Isolation of Stigmasterol from Parthenium hysterophorous plant

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Abstract
Parthenium hysterophorous plant of Asteraceae family has chemical constituents such as alkaloids, proteins, saponins, tannins, carbohydrates, glycosides, terpenoids, steroids, volatile oils, amino acids, flavonoids. In this study, Stigmasterol, a well-known phytosterol was isolated from Parthenium hysterophorous plant and purified by fractional distillation and column chromatography using n-hexane as extracting solvent and reported first time. Isolated compound (Stigmasterol) was characterized by spectral techniques IR, MS, 1H NMR, 13C NMR studies and determined the structure.

Keywords: parthenium hysterophorous, stigmasterol, saponification, asteraceae, NMR

Introduction
Parthenium hysterophorous plant of Asteraceae family known as Gajarghas in south India and has medicinal potential. Chemical investigation of Parthenium hysterophorus plant revealed the presence of chemical constituents such as alkaloids, proteins, saponins, tannins, carbohydrates, glycosides, terpenoids, steroids, volatile oils, amino acids, amino sugars, lignans, phenolic compounds, flavonoids, metallic elements, organic acids, terpenoids and others [1-5]. Major component of the Parthenium hysterophorous plant is Parahtin. Literature review of Parthenium hysterophorous plant directed me to explore the phytosterols of the plant. Stigmasterol is the major phytosterol which is isolated from medicinal plants. Stigmasterol is the precursor for synthesis of progesterone and is an intermediate in the biosynthesis of estrogens, androgens and corticoids [6] and in the synthesis of vitamin D3 [7]. Hence the objective of the research is to isolate stigmastanol from Parthenium hysterophorous plant and determine the structure by 1D and 2D NMR studies.

Materials and Methods
The Chemical reagents used in the analysis are of Analytical grade, Merck India Co. Ltd.

Plant material
Leaves of Parthenium hysterophorous 10kgs were collected from the forest of Tirumala, Andhra Pradesh, India. It was identified by Dr. K. Madhava Chetty, Department of botany, Sri Venkateswara University, Tirupati. A voucher specimen of the plant was deposited in Herbarium, Department of Botany, with the accession number 1216.

Extraction and Isolation
Air dried (in shade) leaves of Parthenium hysterophorous was powdered. 50gms of powdered Parthenium hysterophorous plant material was weighed and transferred into round bottomed flask of Soxhlet extractor. Then 250 ml of hexane was added and the plant material was soaked in hexane for 24hrs at room temperature. Then the hexane extract of plant (dark green colored) was filtered using Wattman No 1 filter paper. This crude extract was used for further analysis.

Identification of Sterols
Test for Alcohol
Small amount of Parthenium plant extract was dissolved in 0.5 ml of dioxane. The obtained solution was added to 0.5 ml of ceric ammonium nitrate reagent. Then about one ml of dioxane was added and shaken. Presence of alcoholic hydroxyl group [8] was indicated by the color change from Yellow to red color formation.
**Libermann-Burchard Test**
Few drops of acetic anhydride were added to the Parthenium plant extract and boiled. After cooling, Concentrated Sulphuric acid was added. Formation of brown ring at the junction of two layers and green color in upper layer [9] confirmed the presence of sterols in the Parthenium plant extract.

**Saponification**
Parthenium crude extract (dark green colored) was dried at room temperature and successively extracted with solvents of increasing polarity. Extract is concentrated under vacuum and labelled. Petroleum ether extract was saponified with alcoholic potassium hydroxide (1M) to remove the fatty material. Unsaponified matter was extracted in petroleum ether and removed the solvent. Small amount of unsaponified extract was dissolved in chloroform and tested with Thin Layer Chromatography (TLC) by spotting on silica gel 60 F254 pre-coated with aluminium. By repeating the tests, it was noted that mixture of n-hexane and ethyl acetate mixture with 8:2 ratio was the best eluent.

Presence of six different molecules with steroidal nucleus was confirmed by spraying the chromogenic agent (5% Sulfuric acid in methanol) and development of chromatograms in iodine chamber. Developed chromatogram in iodine chamber yielded five to six un-detached and continuous spot patterns in the Rf range (0.4 to 0.8). Less number of TLC spots for unsaponified matter shows the presence of fewer compounds in it compared to that of above combined petroleum extracts.

**Fractional crystallization for separation of stigmasterol from sitosterol mixture**
Stigmasterol and β-sitosterol can be separated based on the solubility variation with respect to temperature [10, 11]. By considering this factor, fractional crystallization procedure was followed as evident in the literature by Xu *et al.* [12]. Cyclohexanone and n-Pentanol are the proven solvents for effective separation and purification of stigmasterol and β-sitosterol.

25g of extract was dissolved in 75ml of cyclohexane by stirring for an hour at 60 °C. Upon cooling the solution, Stigmasterol is precipitated. Filtered and dried the precipitate in vacuum drying at 60 °C. Fractional crystallization procedure was repeated five times to obtain pure stigmasterol which is evident from reasonably separated spots on TLC plates.

**Column chromatographic separation**
After fractional crystallization, precipitate obtained was subjected to column chromatography for purification. It was conducted by packing silica gel (Mesh 60-120) using hexane for making wet packing method. Gradient elution technique was followed in which the polarity of solvent mixture was increased by addition of ethyl acetate (0%-100%) to hexane. The eluates were monitored using TLC as mentioned above. Out of the collected 170 eluates, similar fractions were pooled together. Preparative TLC was used for auxiliary purification. Spots with same Rf value were scraped and extracted into petroleum ether as a solvent. Presence of sole phytocchemical was confirmed from single spot even after subjecting TLC using different solvent mixtures like chloroform: ethanol (9.5:0.5), ethyl acetate: ethanol (9.5: 0.5), chloroform: ethyl acetate (4:1). After recrystallizing in acetone, a white crystalline powder was obtained with a melting point of 172°C and Rf value of 0.7 (hexane: ethyl acetate=8:2/v/v) [13].

**Characterization of isolated compound by Spectral Techniques**

**Mass Spectroscopy**
Mass spectra was recorded with mass spectrometer Perkin Elmer. Mass (ES-IMS) (m/z): 413.3 (M+H) + 399, 328.9, 289.1, 261.2, 257.1, 169.1

**IR spectroscopy**
The Infrared spectrum was recorded on FTIR Perkin Elmer. I.R: vmax 3416, 3025, 2936, 2867, 1464, 1051 cm⁻¹

**NMR Spectroscopy**
1H - NMR and 13C -NMR spectra were recorded using CDCl3 as solvent on Bruker NMR spectrometer.

**1H NMR (CDCl3)**
1H NMR has given signals at δ 5.30 (1H, brd, J=5.0, 4.1 Hz, H-6), 5.22 – 4.91 (2H, m, H-22, H-23), 3.48 (1 H, M, H-3), 2.32 – 2.16 (3H, M, H-1 α, H-1β, H-20), 2.13 – 1.91 (3 H, m, H-12β, H-14 α, H-16 α), 1.90 – 1.72 (4 H, m, H-16 β, H-4β, H-7 α, H-7β), 1.68 – 1.32 (10 H, m, H-2 α, H-2β, H-4 α, H-9 α, H-9β, H-11 α, H-11β, H-12 α, H-20, H-25), 1.30 – 1.09 (6, H, H, H-24 α, H-15 α, H-15β, H-17 α, H-28 α, H-28β), 1.03 (3 H, d, J = 6.0Hz, Me-21), 1.00 (3 H, s, Me-19), 0.85 (3 H, d, J=5-1 Hz, Me-26), 0.82 (3 H, t, J=4.6 Hz, Me-29), 0.79 (3 H, d, J=5.0 Hz, Me-27), 0.70 (3 H, s, Me-18)

**13C NMR (CDCl3)**
1H NMR has given signals at δ=140.8 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14), 56.0 (C-17), 51.2 (C-24), 50.2 (C-9), 42.3 (C-4, C-13), 40.4 (C-20), 39.7 (C-12), 37.3 (C-1), 36.5 (C-10), 31.9 (C-7, C-8, C-25), 31.7 (C-2), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-26), 21.1 (C-21), 21.0 (C-11), 19.4 (C-19), 19.0 (C-27), 12.2 (C-29), 12.0 (C-18).

**Results and Discussion**
Polarity of solvents influences the nature of extractable phytocchemical. n-Hexane extracts were taken in the present study. Presence of sterols in the crude extract is indicated with positive results with alcohol test [6, 9]. The molecular ion peak in mass spectrum at m/z 413.3 (M+H) + corresponds to molecular formula C29H48O which is equivalent to the formula of stigmasterol, a well-known phytosterol. The IR signal absorption band observed at 3416 cm⁻¹ is characteristic of O-H stretching. Absorption at 3025 cm⁻¹ is typical olefinic=CH- stretching while the absorption at frequencies such as 1464 cm⁻¹ and 1051 cm⁻¹ are of C=O absorption respectively.

1H NMR spectrum consists of peaks primarily in the upfield region. However, two signals corresponding to olefinic region were observed with high chemical shifts values. A multiplet at δ 5.35 (1H, m, H-3) is characteristic to a carbonylic proton of sterol moiety. Peaks in low absorption field i.e., at δ 5.35 (1H, d, J=4.62 Hz, H-22), 5.15 (1H, q, J=7.86 Hz, H-23) and 5.01 (1H, q, J=7.85 Hz H-6) correspond to two and one ethylene protons respectively present on C22=C23 and C26. High intensity Peaks at δ 1.02 and 0.82 are corresponding to methyl groups (Me-19, Me-18, Me-26, Me27 and Me-29)21.2 (C-26), 21.1 (C-21), 21.0 (C-11), 19.4 (C-19), 19.0 (C-27), 12.2 (C-29), 12.0 (C-18).
13C NMR spectrum shows the presence of 6 methyl, 9 methylene, 11 methine and 3 quaternary carbons. Signals at δ140.80 and 121.7 correspond to double bond between carbons C5 and C6 as in the case of Δ5 spirostene [14]. Attachment of β-hydroxyl group to C3 is visible from a peak at δ 71.81 [15]. High intensity peak at δ 21.20 represent angular methyl carbons – C19 and C18. γ-Gauche interaction enhances the screening of C-18 leading to lower chemical shift (δ) as observed for C-18[16]. By using Mass (ESI-MS), IR, 1H-NMR and C13-NMR techniques, structure was elucidated as stigmasterol (Figure 1).

![Fig 1: Stigmasterol (C29H48O)](image)

It confirms that the isolated molecule is stigmasterol due to presence of a sterol skeleton with one hydroxyl group (at C-1). Further the physical, chemical and spectral (L.R., 1H NMR, 13C NMR and ESI-MS) data of the isolated fraction are in consistent with those available in the literature for the major phytosterol i.e., stigmasterol.

**Conclusion**

Parthenium Hysterophorus plant is a reputed drug in Homeopathy due to various bioactive molecules in the plant. In this study, Stigmasterol, a well-known phytosterol was isolated for the first time using n-hexane as extracting solvent followed by saponification, fractional crystallization and purification by column chromatography. Crystalline powder obtained was characterized by spectral techniques IR, MS, H1NMR, H13NMR and confirmed as Stigmasterol and the structural data is in good agreement with the established structure. This will find applications in pharmaceutical industry.

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**References**