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ISOLATION, PURIFICATION AND STRUCTURAL ELUCIDATION OF NOVEL BIOACTIVE PHYTOCONSTITUENTS FROM *CRATAEVA NURVALA* BUCH-HAM STEM BARK CHLOROFORM FRACTION

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Abstract: The objective of the study was to isolate and characterize the bioactive phytoconstituents from *Crataeva nurvala* Buch-Ham stem bark. For isolation of the compounds, ethanolic extract of *Crataeva nurvala* stem bark was fractionated with chloroform and the chloroform fraction was subjected for conventional column chromatography. Three compounds (CN-01, CN-02 and CN-03) were isolated by gradient elution technique and purified with methanol. The subsequent structures of isolated compounds were elucidated by various spectrophotometric analysis. Mass spectrum of CN-01, CN-02 and CN-03 showed a parent molecular ion (M^+) peak at m/z 415.4 gm/mol correspond to the molecular formula $C_{29}H_{50}O$, 451.4 gm/mol correspond to $C_{29}H_{48}O$ and 465.3 gm/mol correspond to $C_{30}H_{50}O$ respectively. In the ^{1}H- NMR spectrum of CN-01, H-3 proton appeared as multiplet (m) at δ 3.5, H-6 proton appeared as doubly doublet (dd) at δ 5.35, eighteen methyl protons appeared as multiplet (m) between δ 0.6765-1.07 where as in the ^{1}H- NMR spectrum of CN-02, H-3, H-6 and H-22 protons appeared as singlet (s) at δ 2.6, 4.5 and 4.34 respectively, H-23 proton appeared as multiplet (m) at δ 4.35, nine methyl proton appeared as singlet (s) at δ 0.68, 0.97 δ 0.72. In the ^{1}H- NMR spectrum of CN-03, 4-β-CH_3 peak appeared as singlet (s) at δ 0.7608, at δ 0.8297 & 0.7882 two sharp singlet (s) peak correspond to 17-β-CH_3 & 4-α-CH_3; another singlet (s) peak appearing at δ 0.9085 depicted presence of 14-α-CH_3, multiplet (m) appearing at δ 1.1749 assigned for 10-α-CH_3; singlet (s) appearing at δ 2.3848 corresponds to –OH gr, vinylic protons appeared at δ 4.6843 & 4.6901 as singlet (s), H-3 axial portion appeared at δ 3.2078 as multiplet (m) and singlet (s) peak at δ 1.0301 accounted for br. -CH_3. From the physical, chemical and spectral characteristic CN-01, CN-02 and CN-03 were concluded as β-sitosterol, stigmasterol and lupeol.

Keywords: *Crataeva nurvala;* Isolation; β-Sitosterol; Stigmasterol; Lupeol

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INTRODUCTION

India has rich ancient heritage of traditional medicine [1]. From last two decades, the utility of medicinal plants have been phenomenally increased due to their vast chemical biodiversity as World Health Organization advocated traditional medicines as safe remedies [2]. The conventional therapeutic experiences of an array of bioactive phytoconstituents from those species, over hundreds years are considered as valuable remedial recipe to treat various acute and chronic disorders. Among them *Crataeva nurvala* (*C. nurvala*) Buch-Ham (Family: Capparidaceae) commonly known as Varuna, is a well explored traditional Indian medicinal plant used to treat various ailments in particular urolithiasis [3]. It is a medium sized branched deciduous plant distributed throughout the river banks of Westernghat region of southern India, wild or cultivated [4]. Vedic literatures described its potentiality as blood purifier and to maintain homeostasis [5]. Traditionally the stem bark is used as stomachic, laxative, anthelmentic, expectorant and anti-pyretic [6]. Moreover, pharmacological study reveals the potentiality of *C. nurvala* extract and its active principle, particularly lupeol as diuretic, anti-inflammatory, antioxidant, cardio-protective, hepatoprotective, lithonotriptic, anti-rheumatic, anti-periodic, contraceptive, anti-protozoal, rubifacient and vesicant [7]. Preliminary phytochemical screening reveals the plant is rich in secondary metabolites like alkaloids, saponins, triterpenes, tannins, flavanoid glycosides, glucosinolates and phytosterols [8]. Phytoconstituents like ceryl alcohol, friedelin, cadabicine diacetate, betulinic acid and diosgenin have already been isolated from the stem bark [9]. Since, the plant posses diverse medicinal properties, the present work had been designed to isolate and characterize novel bioactive phytoconstituents from chloroform fraction of *C. nurvala* stem bark.

MATERIALS AND METHODS

Collection and authentication of plant material

The stem bark of *C. nurvala* was collected from the stream sides of Westernghat, India and authenticated by Dr. K.V. Nagalakshamma, Professor and Head, Department of Biotechnology (UG) of St. Aloysius College, Mangalore, India. The herbarium (voucher specimen no. NGSMIPS/Hb-04/2011) was preserved in the institutional department.

Extraction and fractionation

1 kg coarsely powdered raw material of *C. nurvala* stem bark was extracted by cold maceration with ethanol and concentrated through rotary flash evaporator at 40^0C under reduced pressure and stored in deep freezer at -20^0C [10]. The yield was found to be 17 % w/w. The concentrated ethanolic extract (60 gm) was defatted with petroleum ether (4 x 100 ml) and

fractionated with chloroform (4 x 100 ml) successively. The combined chloroform fraction was concentrated under reduced pressure to afford chloroform soluble light brownish residue (14 gm). As mentioned in our previous article, chloroform fraction was enriched with maximum phytoconstituents, we had chosen chloroform soluble part for isolation of bioactive phytosteroids [11]. A flow chart of detailed method of extraction and fractionation is given in figure 1.

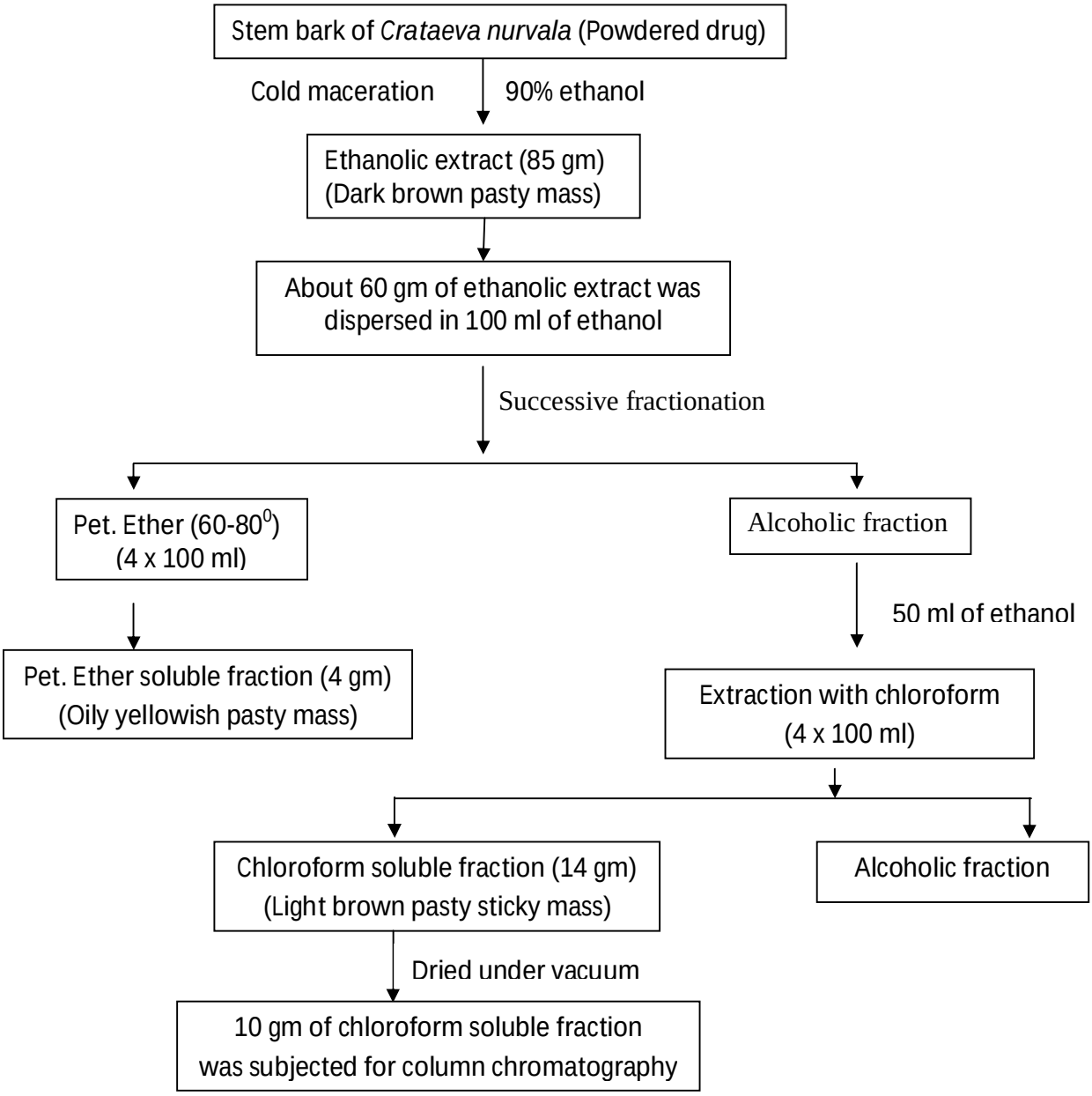

Figure 1: Schematic diagram of extraction and fractionation of stem bark of *C. nurvala*

Isolation and purification of compounds

The compounds were isolated from chloroform fraction (10 gm) through column chromatography using gradient elution technique [12]. The progress of separation was monitored by TLC (silica gel G 60 F_{254} plates, Merck). Fractions eluted with petroleum ether: chloroform (70:30) resulted an amorphous yellowish white residue which after crystallization with methanol provides colorless crystalline substance (36 mg) termed as CN-01**.** TLC chromatogram developed with petroleum ether: chloroform (2:8) was homogenous with R_f 0.52. Elution with petroleum ether: chloroform (20:80) resulted another cream coloured amorphous residue which after crystallization converted to pearl white crystals (48 mg) termed as CN-02**.** The homogeneity of isolated compound was determined by TLC where the chromatogram was developed with petroleum ether: chloroform: ethyl acetate (2:5:1). The R_f value of the compound was found to be 0.69. Further, elution with chloroform: ethyl acetate (10:90) yielded another light brownish amorphous substance which after crystallization converted to colourless crystals (42 mg) termed as CN-03. The purity of the isolated compound was determined by TLC using solvent system of chloroform: ethyl acetate: methanol (3:6:1). The R_f value of the compound was found to be 0.60.

Qualitative analysis

Libermann-Burchard test

Few crystals of CN-01, CN-02 and CN-03 were dissolved in chloroform separately and a few drops of conc. H_2SO_4 were added to the solution followed by addition of acetic anhydride. The solutions turned violet to deep green color [13].

Salkowski test

Few crystals of CN-01, CN-02 and CN-03 were dissolved in chloroform separately and a few drops of conc. H_2SO_4 were added to the solution. The solutions turned blood red color [13].

Tests for alcohol

4 gm of cerric ammonium nitrate was dissolved in 10ml of 2N HNO_3, on mild heating. A few crystals of CN-01, CN-02 and CN-03 were dissolved in 0.5ml of dioxane and added to 0.5ml of cerric ammoinium nitrate reagent. The developed yellow to red color indicates the presence of an alcoholic hydroxyl group [13].

Structural characterization of compounds

The structures of the isolated compounds were elucidated by spectroscopic methods viz. UV (Shimadzu UV-1700 Pharmac-spec UV-Vis spectrophotometer) and IR (Alpha-Bruker IR spectrophotometer), ^{1}H NMR & ^{13}C NMR (Bruker Advance II 400 NMR spectrophotometer), mass (TOF MS ES - 3.26e3 spectrophotometer). ^{1}H & ^{13}C NMR were recorded using $CDCl_3$ as solvent and with tetramethylsilane (TMS) as standard.

RESULTS

From the positive qualitative test, CN-01, CN-02 and CN-03 were assumed to be steroid and triterpenoid. The melting point of CN-01 was 136.2^{0}C, UV- λmax of CN-01 was 248.5 nm. Mass spectrum of CN-01 showed a parent molecular ion (M^+) peak at m/z 415.4 gm/mol correspond to the molecular formula $C_{29}H_{50}O$ (Figure 2).

The melting point of CN-02 was 169.0^{0}C, UV- λmax of CN-02 was 250.3 nm. Mass spectrum of CN-02 showed a parent molecular ion (M^+) peak at m/z 451.4 gm/mol correspond to the molecular formula $C_{29}H_{48}O$ (Figure 3).

The melting point of CN-03 was 213.0^{0}C, UV- λmax of CN-03 was 350.0 nm. Mass spectrum of CN-03 showed a parent molecular ion (M^+) peak at m/z 465.3 gm/mol correspond to the molecular formula $C_{30}H_{50}O$ (Figure 4). The spectral data of CN-01, CN-02 and CN-03 were summarized in table 1, 2 and 3 respectively.

The IR spectrum of CN-01 showed presence of branched -OH str. at 3757 cm^{-1}, CH_2 and -CH_3 str. at 2941, 2894 cm^{-1}, C=C str. at 1645 cm^{-1}, C-H bending at 1465 cm^{-1}, gem dimethyl str. at 1371 cm^{-1}, O-H bending at 1213 cm^{-1}, C-O str. at 1060 cm^{-1} and str. of tri-substituted double bond at 805 cm^{-1}. The ^{1}H NMR spectrum showed a number of characteristic signal viz. δ_H 5.35 (dd, 1H, H-6), δ_H 3.5 (m, 1H, H-3), δ_H 1.09-2.03 (29H, -CH and –CH_2 gr.) and δ_H 0.6768-1.07 (m, 18H, 6-CH_3 grs.). Moreover, ^{13}C NMR spectrum revealed the presence of total 29 carbon atom; δppm 140.36 and 121.72 at C-5 and C-6 revealed presence of double bond between them where as δppm 71.78 at C-3 revealed presence of OH gr. These arrangements were good assignment for the structure of β-sitosterol [13, 14, 15]. (Figure 5)

The IR spectrum of CN-02 showed presence of branched -OH str. at 3747 cm^{-1}, -CH_2 and -CH_3 str. at 2933, 2894 cm^{-1}, C=C str. at 1700 cm^{-1}, C-H bending at 1459 cm^{-1}, Gem dimethyl at 1374 cm^{-1}, O-H bending at 1214 cm^{-1}, C-O str. at 1058 cm^{-1} and str. of tri-substituted double bond at 805 cm^{-1}. The ^{1}H NMR spectrum showed a number of characteristic signal viz. δ_H 2.6 (s, 1H, H-3), δ_H 4.5 (s, 1H, H-6), δ_H 4.34 (s, 1H, H-22), δ_H 4.35 (m, 18H, H-23), δ_H 0.97 (3H, -CH_3 at 19), δ_H 0.72 (3H, -CH_3 at 21), δ_H 0.69 (3H, -CH_3 at 29), δ_H 1.02 (6H, 2 -CH_3 at 26, 27), δ_H 0.68 (3H, -CH_3 at 18), δ_H 1.2-1.48 (25H, -CH and-CH_2 grs.). Moreover, ^{13}C NMR spectrum revealed the presence of total 29 carbon atom; δppm 140 and 120.5 at C-5 and C-6 revealed presence of double bond between them where as δppm 70.38 at C-3 revealed presence of –OH gr. These arrangements were good assignment for the structure of stigmasterol [17, 18, 19]. (Figure 6)

IR spectrum of CN-03 showed presence of branched -OH str. at 3620 cm^{-1}, -CH_2 and -CH_3 str. at 2933 cm^{-1}, C-O str. at 1700 cm^{-1}, C=C str. at 1646 cm^{-1}, CH_3 & CH_2 deformation at 1459 cm^{-1}, Gem dimethyl at

1374 cm^{-1}, O-H bending at 1214 cm^{-1}, and C-H out of plane deformation at 1058 cm^{-1}. The ^{1}H NMR spectrum showed a characteristic singlet peak at δ_H 0.7608 which correspond to 4-β-CH_3; at δ_H 0.8297 & 0.7882 two sharp singlet peak correspond to 17-β-CH_3 & 4-α-CH_3; another singlet peak appearing at δ_H 0.9085 depict presence of 14-α-CH_3 ; the multiplets appearing at δ_H 1.2555-1.6808 accounted for 26 H of CH_2 and CH; another multiplet appearing at δ_H 1.1749 assigned for 10-α-CH_3; singlet appearing at δ_H 2.3848 corresponds to –OH gr; vinylic protons appear at δ_H 4.6843 & 4.6901 as singlet; The H-3 axial portion appeared at δ_H 3.2078 as a multiplet where as singlet at δ_H 1.0301 accounted for br. -CH_3. Moreover, ^{13}C NMR spectrum revealed the presence of total 30 carbon atom in the molecule. The spectrum revealed the presence of seven methyl, eleven methylene, six methine and six quaternary carbons. Two signals at δ_{ppm} 151.00 and 109.32 were due to two olefinic carbons of C-20 and C-29 respectively. The carbon bonded to -OH group at C-3 appeared at δ_{ppm} 79.02. These arrangements were good assignment for the structure of lupeol [20, 21]. (Figure 7)

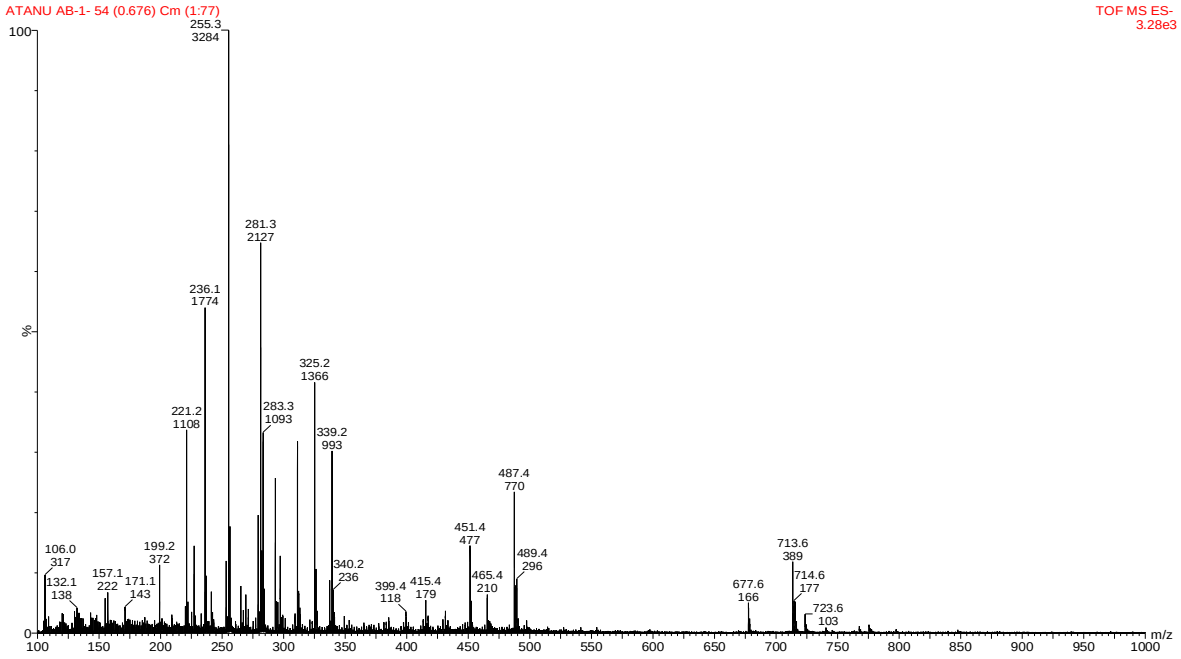

Figure 2: Mass spectrum of isolated compound CN-01

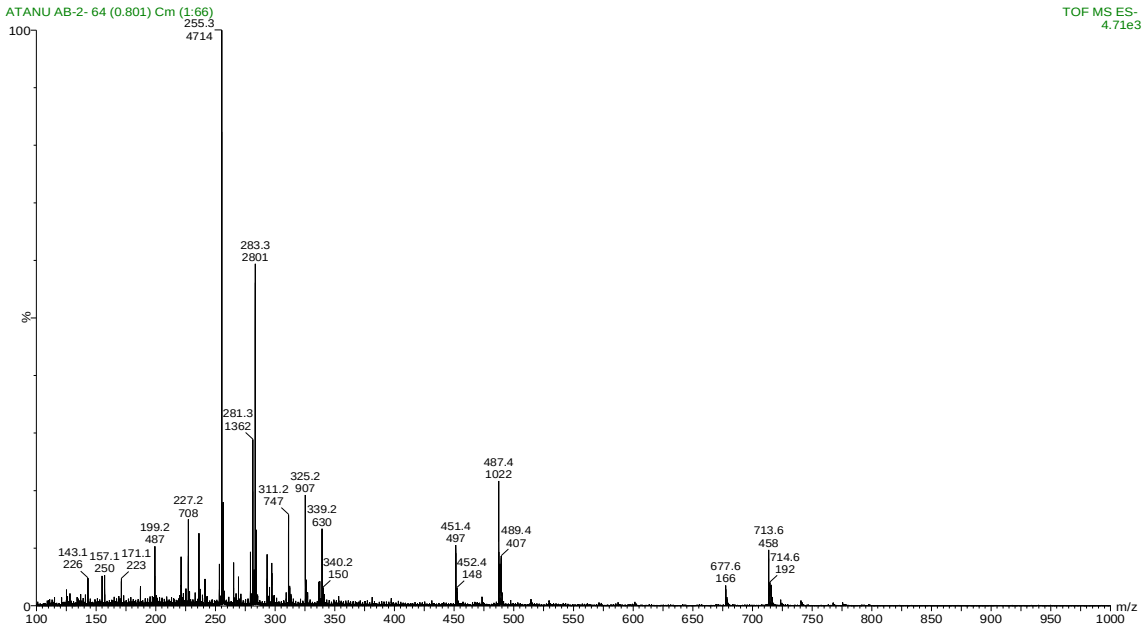

Figure 3: Mass spectrum of isolated compound CN-02

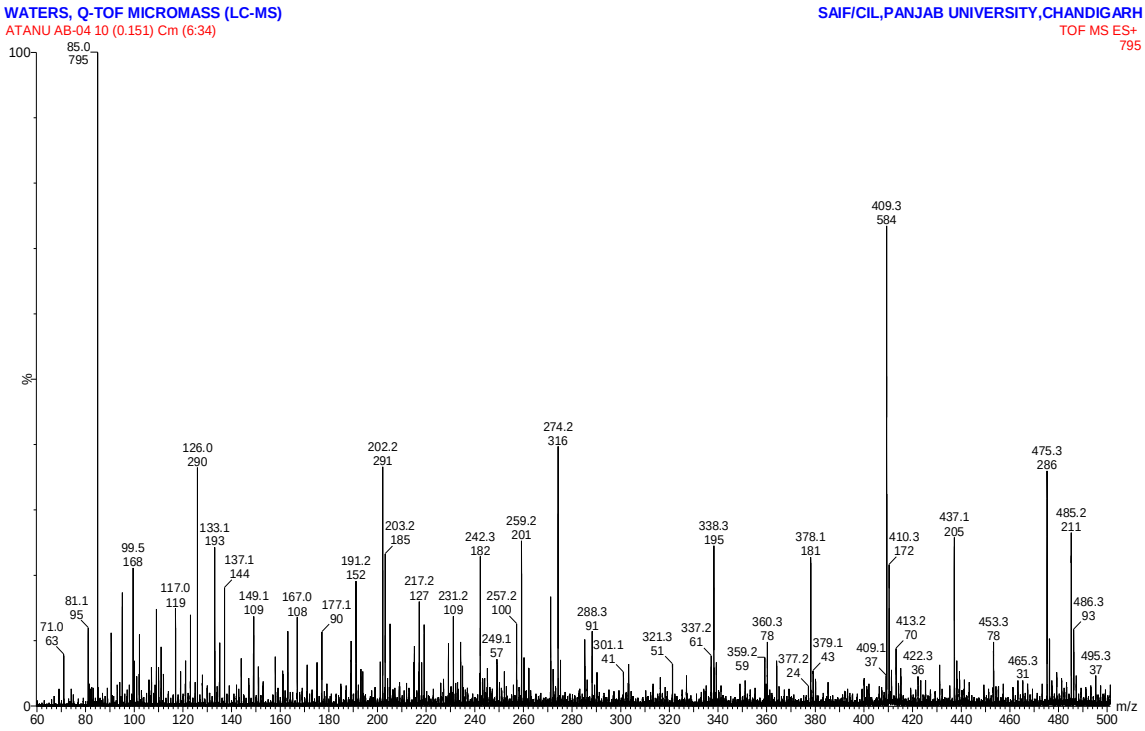

Figure 4: Mass spectrum of isolated compound CN-03

Table 1: Spectral characteristic of CN-01

IR (ATR)	**3753, 2941, 2894, 1645, 1465, 1371, 1213, 1060, 805 cm^{-1}**
^{1}H NMR δ (ppm)	δ 0.6768 – 1.07 (m, 18 H, 6 -CH3 gr. at 18, 19, 21, 26, 27 and 29) δ 1.09 – 2.03 (29 H, -CH and –CH2 gr.), δ 3.5 (m, 1 H, H-3), δ 5.35 (dd, 1 H, H-6)
^{13}C NMR δ (ppm)	37.26 (C-1), 28.03 (C-2), 71.78 (C-3), 42.32 (C-4), 140.36 (C-5), 121.72 (C-6), 31.65 (C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.78 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 28.26 (C-16), 56.1 (C-17), 11.9 (C-18), 19.42 (C-19), 36.2 (C-20), 18.76 (C-21), 35.8 (C-22), 28.03 (C-23), 50.13 (C-24), 31.92 (C-25), 19.4 (C-26), 19.4 (C-27), 23.81 (C-28), 11.9 (C-29)
EIMS (m/z) (%)	415.4 [M+], (5 %),
Relative intensity	399.4 (3 %), 339.2 (30 %), 325.2 (42 %), 281.3 (65 %), 255.3 (100 %), 236.1 (55 %), 221.2 (34 %), 199.2 (12 %)

Table 2: Spectral characteristic of CN-02

IR (ATR)	**3747, 2933, 2894, 1700, 1646, 1459, 1374, 1214, 1058, 756 cm^{-1}**
^{1}H NMR δ (ppm)	δ 2.6 (s, 1 H, H-3), δ 4.5 (s, 1 H, H-6), δ 4.34 (s, 1 H, H-22), δ 4.35 (s, 1 H, H-23), δ 0.68 (s, 3 H, - CH3 at 18), δ 0.97 (s, 3 H, - CH3 at 19), δ 0.72 (s, 3 H, - CH3 at 21), δ 1.02 (s, 6 H, 2 - CH3 at 26, 27), δ 0.69 (s, 3 H, - CH3 at 29), δ 1.2-1.48 (25 H, -CH and –CH2 grs.)
^{13}C NMR δ (ppm)	36.8 (C-1), 31.3 (C-2), 70.38 (C-3), 39.78 (C-4), 140 (C-5), 120.5 (C-6), 31.4 (C-7), 36.81 (C-8), 49.57 (C-9), 36.0 (C-10), 20.76 (C-11), 39.9 (C-12), 41.8 (C-13), 56.18 (C-14), 31.09 (C-15), 28.37 (C-16), 56.28 (C-17), 11.56 (C-18), 18.9 (C-19), 40.2 (C-20), 20.65 (C-21), 137.8 (C-22), 128.61 (C-23), 50.62 (C-24), 28.37 (C-25), 20.49 (C-26), 18.51 (C-27), 24.85 (C-28), 11.8 (C-29)
EIMS (m/z) (%)	451.4 [M+], (10 %),
Relative intensity	339.2 (14 %), 325.2 (19 %), 311.2 (16 %), 283.3 (60 %), 255.3 (100 %), 227.2 (15 %), 199.2 (11 %), 157.1 (5 %)

Table 3: Spectral characteristic of CN-03

IR (ATR)	**3620, 2933, 1700, 1646, 1459, 1374, 1214, 1058, 968 cm^{-1}**
^{1}H NMR δ (ppm)	δ 0.7608 (s, 3 H, 4-β-CH_3), δ 0.8294, 0.7882 (s, 6 H, 17-β-CH_3 & 4-α-CH_3), δ 0.9085 (s, 3 H, 14-α-CH_3), δ 1.0187 (s, 3 H, 8-β-CH_3), δ 1.2555-1.6808 (m, 25 H, 10 x -CH_2 + 5 x -CH), δ 1.1749 (m, 3 H, 10-α-CH_3), δ 2.3848 (s, 1 H, -OH), δ 4.6843 & 4.6901 (s, 2 H, vinylic proton), δ 3.2078 (m, 1 H, 3-α-CH), δ 1.0301 (s, 3 H, br. -CH_3)
^{13}C NMR δ (ppm)	38.06 (C-1), 27.45 (C-2), 79.02 (C-3), 38.87 (C-4), 55.30 (C-5), 18.33 (C-6), 34.29 (C-7), 40.84 (C-8), 50.44 (C-9), 37.18 (C-10), 20.93 (C-11), 25.15 (C-12), 38.71 (C-13), 42.84 (C-14), 27.43 (C-15), 35.59 (C-16), 43.01 (C-17), 48.00 (C-18), 48.31 (C-19), 151.00 (C-20), 29.85 (C-21), 40.01 (C-22), 28.00 (C-23), 15.38 (C-24), 16.13 (C-25), 15.99 (C-26), 14.56 (C-27), 18.01 (C-28), 109.32 (C-29), 19.32 (C-30)
EIMS (m/z) (%)	465.3 [M^{+}], (5 %),
Relative intensity	81.1 (4 %), 85.0 (37 %), 99.5 (9 %), 126.0 (16 %), 133.1 (10 %), 202.2 (14 %), 274.2 (19 %), 288.3 (4 %), 338.3 (13%), 409.3 (28%), 437.3 (15%), 507.2 (100%), 508.2 (28%), 663.4 (30%), 685.4 (32%), 686.4 (14%)

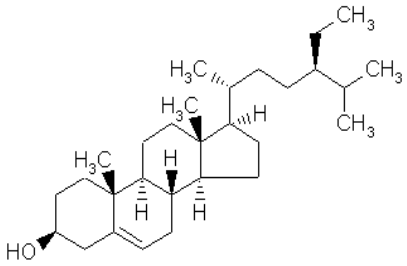

Figure 5: β-Sitosterol

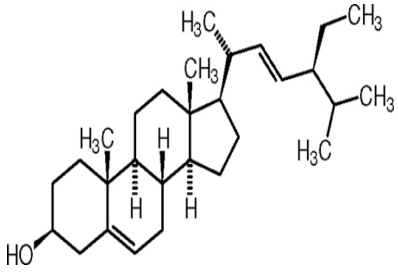

Figure 6: Stigmasterol

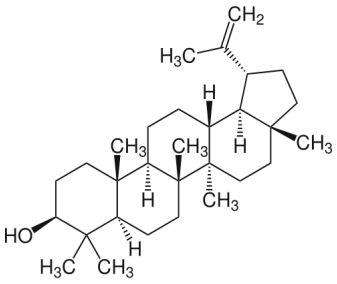

Figure 7: Lupeol

DISCUSSION

β-sitosterol, one of the major phytosterol in higher plants has immense therapeutic potential. In animals, it exhibits potent anti-inflammatory, anti-pyretic, anti-ulcer and immune-modulating activity [22]. Moreover, studies showed a positive effect on male hair loss [23]. In Europe, β-sitosterol is routinely prescribed against benign

prostatic hypertrophy. It is also used for the treatment of prostatic carcinoma and breast cancer [24]. β-sitosterol is an antioxidant able to reduce DNA damage, by decreasing the level of free radical in cells and increase in the level of typical antioxidant enzymes [25].

Stigmasterol, another important phytosterol in higher plants is a potent anti-osteoarthritic, analgesic and anti-inflammatory agent [19]. It was reported to inhibit cholesterol biosynthesis via inhibition of sterol Δ_{24}-reductase in human Caco-2 and HL-60 cell lines, suppressing hepatic cholesterol and thus acts as anti-hyperlipidemic agent. Stigmasterol was found to inhibit the lyase activity of DNA polymerase β in cultured A549 cells and thus acts as anti-tumor drug. Moreover, it had showed decreased in hepatic lipid peroxidation and increase in the activities of catalase, superoxide dismutase and glutathione level and thereby showed its antioxidant property [26].

Lupeol, one of the major triterpenoid has immense therapeutic potential. In animals, it exhibits potent anti-inflammatory, anti-lithotropic, anti-pyretic, anti-atherosclerotic, anticancer and immune-modulating activity [27]. The anti-inflammatory activity of lupeol is mediated via decrease in IL-4 production by Th2 cells, myeloperoxidase levels (neutrophil specific marker) and thus causing reduction in cell infiltration into inflamed tissues in rodents [28]. Lupeol showed powerful antioxidant property via stimulating antioxidant enzymes and reducing lipid peroxidation *in-vivo* [29]. The anti-lithotropic activity of lupeol in animals might be associated with prevention of oxalate and crystal-induced per-oxidative changes in renal tissues [30]. Lupeol's cytotoxic activity was attributed to its ability to inhibit topoisomerase II and lyase, essential enzymes that regulate the breaking of double helix of DNA [31]. Lupeol and its acetate showed marked hypotensive, hypolipidemic potential which might be associated with its cardioprotective activity [32].

CONCLUSION

A new method for isolation of novel bioactive phytosteroids and tripterpenoids viz. β-sitosterol, stigmasterol and lupeol had been developed which might be extremely suitable for use as marker compounds for the standardization of commercial extract and herbal-preparation containing *C. nurvala*.

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CONFLICT OF INTEREST:

We declare that we don't have any conflict of interest

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