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Isolation of Stigmasterol from petroleum ether extract of aerial parts of *Bryophyllum pinnatum* (Crassulaceae)

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ABSTRACT

General phytochemical screening of the aerial parts of *Bryophyllum pinnatum* (Crassulaceae) revealed the presence of bufadienolides, flavanoids, polyphenols, triterpenoids, phytosterols, glycosides and organic acids. The aim of this study is to identify and characterize the bioactive principle from the aerial parts of the plant. It has wide folk medicinal use. For isolation of the compound, the dried aerial parts powder of *Bryophyllum pinnatum* was subjected to hot extraction with petroleum ether; this Extract was saponified with alcoholic KOH and subjected to chromatography. The isolation and purification afforded white crystalline powder which was subjected to physical, chemical and spectral identification by IR, ^{1}H-NMR, ^{13}C-NMR and GC-MS. The compound was concluded as stigmasterol.

Keywords: *Bryophyllum pinnatum*, phytochemical, stigmasterol, organic acids.

INTRODUCTION

Research studies leading to extraction, isolation, identification and biological study of plant constituents have now formed the major field of the study. The genus Bryophyllum (Crassulaceae) comprises about hundred species, most of them native from Madagascar. *Bryophyllum pinnatum* is a perennial medicinal herb popularly used as folkloric medicine in tropical Africa, India, China, Australia and tropical America [15] and other parts of the world to treat various inflammatory diseases. The leaves of the plant have great medicinal value and possess various properties like haemostatic, refrigerant, emollient, mucilaginous, vulnerary, depurative, anti-inflammatory, disinfectant and tonic. It is also employed for kidney stones, gastric ulcers, skin disorders and edema of the legs. It contains substances such as triterpenoids, glycosides, flavonoids, steroids, bufadienolides, lipids and organic acids. [1, 3- 5]

It is a succulent perennial plant that grows 1-1.5 m in height and the stem is hollow four-angled and usually branched. Leaves are opposite, decussate, succulent, 10-20 cm long, distributed all over India. In traditional medicine, the leaves of this plant have been used for antimicrobial, antifungal, antiulcer, anti-inflammatory, analgesic, antihypertensive, potent anti-histamine and anti-allergic activity. [11-14]

But these studies are not enough for identifying and characterizing the bioactive compounds in this plant. The purpose of this study is to identify and characterize the bioactive principles from the aerial part of *Bryophyllum pinnatum.*

EXPERIMENTAL

Collection, Identification and Preparation of Plant materials

The aerial parts of the plant were collected from Herbal Nature Park, Chuharpur, Yamuna Nagar, Haryana in the month December 2007. The plant was taxonomically identified, authenticated by Professor Dr. J. S. Sodhi, HOD, Botany Department, Guru Nanak Khalsa College, Yamuna Nagar, Kurukshetra University, Haryana and deposited with A. R. College of Pharmacy, Vallabh Vidhya Nagar, Gujarat. The aerial parts of the plant were manually separated was air dried, powdered, sieved, weighed and stored in air tight container and subsequently referred to as powdered drug.

Extraction and Isolation

Powdered (400g) aerial parts of *Bryophyllum pinnatum* were defatted exhaustively with petroleum ether (60-80°c) in a soxhlet extractor. The solvent was recovered under pressure to obtained dark greenish brown oily mass (5g), which was labeled as petroleum ether extracts (PEE) and kept in the refrigerator. The resulting marc was air dried at room temperature and then exhaustively extracted successively with solvents with increased polarity and concentrated under reduced pressure and labeled accordingly. The petroleum ether extract of aerial parts of the plant was saponified using 1M alcoholic KOH, to remove fatty material and then subsequently picked up in petroleum ether and the solvent was evaporated to yield 2.5g of unsaponified matter. This fraction contains lesser number of components than the unsaponified extract. [18]

Chromatographic Separation

A small quantity of unsaponifiable matter was dissolved in chloroform and this solution is spotted on TLC plates using pre-coated aluminium with silica gel 60 $F_{254.}$ Then the TLC plates were run by specific solvent system and viewed individually under UV light and also methanol-H_2SO_4 reagent. Through several pilot experiments it was found that the compounds of unsaponifiable fraction were separated by the solvent system of chloroform and ethanol in the proportion of 9.8:0.2. The chromatograms when developed in iodine chamber yielded six to seven spots respectively and spots at Rf (0.45) becomes reddish brown soon turns to purple or violet indicate zones for steroidal nucleus. The unsaponifiable fraction was subjected to column chromatography using silica gel (Mesh 60-120) that was packed using wet packing method in hexane. The column was run using hexane, chloroform and methanol by gradient elution technique. TLC was used to monitor the eluates. A total of 70 eluates were collected. Similar fractions were pooled together. Further purification is carried out using preparative TLC. Spots were identified, scraped and eluates using petroleum ether and chloroform as solvents. [17, 18]

Finally eluate BST yielded a single spot when subjected to TLC using several solvent systems including chloroform: ethanol (9.8:0.2), ethyl acetate: ethanol (9.8: 0.2), chloroform: ethyl acetate (4:1) and it showed to be homogenous compound. BST a white crystalline powder (50mg) with melting point (144-146°C) was further subjected to chemical tests and spectral characterization by IR, Proton NMR (400MHz), Carbon-13 NMR (100 MHz) and GC-MS to ascertain the chemical structure.

Test for Alcohol:

4g of cerric ammonium nitrate was dissolved in 10ml of 2N HNO_3, on mild heating. A few crystals of isolated compound were dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of cerric ammoinium nitrate reagent and diluted to 1ml with dioxane and shaken well. The developed yellow to red color indicates the presence of an alcoholic hydroxyl group. [9]

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Tests for Steroid:

Salkowski reaction: A few crystals were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to the solution. A reddish color was seen in the upper chloroform layer. [9]

Liebermann-burchardt reaction: A few crystals were dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by addition of 2-3 drops of acetic anhydride. Solution turned violet blue and finally green. [9]

Spectroscopic Characterization:

Different spectroscopic methods were used to elucidate the structure of isolated compound BST. Among the spectroscopic techniques IR, ^{1}H-NMR, ^{13}C-NMR and GC-MS were carried out. The infra-red spectrum was recorded on FTIR Perkin Elmer, ^{1}H-NMR and ^{13}C-NMR spectra were recorded using $CDCl_3$ as solvent on Bruker Advance II 400 NMR spectrometer SAIF Panjab University, Chandigarh and GC-MS spectra were recorded at high resolution on a mass spectrometer (Perkin Elmer Autosystem) at Sophisticated Instrumentation centre for Applied Research and Technology, Anand, Gujarat, India and the data are given in m/z values.

. The IR absorption spectrum showed absorption peaks at 3393.8cm^{-1} (O-H str.); 2927.16 cm^{-1}and 2852.19cm^{-1} (aliphatic C-H str.); 1640.6cm^{-1} (C=C absorption peak); other absorption peaks includes 1462.3cm^{-1} (CH_2); 1381.1cm^{-1} (OH def) and 1041.79cm^{-1} (cycloalkane).

^{1}H-NMR ($CDCl_3$, 400MHz) of BST: ^{1}H-NMR has given signals at d 5.35 (1H, m, H-6), 5.18 (1H, m, H-22), 5.00(1H, m, H-23), 3.52(1H, m, H-3), 2.28(1H, m, H-21), 0.68(s), 1.0(s), 0.91 (d, j=4.64), 0.93 (d, j=8.04), 0.90 (s) 1.8-2.2 (5H, m) ppm. Other peaks are observed atδ 0.68, 0.79, 0.82, 0.85, 0.91 and 1.02 ppm.

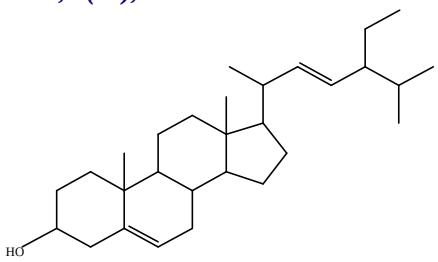
^{13}C-NMR ($CDCl_3$, 100MHz) of BST: ^{13}C-NMR has given signal at 140.77 (C-5), 138.33 (C-22), 129.28 (C-3), 121.73 (C-6), 71.82 (C-3), 56.88(C-14), 56.06 (C-17), 51.25(C-24), 50.14 (C-9), 45.84(C-25), 42.2 (C-13), 40.5 (C-20), 39.7 (C-12), 37.2 (C-1), 36.5 (C-10), 33.96 (C-8), 31.9 (C-7), 29.16 (C-16), 28.2 (C-2), 26.08 (C-11, 26), 25.4 (C-28), 24.31(C-15),21.22(C-21), 20.21 (C-27), 19.4 (C-26), 19.0(C-19), 12.26 (C- 29), 12.05(C-18).

FAB-MS spectroscopy showed the molecular ion peaks at 412 that correspond to molecular formula $C_{29}H_{48}O$. Ion peaks were also observed at m/z 327, 271, 255, 229, 213, 199, 159, 145, 133, 121, 105, 91, 83, 81, 69, 55, 41.

RESULTS AND DISCUSSION

The BST is white crystalline needles like substance with melting point 144-146^{o} c. From the positive tests for steroid and alcohol given by compound BST, it was assumed to be a sterol. On subjection to IR spectroscopic analysis, the observed absorption bands are 3393.78cm^{-1} that is characteristic of O-H stretching. Absorption at 2927.16 cm^{-1}and 2852.19 cm^{-1} is due aliphatic C-H stretching and bending vibrations of methyl group. Other absorption frequencies include 1640.6 cm^{-1} as a result C=C stretching however this band is weak, at 1462.29 cm^{-1} is a bending frequency for cyclic (CH_2)n and 1381.11cm^{-1} for C-H bending. The absorption frequency at 1041.79 cm^{-1} due to C-C vibration signifies cycloalkane. The out of plane C-H vibrations of unsaturated part was observed at 881 cm^{-1}. These absorption frequencies resemble the absorption frequencies observed for Stigmasterol. The proton NMR showed the proton of H-3 appeared as a multiplet at d 3.52 and H-6 olefinic proton showed a multiplet at d 5.35. Two olefinic protons appeared at d 5.15(m) and 5.01(m) which were identical with chemical shift of H-22 and H-23, respectively of stigmasterol. Angular methyl proton appeared at 0.68(s), 0.69(s) and 1.02(s) corresponds to C_{18} and C_{19} proton respectively. Spectra showed the presence of six methyl groups that appeared at d 0.68, 0.79, 0.82, 0.86, 0.92 and 1.02 (3H each, s, CH_3).

The ^{13}C-NMR has shown recognizable signals 140.77 and 121.7 ppm, which are assigned C_5 and C_6 double bonds respectively as in $\triangle^5$ spirostene. [2] The δ value at 71.82 ppm is due to C.3 β hydroxyl group. The signal at 19.4 and 11.99ppm corresponds to angular carbon atom (C_{19} and C_{18} respectively). The value for C_{18} is lower due to γ-gauche interaction that increases the screening of the C_{18} hence lower chemical shift. However, the loss of H in C_6 results in decrease in screening of the C_{19} leading to increase in C_{19} chemical shift to higher frequency. This is also tenable as in chemical shift of 21.22 and 11.99 ppm (for C_{19} and C_{18} respectively). The alkene carbons appeared at d140.77, 138.33 and 121.73.The weak molecular ions were given at m/z 412 and the characteristic peaks are m/z 271 due to the



Stigmasterol
$C_{29}H_{48}O$
Mol.Wt. 412.69

formation of carbocation by ß bond cleavage of side chain leading to the loss of $C_{10}H_{21}$ that corresponds to the M-141. The molecular weight and fragmentation pattern indicate that the compound presenting BST is stigmasterol. Further the loss of C-18 CH_3 and H 16 gives rise to a stable carbocation showing a molecular peak at m/z of 255 which on successive dealkylation would yield ions at m/z 229, 213, 199, 159, 145, 133, 121, 119, 105, 91, 83, 81, 69, 55, 41. The fragment at m/z 271 will lose C_4H_9=$^{+}$OH to yield fragment at m/z 199 which on further dealkylation would yield fragments at m/z 199 which on further dealkylation would yield fragments at m/z 173, 145 and 119. Another pattern is from m/z 412 to m/z 327 (M-85) then to m/z 300.

The above IR, ^{1}H-NMR, ^{13}C-NMR and GC-MS spectral data and their comparison with those described in the literatures showed the structure of BST to be stigmasterol. [6, 7, 9, 10, 16]

CONCLUSION

From the above physical, chemical and spectral evidences, the compound isolated from petroleum ether extract of the aerial parts of *Bryophyllum pinnatum* was confirmed as stigmasterol and chemical structures elucidated respectively. It was carried out by means of various physical (solvent extraction, TLC, Column chromatography) and spectral techniques.

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