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Der Pharmacia Lettre, 2014, 6 (6):113-116
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ISSN 0975-5071
USA CODEN: DPLEB4

Isolation of phytoconstituents from *Aponogeton natans* (Linn.) Engl & Krause- An important folklore medicine

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ABSTRACT

The objective of the present work is to isolate the phytoconstituents from the active extract of Aponogeton natans (Linn.) Engl & Krause leaf with leaf stalks. The preliminary phytochemical screening and HPTLC analysis were carried for the pet ether, benzene, chloroform and methanol extracts. From the preliminary phytochemical and HPTLC analysis it was found that methanol extract contained carbohydrate, protein, phytosterol, glycoside, saponin, flavonoids and polyphenols. The methanol extract showed 11 peaks having maximum R_f value 0.08, 0.12, 0.18, 0.27, 0.38, 0.47, 0.53, 0.56, 0.62, 0.73 and 0.80. Thereafter the methanol extracts was subjected to column chromatography for the isolation of the phytoconstituents. Two compounds namely ANSD-1 and ANSD-2 were isolated and purified from methanol extract by column chromatography and the structure were determined as stigmasterol and gallic acid by physical, chemical and spectral characteristics. The information gathered from the phytochemical study and HPTLC of Aponogeton natans Linn. delivered the parameters will serves determine the quality of the plant material in the future. The isolation carried determined the presence of two important phytoconstituents of medicinal value which may be responsible for the pharmacological action of the plant.

Keywords: *Aponogeton natans* (Linn.) Engl & Krause, Methanol extract, Stigmasterol and Gallic acid

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INTRODUCTION

At present, evaluation includes method of estimating active constituents present in the crude drug, in addition to its morphological and microscopic analysis. With the advent of separation techniques and instrumental analysis, it is possible to perform physical evaluation of a crude drug, which could be both of qualitative and quantitative in nature.

Aponogeton natans (Linn.) Engl. & Krause.belongs to aponogetonaceae family. The plant occurs in plains, in the ponds and marshy places in Asia, Australia, India and Srilanka. Leaf pastes are consumed with hot water to treat cuts & wounds (1)**.** Fresh tuber are ground into a paste and boiled with 200 ml of coconut oil and applied on hair before bath for three days to get rid of fungal infection (2)**.** *Aponogeton natans* (Linn.) Engl. & Krause is a important ingredient in preparation an important ayurvedic formulation, Useerasava. This asava is useful for raktapitta (Haemothermia), anaemia, impurity of blood and diabetes (3). A perusal of existing reports reveals that there is no isolation study carried out earlier.

MATERIALS AND METHODS

Fresh parts of *Aponogeton natans* (Linn.) Engl. & Krause.were collected from Salipur, Cuttack, Odisha, India which was identified and authenticated by Prof.P.Jayaraman, PARC, Chennai. The voucher specimen was given the No. PARC/2009/398.

Reagent and chemicals
All reagents and chemicals used for isolation were analytical grade obtained from SRL Chemical, Rankem and Himedia Pvt Ltd.India.

Phytochemical screening (4, 5, 6 and 7)
The dried and coarsely leaves sample was extracted successively with petroleum ether (60-80^{0}C), chloroform, benzene and methanol in a soxhlet extractor by continuous hot percolation. Each time before extracting with the next solvent of higher polarity the powder drug (marc) was dried in a hot air oven below 50^{0}C for 10 minutes. Each extract was concentrated by distilling off the solvent, which was recovered subsequently. The successive extract, as mentioned above, were subjected to various qualitative phytochemical test and HPTLC fingerprinting analysis for the identification of chemical constituents present in the plant material.

Extraction and isolation
The leaf with leaf stalks of *Aponogeton natans* Linn. were shade dried, ground and extracted with methanol. The methanol extract was evaporated to dark greenish mass (700 gm). The methanol extract was initially partitioned between n-hexane and water. The aqueous fraction was successively partitioned with chloroform, ethyl acetate and n-butanol.

Chromatography separation
The chloroform soluble fraction (125gm) was subjected to column chromatography over silica gel eluting with n-hexane, n-hexane-chloroform, chloroform, chloroform-methanol and methanol in increasing polarity (0-100%). The chloroform soluble fraction residue which was chromatographed successively with n-hexane: chloroform mixture by increasing polarity gave 71 numbers of eluents (500ml each). The eluent no. 44-49 obtained from n-hexane: chloroform (3:7) mixture gave a single spot on TLC which on PTLC using solvent system n-hexane: ethyl acetate (4.5:5.5) provided ANSD-1.

The ethyl acetate soluble fraction (95gm) was subjected to column chromatography over silica gel eluting with n-hexane, n-hexane-ethyl acetate, ethyl acetate, ethyl acetate -methanol, and methanol in increasing polarity (0-100%). The ethyl acetate soluble fraction residue which was chromatographed successively with n-hexane: ethyl acetate mixture by increasing polarity gave 66 numbers of eluents (500ml each). The eluent no. 48-53 obtained from n-hexane: ethyl acetate (2:8) mixture showed a single spot on TLC which on PTLC using solvent system n-hexane: ethyl acetate (1:9) provided ANSD-2. ANSD-1 and ANSD-2 were further subjected to FTIR, ^{1}HNMR, ^{13}CNMR and EI mass spectrometry to ascertain the chemical structure.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis
All the four extracts were screened for phytochemical investigation by different phytochemical tests to check the presence or absence of a group of phytochemical constituents. These phytochemical tests showed the presence of proteins, carbohydrates, alkaloids, saponins, tannins, flavonoids, steroids, triterpenoids etc. Petroleum ether extract gave positive tests for fats and phytosterol; benzene extract gave positive results for fats, phytosterol and glycoside; chloroform extract shown positive tests for phytosterol, glycoside, flavonoids and alkaloids; methanol extracts were found to contain carbohydrate, protein, phytosterol, glycoside, saponin, flavonoids and polyphenols.

High performance thin layer chromatography
High Performance Thin Layer Chromatography (HPTLC) technique is most simple and fastest separation technique available today which gives better precision and accuracy with extreme flexibility for various steps. The methanol extract showed 11 peaks having maximum R_f value 0.08, 0.12, 0.18, 0.27, 0.38, 0.47, 0.53, 0.56, 0.62, 0.73 and 0.80 with n-butanol:water:acetic acid (4:5:1) as solvent system.

Characterization of isolated compounds
The structures of the isolated compounds ANSD-1 and ANSD-2 were established by melting point, FTIR, ^{1}HNMR, ^{13}CNMR and EI mass spectrometry.

Characterization of Compound (ANSD-1) stigmasterol colourless crystalline solid **M.P:** 170-172°C; **M.F.:** C29H48O;**FTIR** ν max cm-1 (KBr): 3348(O-H stretching), 2935(CH3 Stretching), 2866(CH2 stretching), 1667(C=C stretching), 1459(CH3 stretching), 1387 CH2 stretching of overlap doublets), 970 (CH out plane bending);**^{1}HNMR** (CDCl3) (300MHz) (δ ppm): δ 7.26(s,1H,OH), 5.34-5.35(d,1H,CH), 5.11-5.14(d,1H, CH), 4.97-5.02(t,1H,CH), 1.31(s,3H, CH3), 1.35-1.39(q,3H,CH3), 1.40-1.44(q,3H,CH3), 1.48-1.52(q,2H,CH2), 1.64-1.69(q,3H,CH3), 1.48-1.52(q,2H,CH2), 1.64-1.69(q,2H, CH2), 1.70-1.73(t,2H,CH2), 1.77-1.82(t,2H, CH2), 1.86-

1.98(m,1H,CH), 2.00-2.08(m,1H,CH), 2.19-2.32(m,1H,CH), 3.47-3.57(m,1H,CH), 0.69(s,3H,CH3), 0.78-0.88(m,2H,CH2), 0.92-1.03(m, 3H,CH2); **^{13}C-NMR** (CDCl3) (300MHz) (δppm): 140.72 (C-5), 138.3 (C-22), 129.23(C-23), 121.66 (C-6), 71.74 (C-3), 56.83 (C-14), 55.91 (C-17), 51.21 (C-9), 50.11 (C-24), 42.17 (C-13), 42.26 (C-4), 40.50 (C-20), 39.65 (C-12), 37.23 (C-1), 36.48 (C-10), 31.86 (C-8), 31.61 (C-25), 28.91 (C- 16), 25.39 (C-28), 24.34 (C-15), 21.21 (C-11), 21.08 (C-21),21.04 (C-19), 19.38 (C-26), 18.96 (C-27), 12.02 (C-18), 12.24 (C-29); **EI-MS** m/z (rel int. %): 412[C29H48O] (100), 369 [C24H4O,M-C3H7] (40), 351 [C26H39,M-C3H9O] (45), 300 [C21H32O, M-C8H16] (75), 271 [C19H27O, M-C10H20] (75), 255 [C19H27, M-C10H21O] (90), 83[C6H11, M-C23H37O] (70), 55 [C4H7, M-C25H41O] (65) **Figure 1**.

Compound ANSD-1 was isolated as colourless needles from the chloroform soluble fraction. The molecular formula C29H48O was established through EI-MS showing molecular ion peak at m/z 412.

The IR spectrum (3348 cm-1) indicated the nature of oxygen to be hydroxyl. Presence of olefinic double bond was confirmed by a band at 1667 cm-1. The 1H-NMR spectrum of ANSD-1 corresponded to the data for stigmasterol. It displayed signals for two tertiary methyl groups (δ 0.83, 0.68), two multiplets for three olefinic protons at δ 5.34-5.35 (1H) and 5.11-5.14 (2H) and a multiplet for the carbonylic proton at δ 3.47-3.57. The 13C-NMR spectrum of ANSD-1 disclosed the presence of twenty nine signals for six methyl, nine methylene, eleven methane and three quaternary carbon atoms. The mass spectrum showed characteristic fragmentation pattern of Δ5, 22 sterol. The above data was compared with the literature (8) and showed complete agreement to those of stigmasterol.

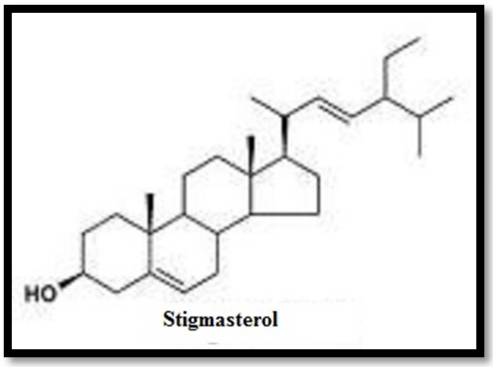

Figure 1: Stigmasterol (ANSD-1)

Characterization of Compound (ANSD-2) Gallic acid White leaflets; **M.P:** 236-238 °C; **M.F.:** C7H6O5; **FTIR** ν max cm-1 KBr): 3495(O-H stretching), 3283(O-H stretching with hydrogen bonding), 1704(C=O at COOH stretching), 1614(C=C stretching at ring), 1541, 1437(Ar-CH stretching), 1310(C-O stretching at dimer), 1250(OH bending), 1028(C-O-C stretching); **^{1}HNMR** (CD3OD) (300MHz) (δ ppm): δ 9.11 (S, 1H, COOH), 7.08-7.14 (S, 2H, Ar-CH), 4.96(S, 1H, OH); **^{13}C-NMR** (CD3OD) (300MHz) (δppm): δ 170.72 (s, C-7 of COOH), 146.61 (s, C-3,5), 139.85(s, C-4), 122.16 (s, C-1) and 110.60 (s, C-2,6); **EI-MS** m/z (rel int. %): 170[C7H6O5](100%), 153[M-HO, C7H4O4] (75%), 129(M-CHO, C5H5O4)(15%), 79[M-C2H3O4, C5H3O](15%), 51[M-C3H3O5, C4H3] (9%) **Figure 2**.

Compound ANSD-2 was isolated as colourless substance from the ethyl acetate soluble fraction. The FTIR spectrum showed the absorption bands at 3495 (O-H), 1704 (C=O) and 1614 (aromatic).

The 1H-NMR spectrum of ANSD-2 displayed only a singlet in aromatic region at δ 9.11 (S, 1H, COOH), 7.08-7.14 (S, 2H, Ar-CH), 4.96(S, 1H, OH). . The 13C-NMR spectrum of compound 67 disclosed the presence of five carbon signals for one methane and four quaternary carbon atoms. The downfield signals at δ 170.72, 146.61 and 139.85 were assigned to acid carbonyl and aromatic oxygenated quaternary carbon atoms whereas other signals in the aromatic region at δ 110.60 and 122.16 were assigned to aromatic methane and aromatic quaternary carbon atoms. The EI-MS of compound ANSD-2 gave the molecular ion peak at *m/z* 170 corresponding to the molecular formula C7H6O5 .The above data was compared with the literature (9) and showed complete agreement to those of gallic acid.

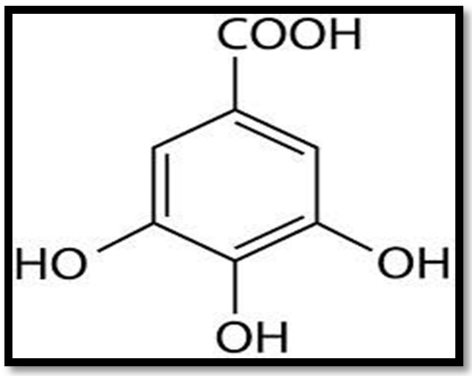

Figure 2: Gallic acid (ANSD-2)

Methanol extract of *Aponogeton natans* (Linn.) leaf with leaf stalks resulted in column chromatographic separation of **ANSD-1** (Stigmasterol) and **ANSD-2** (gallic acid) which are confirmed by above physical and spectral data given above.

CONCLUSION

The present phytochemical data emphasize the knowledge of chemical constituents present in *Aponogeton natans Linn.* The compounds isolated were characterized by using modern spectral analytical method. The active isolated compounds in future can be studied and related for their pharmacological activities.

Acknowledgements
The authors are greatly thanks to Prof.P.Jayaraman, Plant Anatomy Research Centre, 2nd street, Shakti Nagar, West Tambaram, Chennai-600045, India, who identified and authenticated the plant specimen for the present work.

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