon concentration under reduced pressure left a dark green sticky and oily residue (5.7 %). It gave positive reaction when titrated against sodium hydroxide using phenolphthalein as an indicator. In TLC examination of the extract showed eight spots (solvent system:petroleum ether : ethyl acetate in the ratio of 3.75:1.25) on spraying with vanillin sulphuric acid. The ethanolic extract of plant was subjected to column chromatography over silica gel (60-120 mesh size) using petroleum ether:ethyl acetate, in the ratio of 3.75:1.25 taking 250 mL fraction. Then purification was done by fractional crystallization using acetone and methanol yielded 20 mg of compound.

Phytochemical screening: Qualitative phytochemical screening was performed as per the standard protocol to identify the different classes of compounds present in the crude extract[10].

Thin layer chromatography: Analytical TLC was performed on the precoated aluminum TLC plates with silica gel 60 F254 (Merck, 0.25 mm) spots were detected by UV (254, 366 nm) or by vanillin-H_2SO_4 (1.2 g of vanillin dissolved in 212.48 mL MeOH + 25 mL acetic acid + 11 mL H_2SO_4 drop by drop) and 1 % $Ce(SO_4)_2$-10 % aqueous H_2SO_4 followed by heating. The different solvent system was repeatedly used to select the best solvent system for the isolation of compounds present in the plant extract.

Column chromatography: Column chromatography was carried out on silica gel 60-120 mesh (Merck), sephadex LH 20 (25-100 μm) (Merck); column size: 90 cm length and 3 cm width. The solvent system was used as petroleum ether:ethyl acetate in the ratio of 3.75:1.25. Flow rate of the solvent 30 mL/min. After collecting the fractions TLC was performed for finding the compound identification and elucidation.

UV spectrum was measured in the UV Shimadzu 1800 series in the wavelength range of 250 to 400 nm using ethanol as a solvent. IR spectroscopy was measured on a Bruker IFS 28 infrared spectrophotometer as KBr pellets. The wavelength is indicated in cm^{-1}. The IR spectra were measured in the wavelength range of 4000-500 cm^{-1}. The ^1H and ^{13}C NMR spectrum was recorded by using deuterated chloroform as a solvent tetramethyl silane (TMS) was used as an internal standard using Astra OH0210 model. The intensity of each peak was measured.

Melting points were obtained on a VMTG apparatus (Leica, Germany). The specific rotation was measured with a JASCO DIP-1000 polarimeter. LC-MS spectroscopy measurements were performed in LC-MS-2010, Shimadzu, Japan. The following conditions were maintained for analysis of the sample. The elemental analysis was performed in a Flash EA 1112 series using 1.328 mg of the amorphous crystalline. Every elements has been measured their percentage and the retention time.

HPLC conditions: (a) Column: C18; (b) Mobile phase: methanol:water in the ratio of 90 :10; (c) Flow rate: 0.2 mL/min; (d) Injected volume: 5 μL; (e) The sample is dissolved in methanol; UV visible absorbance wave number 254 nm.

Mass spectroscopy conditions

APCI: Atmospheric pressure chemical ionization mainly used to analyze the non polar compounds.

ESI: Electron spray ionization mainly used to analyze the polar compounds. Positive ionization gives protonated M+1 value; negative ionization gives deprotonated M-1 values. Positive or negative ionization and the type of probe can be identified in data file name. In presence of halogens the values will show M and M+2 in positive, M and M-2 in negative.

Adduct ions: In presence of sodium salts are M+23 adduct ions; methanol as mobile phase are showed that M+32 adduct ions. In some cases both M+55 will form the adduct ions mainly showed in electron spray ionization (ESI); dimerization shows 2M+1 or 2M+23.

Antibacterial activity of EELP elucidated amorphous crystalline: In the present study, the pathogenic microbial strains scuh as *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aerogenosa*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Salmonella typhi*, *Salmonella paratyphi*, *Bacillus polymizia*, *Aeromonas hetrophylla* and *Vibrio cholera* were taken for performing the antibacterial activity of the isolated long chain fatty acids.

Disc diffusion method: The disc diffusion test was performed using the procedure described by Jorgensen *et al.*[11]. The inoculum suspension of each microbial strain was swabbed on the entire surface of Mueller-Hinton agar media. The compound was dissolved in dimethyl sulfoxide to get the following concentrations 500 μg/mL, 800 μg/mL, 1000 μg/mL and were impregnated in sterile 6 mm filter paper discs and were aseptically placed. Penicillin G (10 unit/mL) was used as standard drug. The plates were left at ambient temperature for 15 min to allow excess pre-diffusion of the solution of drug prior to incubation at 37 °C for 24 h. Diameters of zones of inhibition were measured and it was done in triplicate.

RESULTS AND DISCUSSION

Phytochemical evaluation gives basic foundation on the structure elucidation of compounds present in the plant extracts. The qualitative phytochemical evaluation was done as per the standard procedure. The ethanolic extract of *Limnophila polystachya* contains alkaloids, glycosides, flavanoids, steroids, terpenoids, gums and mucilage, reducing sugars and starch reported to possess various therapeutic properties. The presence of wide range of phytochemical constituents indicates that plant could serve as lead for the development of novel agents for various pathological disorders[12]. Contents of phytoconstituents in ethanolic extract of the plant varied with the plant organ and solvent system used. The presence of phytoconstituents in the ethanolic extract of *Limnophila polystachya* was confirmed through TLC. The solvent system was selected based on the number of phytoconstituents separated on the TLC plate[13]. On the other hand, the running distance depends on the solubility of the component in the solvent. Since TLC employs a high non-polar solvent (petroleum ether), the components that are

least polar (long chain fatty acids) will be the best solvent in the non-polar solvent ethyl acetate and thus have the largest running distance. From the selected solvent system petroleum ether: ethyl acetate in the ratio of (3.75:1.25) was selected as a best solvent system, because of more number of phytoconstituents were separated in the form of bands. After the collection the first fraction in TLC was concluded that only single compound is separated range 0.8666. The different components in the sample mixture pass through the column at different rates due to various partitioning behaviour between the mobile liquid phase and the stationary phase. Then the fraction was further purified by fractional crystallization yielded colourless crystals. The structure of the isolated compound as crystals were elucidated based on the chromatographic and spectral data[14]. The UV spectrum of the isolated crystals showed that λ_{max} at 246 nm and 320 nm for a $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transition indicated the presence of a chromophoric group probably (C=O). The $\pi \rightarrow \pi^*$ and the $n \rightarrow \pi^*$ transitions occurred in the near UV and visible region (180-700 nm). Molecule exhibits the $n \rightarrow \pi^*$ transitions were found in the UV and visible region, the transitions suggest that the presence of C=O, N=O, N=N in the elucidated crystals. The IR spectrum showed a C-H stretching bands at 2920, 2850 cm^{-1} and C-H bending bands at 1464, 1350 cm^{-1} that are characteristic indication of presence of aliphatic hydrocarbon; O-H carboxylic stretching bands at 3373 cm^{-1} that are characteristic indication of presence of acid group. The ^1H NMR exhibits a peak at δ 0.851 showing the presence of methyl groups. The strong peak at δ 1.254 indicated the presence of a long chain of methylene groups and at the least a peak at δ 2.31 is due to methylene groups adjacent to carbonyl group. Although, the ^{13}C NMR showed the evidence of peak at δ 14.83 for a methyl group and the peaks at δ 20.12, 23.07, 29.74, 32.31 are due to long chain methylene groups. Taking all these in consideration, it appears that the compound is a long chain hydrocarbon and a fatty acid. The structure of the long chain hydrocarbon may be CH_3-$(CH_2)_{30}$-CH_3.

Characterization of isolated amorphous crystalline from the ethanolic extract of *limnophila polystachya*: From above study, the colourless amorphous crystalline and the confirm functional group by various determinations. The isolated crystals possessed its melting point is 95 ºC and the optical rotation +29.1 which showed the molecular weight of the compound 480 obtained from APCI-LCMS on negative mode spectrum and m/z 479 for $[M-H]^-$ ion of the mass spectrum; simultaneously, the APCI-LCMS on positive mode spectrum and m/z 481 for $[M+H]^+$ ion; such that the LCMS predictions determine the molecular formula: CH_3-$(CH_2)_{30}$-COOH (Fig. 1).

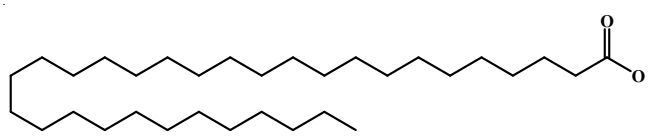

Fig. 1. Isolated amorphous crystalline structure determination (lacceroic acid)

The structural formula was corroborated by the ^{13}C NMR spectrum which showed 32 carbon resonances. Eventhough, the isolated crystals showed that maximum UV absorption in ethanol at 246 and 320 nm and gave pink colour in Liebermann Burchard test suggested that the isolated crystalline was containing acid group[15].

Lacceoroic acid showed antibacterial activity (Table-1) against *Escherichia coli* (gram-negative), *Pseudomonas aerogenosa* (gram-negative), *Salmonella paratyphi* (gram-negative), *Vibrio cholerae* (gram-negative)in the concentration of 600 µg/mL,1000 µg/mL. *Pseudomonas aeruginosa* shows higher zone of inhibition at 1000 µg/mL using amphotericin-B as a standard[16]. The growth of *Escherichia coli* (gram-negative), *Pseudomonas aerogenosa* (gram-negative), *Salmonella paratyphi* (gram-negative), *Vibrio cholerae* (gram-negative) were controlled by the lacceroic acid which indicated that the compound could inhibit the action of bacteria. Lacceroic acid obtained from *Limnophila polystachya* showed various level of antibacterial activity when tested by paper disc diffusion method. The results clearly indicated that the concentrations of the maximum zone of inhibition was observed at higher dose (1000 µg/mL). The maximum zone of inhibition was observed against *Pseudomonas aeruginosa* when compared to standard drug.

TABLE-1
ANTIBACTERIAL ACTIVITY ON DO-TRIACONTANOIC ACID (Syn: LACCEROIC ACID) USING DISC DIFFUSION METHOD

Name of the organisms	Diameter of zone of inhibition (mm)			
	Std. disc	Concentration (µg/mL)		
		200	600	1000
Escherichia coli	15	–	5	10
Proteus vulgaris	20	–	–	–
Pseudomonas aerogenosa	22	–	8	15
Staphylococcus aureus	23	–	–	–
Streptococcus faecalis	20	–	–	–
Salmonella typhi	22	–	–	–
Salmonella paratyphi	17	–	4	9
Bacillus polymizia	15	–	–	–
Aeromonas hetrophylla	19	–	–	–
Vibrio cholerae	14	–	4	6

Conclusion

The present study concluded that the spectral data's like UV, IR, ^1H, ^{13}C NMR and LC-MS, suggested and elucidated that structure was long chain fatty acid found as lacceroic acid do-triacontanoic acid having the structural formula CH_3-$(CH_2)_{30}$-COOH. Also it could be found that significant antibacterial activity against life threatening pathogenic microorganism.

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REFERENCES

1. S.P. Ambasta, The Useful Plants of India, PID, CSIR, New Delhi, p. 329 (1986).
2. S. Arunya, B. Nuntavan, V. Opa and W. Hiroshi, *Thai J. Phytopharm.*, **11** (2004).
3. A. Suksamrarn, *Arch. Pharm. Res.*, **26**, 816 (2003).
4. U.A. Kukongviriappan and S.A. Luangaram, *Biol. Pharm. Bull.*, **30**, 661 (2003).
5. K.S. Mukherjee, G. Brahmachari, T.K. Manna and P. Mukherjee, *Phytochemistry*, **49**, 2533 (1998).

6. G. Bramachari, D. Gorai, D. Chatterjee, S. Mondal and B. Mistri, *Indian J. Chem*., **43B**, 219 (2004).
7. K.S. Mukherjee, G. Bramhachari, T.K. Manna and P. Mukherjee, *Phytochemistry*, **28**, 1778 (1989).
8. G. Brahmachari, S.K. Jash, L.C. Mandal, A. Mondal, R. Roy, *Rasayan J. Chem.*, **1**, 288 (2008).
9. G. Brahmachari, S.M.A. Sohel, D. Gorai, S. Mondal and B. Mistri, *J. Chin. Chem. Soc.*, **50**, 325 (2003).
10. M.M. Khine, N. Arnold, K. Franke, A. Porzel, J. Schmidt and L. Wessjohann, *Biochem. Systemat. Ecol.*, **35**, 517 (1987).
11. J.H. Jorgensen, J.D. Turnidge and J.A. Washington, in eds.: P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover and R.H. Yolken, Antibacterial Susceptibility Tests: Dilution and Disk Diffusion Methods, Manual of Clinical Microbiology, ASM Press, Washington, DC, edn. 7, pp. 1526-1554 (1999).
12. J. Simanek, D. VrbaPetr, V. Jaroslav and U. Jitka, *Toxicol. in vitro*, **23**, 580 (2009).
13. G.B.S. Reddy, A.B. Melkhani, G.A. Kalyani, J.V. Rao, S. Annie and K. Mohan, *Pharm. Biol.*, **29**, 145 (1991).
15. B. Mabry, E.N. Abou-Zeid and A.M. Rizk, *Chem. Mater. Sci.*, 120 (1970).
16. A.M. Ashraful, H.M. Rowshanul, N. Farjana, R. Matiar and K.M. Rezaul, *Bangladesh J. Scient. Res.*, **43**, 397 (2008).