



Sequestration of Stigmasterol and β -Sitosterol from Ethanolic Extract of Kam Sabut (*Croton caudatus* Geiseler) Leaves

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Croton caudatus (Kam sabut) has been traditionally used to treat cancer in northeastern India and earlier studies reported the anticancer activity of ethanolic extract of *C. caudatus* leaves in HeLa cells *in vitro* and Swiss albino mice transplanted with Dalton's ascites lymphoma. Therefore, isolation of stigmasterol and β -sitosterol from the ethanolic extract of *C. caudatus* Geiseler leaves was carried out. Stigmasterol and β -sitosterol were isolated from the ethanolic extract of dried leaves of *C. caudatus* by column chromatography. The fractions were purified further on Sephadex[®] LH-20 column chromatography and reverse-phase HPLC. The isolated phytosterols were characterized and quantified by IR, ¹H & ¹³C NMR, mass spectroscopy and HPLC. This has led to the separation of 100 mg stigmasterol and 80 mg β -sitosterol. Stigmasterol and β -sitosterol have been isolated from the ethanolic extract of *C. caudatus* leaves for the first time.

Keywords: *Croton caudatus*, Stigmasterol, β -sitosterol, Column chromatography.

INTRODUCTION

Plants and natural products have played an important role in the drug development throughout the human history. The pharmaceutical industry is constantly striving to develop new medicines/drug to facilitate human healthcare. The novel molecules for cancer cure and other diseases have been extracted from different plants/natural products before their actual chemical synthesis [1-3]. The plants and natural products form an important source for the development of new novel compounds and also scaffolds for blockbuster drugs including statins, vinca alkaloids, taxols, several antibiotics and other important drugs, which have been used to treat various disorders including neoplasia, cardiovascular and infectious diseases and inflammation in humans [1-4].

The screening of different plants may lead to the development of new but novel drugs for the treatment of different diseases. Plants synthesize phytochemicals for a variety of reasons, including nutrition, pollination, protection from insect predation and stress tolerance [5]. Over 326,000 phytochemicals

have been isolated from plants/natural products since ancient times. These include terpenoids, alkaloids, glycosides, polyphenolics, steroids and other molecules [6]. The investigation of natural products received a major boost from the fact that in the year 2013 Federal Drug Administration US approved 547 drugs derived from the plants/natural products [7]. The plant/natural products may continue to provide new novel molecules as many of them still remain unexplored. The screening of plants/natural products provides a major avenue for new drug discovery for the human healthcare. This approach is pursued in the hope of getting new bioactive molecules through combinatorial chemistry and computerized molecular modeling [1,8-10]. The important chemotherapeutic molecules derived from plants/natural products include doxorubicin, bleomycins, vinca alkaloids, taxols, epipodophyllotoxins and camptothecins [11].

Despite the fact that the modern chemotherapeutic drugs have been highly successful in treating various kinds of neoplasias, the induction of numerous adverse side effects like myelosuppression, gastrointestinal, reproductive and nephron-

toxicities and hair follicle damage by these drugs are of major concern to realize their full potential [12,13]. The modern chemotherapeutic drugs induce drug resistance during tumor chemotherapy making tumors refractory to the treatment [14-16]. This indicates the continuing need to develop newer biomolecules which can effectively kill cancerous cells and spare normal cells with very low or negligible toxicity.

Kam sabut or *Croton caudatus* Geiseler (Family: Euphorbiaceae), grows abundantly in Northeast India, Indo-Myanmar region, Bhutan, Nepal, Borneo, Pakistan, South East Asia, Sri Lanka, Java, Laos, Malaysia, Philippines, Sumatra, Singapore, Thailand and Vietnam. Kam sabut is traditionally used to treat liver diseases. The root of Kam sabut is purgative and its poultice is used to treat fever and sprains in the North-eastern part of India. The Kam sabut has been reported to possess low toxicity profile and the whole plant is of medicinal use [5,17]. Kam sabut is also used to cure pain and stomach diseases in the Chinese medicine. The Kam sabut stems and leaves are utilized to treat convulsions, ardent fever, numbness, malaria and rheumatic arthritis traditionally [5,17]. The topical application of Kam sabut is useful in protecting cattle wounds against maggots infestation. The different leaf extracts of Kam sabut have been reported to scavenge DPPH, hydroxyl, superoxide and nitric oxide free radicals and increase ferric reducing power earlier [5]. The phytochemical analysis of Kam sabut showed the presence of alkaloids, flavonoids, cardiac glycosides, phytosterols, phlobatannins, phenolics, saponins and terpenoids [10]. The ethanolic extract of Kam Sabut increased the activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase enzymes in mouse liver along with the elevation of albumin, total proteins, and total bilirubin contents. Treatment of Dalton's lymphoma bearing mice with different doses of ethanol extract of Kam sabut increased the effect of γ -radiation [17]. Exposure of HeLa cells to different concentrations of ethanol extract of Kam sabut increased the cell killing effect in a concentration dependent manner. The cytotoxic action of ethanol extract was due to its ability to increase DNA damage, apoptosis, lipid peroxidation and lactate dehydrogenase and alleviate glutathione concentration in HeLa cells [18]. Therefore, the present study was designed to isolate the active compounds from the ethanolic extract of Kam sabut leaves.

EXPERIMENTAL

All the analytical grade chemicals and Milli Q water was used throughout the analysis. The stigmasterol (purity ~ 95%), β -sitosterol (purity \geq 95%) and Sephadex[®] LH-20 were procured from Sigma-Aldrich Chemical Co., India. Methanol, ethanol, chloroform, hexane, ethyl acetate, glacial acetic acid, ferric chloride, bismuth nitrate, petroleum ether, potassium iodide, concentrated sulphuric acid, concentrated hydrochloric acid, anisaldehyde, antimony(III) chloride, platinum chloride, silica gel G, silica gel (60-120 mesh), silica gel (100-200 mesh), HPLC grade methanol, HPLC grade water and TLC plates were supplied by Merck India, Mumbai, India.

Collection and preparation of extract: The non-infected mature leaves of Kam sabut, *Croton caudatus* Geiseler (family:

Euphorbiaceae) were collected from Saikot village, Chura-chandpur District of India in the dry season (May-June). The plant was identified by Prof. Kumar Singh, Manipur University, Imphal, India, vide voucher specimen No. 004101, and was further authenticated by the Botanical Survey of India, Shillong as *Croton caudatus*.

Isolation and separation of pure components from *Croton caudatus*: *Croton caudatus* (100 g leaf powder) was extracted sequentially in petroleum ether, chloroform, ethanol and water in a Soxhlet apparatus for 72 h. The extract was concentrated *in vacuo* in a rotary evaporator, resulting in a brownish residue. The residue was dried for 4 days in a vacuum desiccator with an approximate 27% yield. The residues were stored at -80 °C until use.

Column chromatography: Cotton wool or sintered glass plug was placed at the bottom of the column (15 cm long and 4 cm wide) with an adsorbent packing above. The adsorbent was packed in the column using the wet packing technique. A suitable amount of adsorbent was mixed with the solvent for the mobile phase and poured into the column and the stationary phase was allowed to settle in the column without entrapment of air bubbles [19]. The solvent was eluted at a rate of 2 mL/min.

The crude ethanol extract (40 g) of *Croton caudatus* leaves was eluted in a silica gel (250-125 μ m) column with 100 mL $\text{CHCl}_3/\text{MeOH}$ (60:40), 100 mL MeOH/n -hexane (60:40) and n -hexane/ EtOAc 100 mL (60:40) 2mL/min resulting in the separation of twenty eight fractions (1-28). Each fraction was monitored by TLC (SiO_2 , silica gel 60 F₂₅₄, 15 μ m, 0.2-0.3 mm thick, 20 cm \times 20 cm) $\text{CHCl}_3/\text{MeOH}$ 8:2; Dragendorff reagent, iodoplatinate reagent, anisaldehyde reagent or antimony (III) chloride reagent and the fractions having similar R_f value were pooled together. Fraction (4-8) with R_f values of 0.88, 0.91 and 0.92 and fraction (12-17) with R_f values of 0.80, 0.83 and 0.85 were pooled together. A 2 g of fraction 4-8 was re-chromatographed using silica gel (75-150 μ m) column chromatography and eluted with n -hexane/ EtOAc (90:10 to 60:40) and 15 fractions were collected. Fractions 6-10 with R_f 0.91 were combined and evaporated to dryness, purified on Sephadex[®] LH-20 (25-100 μ m) column and eluted with 8:2 $\text{CHCl}_3/\text{MeOH}$ at a flow rate of 0.5 mL/min resulting in the separation of 100 mg white coloured crystals code named BG. Fractions 12-17 were passed through silica gel (75-150 μ m) column and elution with $\text{CHCl}_3/\text{MeOH}$ (90:10 to 60:40) gave a total of 12 fractions. The fractions 5-9 with R_f values of 0.85 were pooled together and separated by preparative TLC [19].

Preparative TLC: Silica gel G (2-25 μ m) slurry was poured onto a glass plate (15 cm \times 20 cm) to form 1 mm thick layer and served as preparative TLC plates. The plate was heated in an oven (NSW-142, India) at 110 °C for 30 min for activation and removed from the oven and brought to room temperature. The sample was evenly applied onto the TLC plate, inserted into the TLC chamber (20 cm \times 20 cm with glass lid 20 cm \times 10 cm, Labtech) containing n -hexane/ EtOAc (90:10) and allowed to run until the solvent travelled three-fourth of the plate [20]. The plate was removed from the chamber, dried at room temperature. The spots were scrapped and dissolved

in *n*-hexane/EtOAc (90:10). The solvent was evaporated to dryness leading to the separation of 80 mg of white-coloured crystals code-named DP.

Thin layer chromatography: The isolated compounds BG and DP were dissolved in MeOH and applied over the silica gel TLC plates (60 GF₂₅₄, 20 cm × 20 cm; 0.2-0.3 mm thick, Merck) along with the standard stigmasterol (purity ~ 95%) and β -sitosterol (purity \geq 95%) [21]. The TLC plates were run in a *n*-hexane/EtOAc (90:10) solvent mixture, air-dried sprayed with anisaldehyde reagent and visualized in an Ultra-Violet Chromatography Inspection Cabinet (JSGW, India). The TLC led to the separation of BG and DP into two distinct spots of different R_f values whose R_f values exactly matched with R_f values of standard stigmasterol (R_f 0.91; blue coloured) and β -sitosterol (R_f 0.85; blue coloured), respectively (Figs. 1 and 2). Therefore, BG and DP were considered as stigmasterol and β -sitosterol, respectively. Purified isolated compounds were solubilized in acetone and a few drops of MeOH and evaporated for recrystallization and analyzed with UV, HPLC, IR, NMR and mass spectrometers [22,23].

RP-HPLC analysis: The isolated compounds were passed through reverse-phase high pressure liquid chromatographic (Waters 515 HPLC pump, a valve type injector, Waters 2489 UV/Visible Detector), Symmetry[®] C₁₈ (250 mm × 4.6 mm) column with a particle size of 5 μ m (Waters, Singapore). The BG and DP were run in a mobile phase of MeOH and water (HPLC grade) in the ratio of 80:20 with a flow rate of 0.7 mL/min with an injection volume of 5 μ L at ambient temperature. The BG and DP were detected at 366 nm. The stigmasterol and β -sitosterol standards were subjected to HPLC with a similar condition [22]. The samples BG and DP and standards had a retention time of 20.452 and 20.109 for stigmasterol and β -sitosterol, respectively. The identity of BG and DP was thus confirmed as stigmasterol and β -sitosterol, respectively.

Spectroscopic characterization: The BG and DP were subjected to ¹H NMR, ¹³C NMR and mass spectrometry to elucidate their structure. The ¹H NMR and ¹³C NMR spectra were recorded using CDCl₃ as a solvent on Bruker-DPX-300 NMR spectrometer (Switzerland). The mass spectra were recorded at high resolution on a mass spectrometer (Shimadzu Corporation, Japan) at Sophisticated Instrumentation Centre, Indian Institute of Chemical Biology, Kolkata, India and the data were expressed as *m/z* values.

RESULTS AND DISCUSSION

The exact molecular mass of BG and DP was found to be 451 and 412, with a tentative molecular formula of C₂₉H₄₈O and C₂₉H₅₀O, respectively. This has been confirmed by ¹³C NMR and ¹H NMR analyses, which suggest that the number of C and H atoms are near to the formulas C₂₉H₄₈O and C₂₉H₅₀O for BG and DP, respectively. Since the compounds were positive for steroids, therefore all other structures other than steroids were not considered. On considering the nature of oxygen as hydroxyl and the presence of one double bond, the general formula deduced for both the compounds was C_nH_{2n-6}. Therefore, they seem to be tetracyclic compounds. The proposed molecular structure of the isolated compounds BG and DP is shown in Fig. 1.

Identification of stigmasterol (BG): The identity of compound BG was confirmed as stigmasterol, which was isolated as a white powder (100 mg) with a melting point of 174-176 °C. The λ_{max} in MeOH was 206 nm. ¹H NMR (CDCl₃, 300 MHz), ¹³C NMR (CDCl₃, 150 MHz) (Table-1). MS (*m/z*):(EI mode) showed molecular ion peaks at 412 that corresponded to the molecular formula of C₂₉H₄₈O. Ion peaks were further observed at *m/z* 369, 351, 271, 300, 255, 213, 199, 159, 133, 95, 83 and 53. All these data confirmed that BG is stigmasterol (Fig. 2). The separation of active molecules from natural products has been used for the production of new drugs and also forms part of combinatorial chemistry. Herein, the isolation of two phytosterols from the leaves of *Croton caudatus* Geiseler is reported for the first time. Initially, these two compounds (BG and DP) were separated as the white powders. The purification and further characterization by ¹H and ¹³C NMR, IR and mass spectrometry confirmed that the isolates were stigmasterol and β -sitosterols. The mass spectral analysis led to a molecular formula of C₂₉H₄₈O for BG, that is stigmasterol. The ¹³C NMR spectral data confirmed this contention where ¹H NMR spectra of BG indicated that it contains two methyl singlets at δ 0.69, and 1.03; three methyl doublets at δ 0.80, 0.83, and 0.91 and finally, a methyl triplet at δ 0.83. Compound BG also showed protons at δ 4.97, 5.14 and 5.34 suggesting the presence of three protons corresponding to the trisubstituted and disubstituted olefinic bonds. Liebermann-Burchard reaction indicated that compound BG is having a sterol skeleton [24,25]. The proton related to the H-3 of a sterol moiety is a triplet of doublet

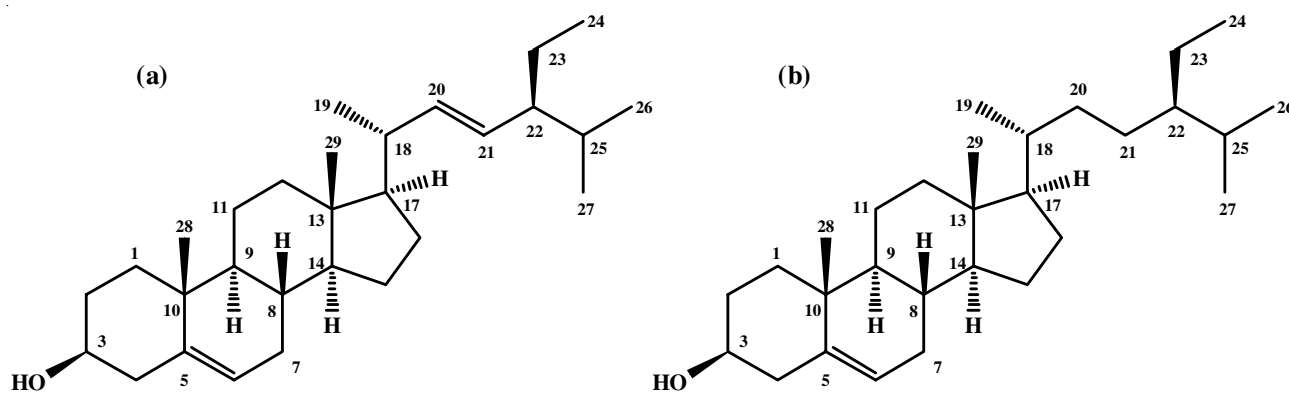


Fig. 1. Proposed chemical structure of isolated compounds BG (a: Stigmasterol) and DP (b: β -sitosterol)

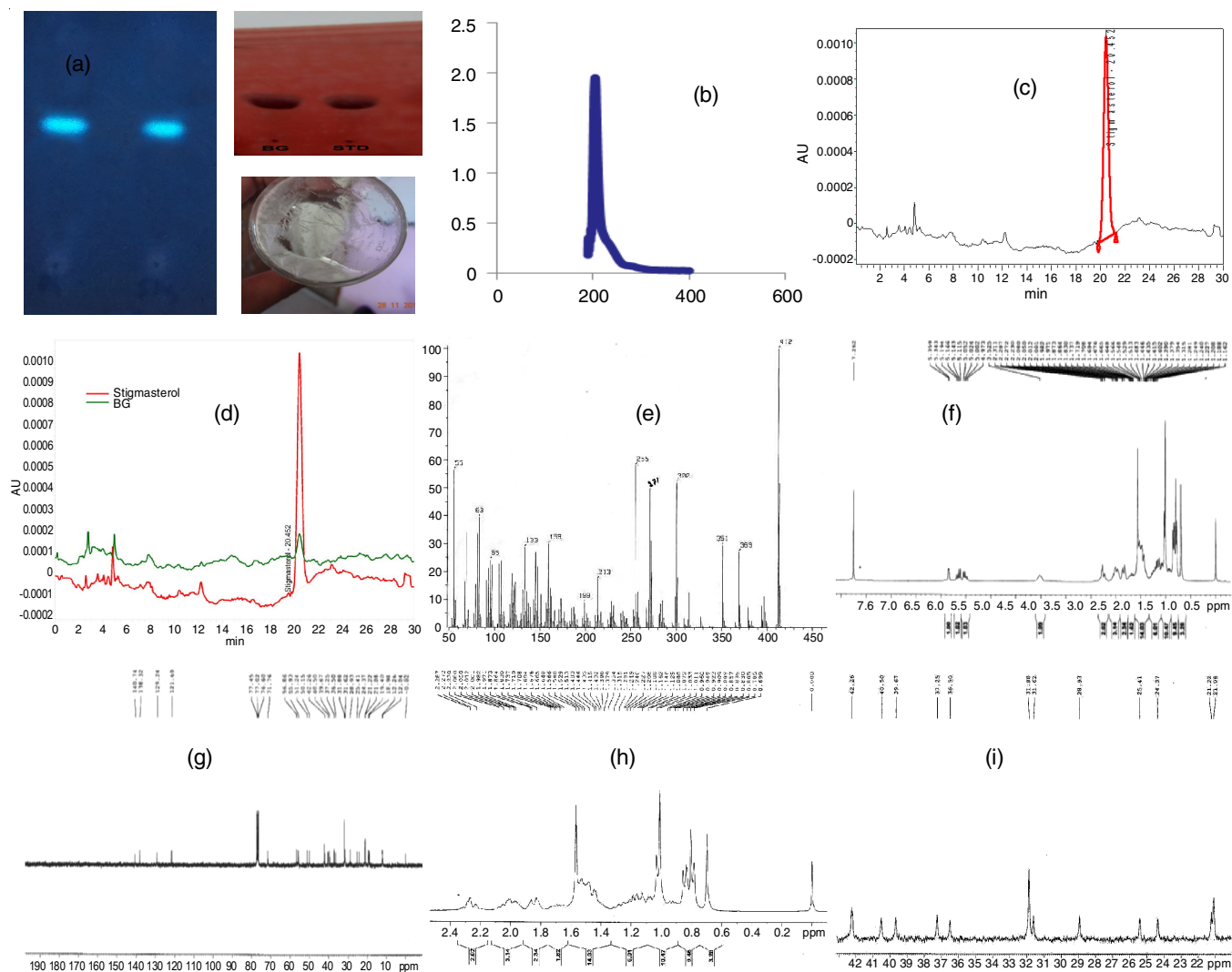


Fig. 2. Isolation of stigmasterol from *Croton caudatus* ethanol extracts (CCE). (a) TLC profile left spot (CCE) right spot: standard stigmasterol and isolated compound (Petridish); (b) IR spectra of unknown (BG) (c and d) HPLC (Solvent; methanol:water (8:2), Flow rate: 0.7 mL/min, Pressure: wavelength: 366 nm) (d) Overlay of BG (green) with standard stigmasterol (red); (e) Mass spectrum of BG; (f) Proton NMR spectra of BG and (g-i) Carbon NMR spectra of BG

TABLE-1
 ^1H AND ^{13}C NMR CHEMICAL SHIFT VALUES FOR COMPOUND BG AND DP IN CDCl_3
 CHEMICAL SHIFT VALUES ARE IN δ (ppm), COUPLING CONSTANTS ARE IN Hz

Position	BG		DP	
	^1H	^{13}C	^1H	^{13}C
1		31.85		31.66
1		31.85		31.66
2		37.25		40.5
3	3.525 (t dd, 1H, $J = 4.5, 4.2, 3.8$ Hz)	71.76	3.525 (t dd, 1H, $J = 4.5, 4.2, 3.8$ Hz)	68.9
4		42.26		44.7
5	5.343 (t, 1H, $J = 6.1$ Hz)	140.74	5.34 (t, 1H, $J = 6.4$ Hz)	140.1
6		121.69		121.9
7		31.88		29.7
8		31.62		34.19
9		50.15		45.81
10		36.50		49.43
11		21.22		25.5
12		39.67		36.9
13		42.26		44.0
14		56.86		56.2
15		24.31		27.3

16		28.93		27.7
17		55.93		50.3
18		40.50		28.93
19	0.909 (d, 3H, $J = 7.1$ Hz)	21.08	0.934 (d, 3H, $J = 6.5$ Hz)	19.5
20	4.973 (m, 1H)	138.32		36.14
21	5.143 (m, 1H)	129.24		19.40
22		51.24		33.9
23		25.41		29.64
24	0.835 (t, 3H, $J = 7.1$ Hz)	12.26	0.847 (t, 3H, $J = 7.2$ Hz)	40.24
25		28.93		28.9
26	0.830 (d, 3H, $J = 6.6$ Hz)	19.40	0.826 (d, 3H, $J = 6.4$ Hz)	31.48
27	0.805 (d, 3H, $J = 6.6$ Hz)	19.39	0.805 (d, 3H, $J = 6.4$ Hz)	12.26
28	0.699 (s, 3H)	18.98	0.681 (s, 3H)	21.20

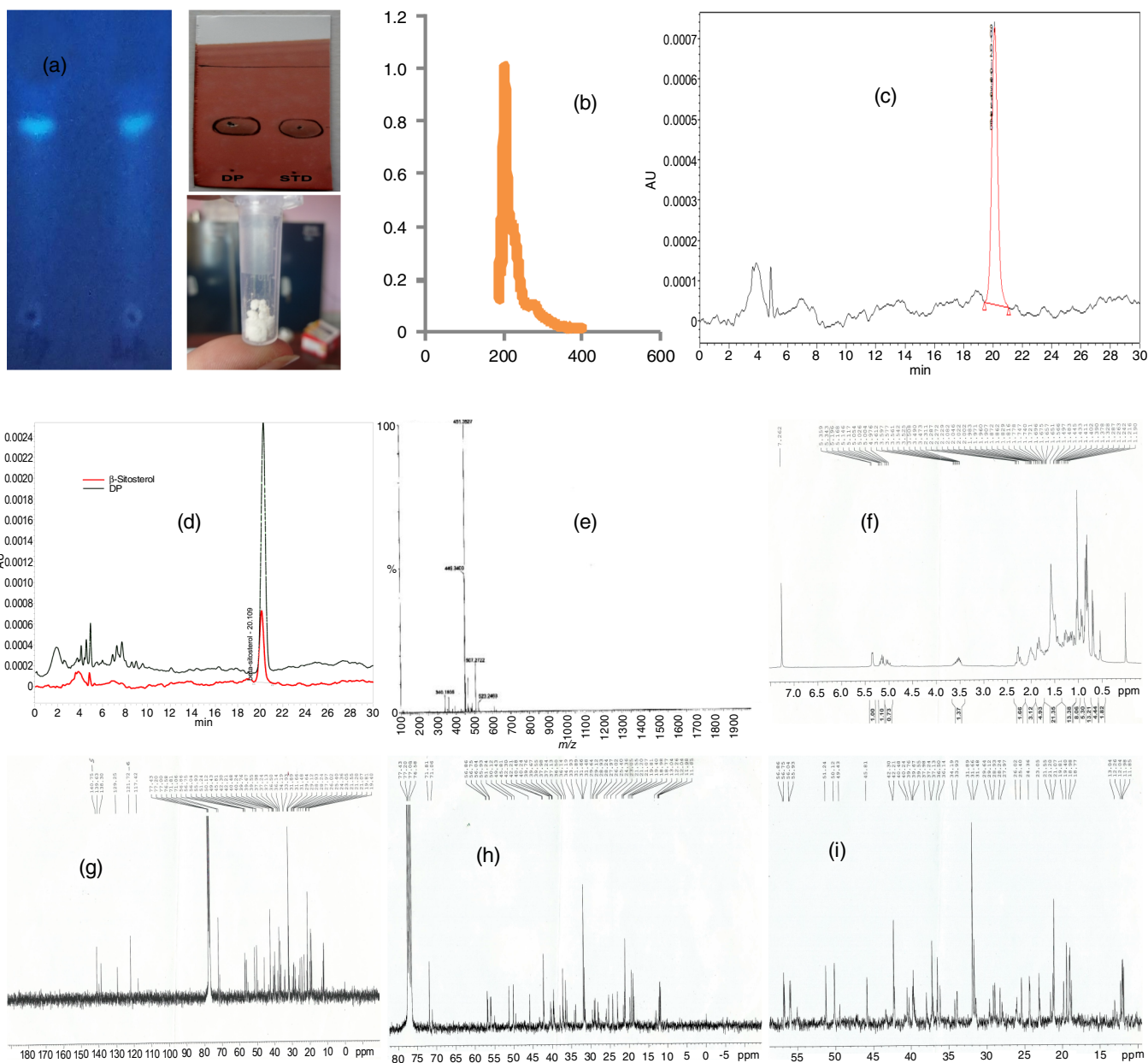


Fig. 3. Isolation of β -sitosterol from *Croton caudatus* ethanolic extracts (CCE). (a) TLC profile left spot (CCE) right spot: standard β -sitosterol and isolated compound (white powder); (b) IR spectra of unknown (DP) (c and d) HPLC (Solvent; methanol:water (8:2), Flow rate: 0.7 mL/min, Pressure: wavelength: 366 nm) (d) Overlay of unknown (green) with standard β -sitosterol (red); (e) Mass spectrum of DP; (f): Proton NMR spectra of DP and (g-i): Carbon NMR spectra of DP

of doublets at δ 3.52. The spectral data (Table-1) revealed that molecular structure of BG consists of a sterol skeleton encompassing a hydroxyl group at C-3 position, two double bonds at C-5/C-6 and C-20/C-21 and six methyl groups, which were confirmed by the key COSY and HMBC correlations. Thus, the structure of BG was identical to the known compound stigmaterol. The physical and spectral data of this study agree with that reported for stigmaterol earlier [26,27].

Identification of β -sitosterol (DP): The other white powder (80 mg) was separated as a second compound from ethanol extract of *Croton caudatus* leaves as DP, which had a melting point of 134-135 °C and λ_{\max} 204 nm in MeOH. The ^1H NMR (CdCl_2 , 300 MHz) and ^{13}C NMR (CDCl_3 , 150 MHz) are given in Table-1. The mass spectrometry in TOF mode showed (m/z): 451(M^+), 523, 507, 449 and 340 (Fig. 3). All these characteristics indicated that DP is β -sitosterol (Fig. 3).

The other isolated chemical DP was also a white powder whose mass spectral data indicated a molecular formula of $\text{C}_{29}\text{H}_{50}\text{O}$. Compound DP also showed a positive Liebermann-Burchard reaction, indicating its sterol nature. The ^1H NMR spectra of compound DP revealed that it consists of six methyl signals, which are two methyl singlets at δ 0.68 and 1.01; three methyl doublets at δ 0.81, 0.82, and 0.93; and a methyl triplet at δ 0.84. The ^1H NMR spectra of compound DP also showed one olefinic proton at δ 5.34. The conspicuous absence of protons related to the double bond between C-20/C-21 in compound DP together with its mass spectral data showed that it is 2 amu higher than the BG. It contained a trisubstituted double bond at C-5/C-6 in its molecular structure. The ^1H NMR spectra of compound DP showed a proton corresponding to the proton connected to the C-3 hydroxyl group and appeared as a triplet of doublet of doublets at δ 3.52. Thus, the structure of DP is akin to β -sitosterol that has been reported in the literature earlier from other plants as well as the stem of *Croton caudatus* Geisel. var. *tomentosus* Hook [26,28].

Conclusion

Two new sterols were isolated from the ethanolic extract obtained from the leaves of Kam Sabut (*Croton caudatus* Geiseler). The structures of the isolated new compounds were identified as stigmaterol (BG) and β -sitosterol (DP) on the basis of spectroscopic ^1H and ^{13}C NMR spectral and MS/MS spectroscopic data and by comparing their physical properties reported in the literature.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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