Annals of Phytomedicine 1(2): 45-51, 2012

ANNALS OF PHYTOMEDICINE
An International Journal

Isolation and characterization of triterpenoids from bark of *Syzygium alternifolium* (Wight) Walp

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Received for publication November 7, 2012; accepted December 2, 2012

Abstract

Natural products from medicinal plants, either as pure phytoconstituents or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. In the present investigation include the extraction, isolation and characterization of active compounds from *Syzygium alternifolium* (Wight) Walp. Isolated phytoconstituents were forwarded to phytochemical screening and chromatographic techniques such as Thin Layer Chromatography (TLC). Crude bark extract of *Syzygium alternifolium* was extracted and forwarded to preliminary phytochemical investigation which shows the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, volatile oils, tannins, proteins and carbohydrates. Based on extractive value, methanolic extract was fractionated with hexane and ethyl acetate. The hexane fraction of methanolic extract was forwarded to column for isolation. Three triterpenoids and sterol, which were isolated and characterized by chromatographical and spectroscopical analysis as friedelane, friedelin 3â-friedelinol and stigmasterol, respectively.

Key words: *Syzygium alternifolium* (Wight) Walp, Myrtaceae, Phytochemical investigation, Phytoconstituents, Triterpenoids

Introduction

Natural products as standardized extracts provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Sasidharan *et al*., 2011). According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation.

Plants are potent biochemists and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, *etc.* or any part of the plant may contain active components. The systematic screening of plant species with the purpose of discovering new bioactive compounds, is a routine activity in many laboratories. Scientific analysis of plant components follows a logical pathway. Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found (Parekh *et al*., 2006). In most of the reported works, underground parts (roots, tuber, rhizome, bulb *etc*.) of a plant were used extensively, compared with other above ground parts in search for bioactive compounds, possessing antimicrobial properties (Das *et al*., 2010).

Syzygium alternifolium (Wight) Walp (Family: Myrtaceae) is a tree or shrubs, evergreen, usually with essential oils containing cavities flowers. Leaves opposite, occasionally alternate, occasionally ternate or pseudo-whorled; inflorescences axillary or terminal, cymose but variously

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arranged. *Syzygium alternifolium* fruit is used for curing stomach ache, ulcers (Baqtiyar *et al*., 2012) and management of rheumatic pains (Venkata Ratnam and Venkat Raju, 2008); seeds as antidiabetic agents (Ramesh *et al*., 2010 and 2012 and Kameshwar and Appa Rao, 2001), leaves to treat dry cough and dysentery and stem bark as antiseptic.

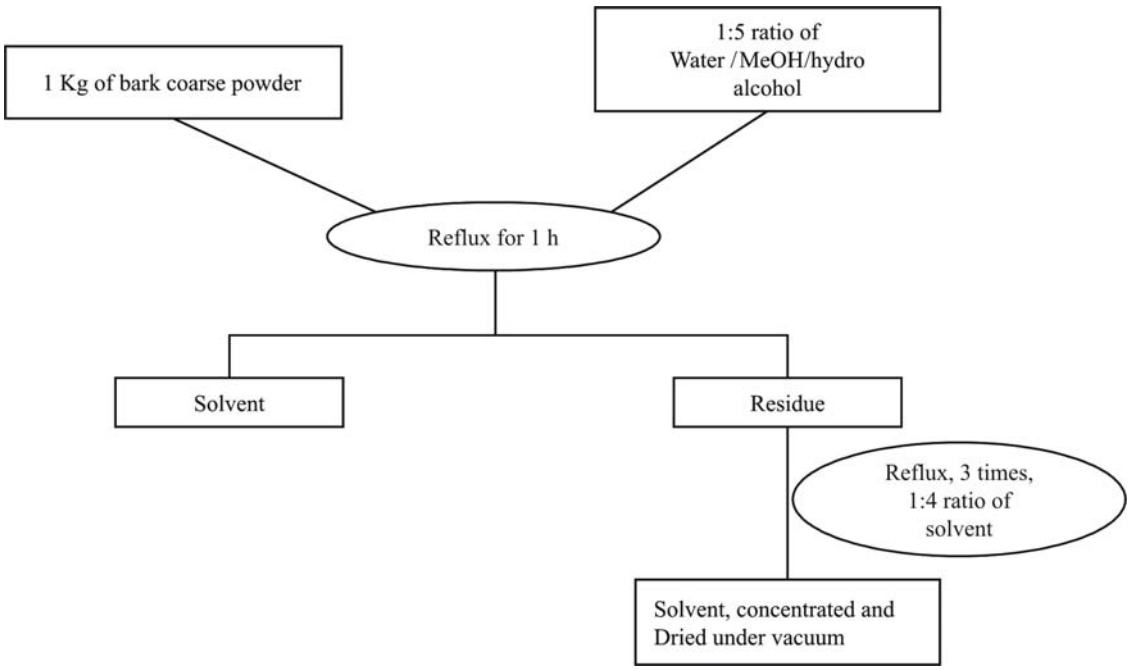
Syzygium alternifolium is a tree endemic to this region. The seed powder in water given orally 3 times a day after food, controls diabetes (Nagaraju and Rao, 1989 and 1997 and Thammanna and Madhava Chetty, 1994). It is locally known as Mogi or Adavinerudu. It is also used for fevers and skin diseases (Thammanna and Nagaraju, 1990). *Syzygium alternifolium* belongs to the family, Myrtaceae and the other species of this genus *S. cumini* L. Skeel is well known for its antidiabetic activity (Teixeira *et al*., 1997). Bark, leaves and seeds of *S. cumini* are astringent. Its berry as a whole is astringent. Juices of these fruits are stomachic, astringent, diuretic and antidiabetic (Nadkarni, 1994).

Materials and Methods

Plant material

The bark of *Syzygium alternifolium* (Wight) Walp were collected from Seshachalam hills, India during july 2011 and it was authenticated by Dr. K. Madhava Chetty, Assistant Professor in the Department of Botany, Sri Venkateshwara University, Tirupati. India.

Flowchart for extraction procedure



Different codes were assigned to different extracts.

- SA1- *Syzygium alternifolium* water extract

General experimental procedures

1H and 13C NMR were recorded on Bruker 400MHz (1H: 400 MHz, 13C: 100 MHz) or Varian 500MHz (1H: 500 MHz, 13C: 125 MHz) NMR spectrometers with tetramethylsilane (TMS) as an internal standard. Standard pulse sequences were employed for the measurement of 2D NMR spectra (1H-1H COSY, HMQC, and HMBC). The solvents used for NMR spectra were deuterated methanol (CD3OD) and deuterated dimethylsulfoxide (DMSO-d6). Mass spectra were measured on either JEOL JMS AX-500 or FT-ICR-MS (Bruker, APEXQ) spectrometers. All the melting points were recorded in a Toshniwal melting point apparatus. IR spectra of the compounds were recorded, using the KBr pellet method on a Perkin Elmer 700 IR spectrophotometer. Column chromatography was carried out on neutral alumina of 70-300 mesh from S.D. Fine Chemicals Pvt. Ltd., Bombay. All the chemicals and reagents used were obtained in high purity from S.D. Fine Chemicals Pvt. Ltd; Bombay, India and E. Merck Pvt. Ltd., Bombay, India.

Extraction and isolation of plant material

The collected bark was dried under shade and size reduced to the fine powder. The shade dried powdered bark (1Kg) was extracted using reflux with methanol, hydroalcohol (60 % methanol in water) and water for one hour to obtain methanol hydroalcohol and aqueous extracts.

- SA2- *Syzygium alternifolium* hydroalcoholic extract
- SA3- *Syzygium alternifolium* methanolic extract

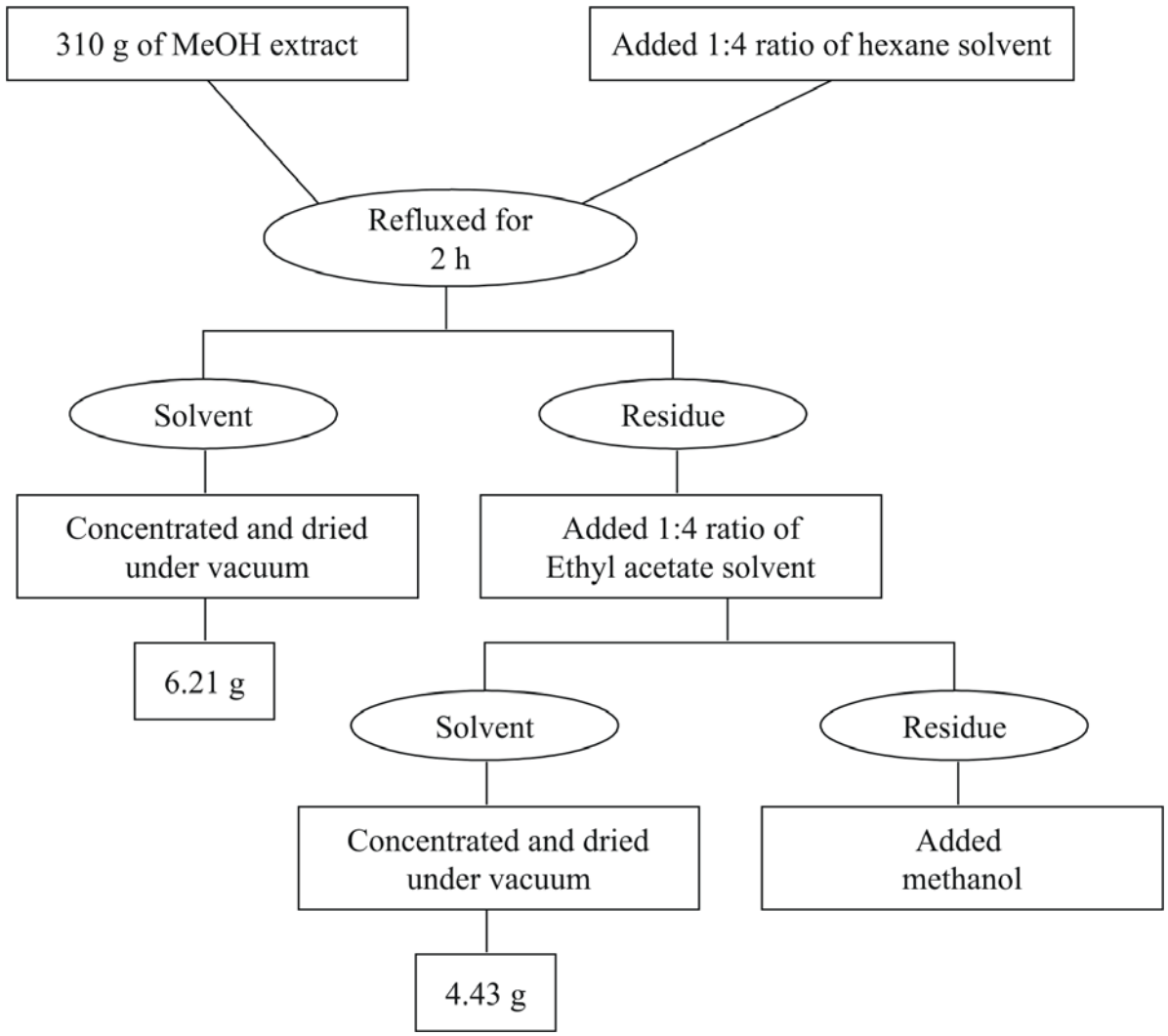
Preliminary phytochemical screening

The preliminary phytochemical studies were performed on for methanolic, hydroalcoholic and aqueous extracts of *Syzygium alternifolium* bark.

Fractionation of methanolic extract and isolation of compounds

310 g of methanol extract (SA3) was partitioned with 1:4 ratio of hexane solvent (1200mL) by refluxing for 2 h. The obtained residue was further fractionated with ethyl acetate solvent in the ration of 1:4.

Flowchart for partitioning of methanol extracts using hexane and ethyl acetate solvents



Codes of extracts were named as below and in the further discussion, the extracts were mentioned as their codes.

- SA 3/01-*Syzygium alternifolium* hexane extract
- SA 3/02- *Syzygium alternifolium* ethyl acetate extract
- SA 3/03- *Syzygium alternifolium* methanolic extract

The weight of hexane extract was found to be 6.21g and ethyl acetate extract weight was found to be 4.43g.

The methanolic extract (310 g) was partitioned with 1:4 ratio of hexane solvent (1200 mL) and then fractionated successively with ethyl acetate. All the fractions were washed with distilled water (30 mL), dried over anhydrous sodium sulphate. The methanolic extract was, thus, fractionated into hexane extract (6.21g), ethyl acetate extract (4.43g). The column was packed with silica gel (100-120) and eluted with ethyl acetate and polarity was slowly increased with methanol. All fractions were allowed for crystallization and crystals were collected from fraction-4 SA3/03/04, fraction-5 SA3/03/05 and fraction-11 SA3/03/11. In TLC, single spot was observed in fraction-5 and fraction-11. The both fractions were further crystallized to obtain the pure compound.

Hexane extract was fractionated by column chromatography using silica gel. 5g of hexane extract was taken and adsorbed with 50 g of silica gel. The fraction-2, fraction-3 and fraction-4 showed a single spot in TLC, so mixed to obtained single compound.

Results and Discussion

Preliminary phytochemical investigation

The proximate chemical analysis showed the presence of alkaloids, glycosides, steroids, flavonoids, terpenoids, carbohydrates, proteins and tannins.

Compound SA3/01/02

The compound **SA3/01/02** (Tables 1, 2, 3) was obtained as white crystalline powder with a MP-258-260°C.The molecular formula of compound **SA3/01/02** was shown to be $C_{30}H_{50}O$ based on the positive ion LC-MS [*m/z* 427(M+H)$^+$ and 449.4 (M+Na)$^+$] analysis. The IR spectrum showed bands at 2944 and 2894(CH Symmetric and Asymmetric Stretch), 1716 (Ketone), 1452 and 1348cm^{-1} Ar-C-H Stretching. The ^{1}H and ^{13}C-NMR spectrum of **SA3/01/02** indicated the presence of 30 carbon resonances. The DEPT spectrum indicated the presence of carbonyl at ä 213.07 including seven quaternary carbons five CH carbons ten CH_2 carbons and eight CH_3 carbons. Since, the molecular formula indicative six units of unsaturation, this compound **SA3/01/02** was concluded to be penta cyclic triterpene with a ketone group. The presence of signals due to one secondary and seven quaternary methyls in the ^{1}H-NMR spectrum suggested the friedelane skeleton. In the ^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) quartet signal integrating for one proton at (δ 2.18q) assigned to H-4. The resonances of eight methyls δ 0.7281.003 (3H, s, H-24), 0.8761.003 (3H, s, H-25), 0.8751.003 (3H, dJ=6.5Hz, s, H-23), 0.9471(s, H-30), 1.013(s, H-29), 1.003(s, H-27), 1.005(s, H-26) and 1.180(s, H-28). The signals at δ2 and 2.261, δ1.956 and1.65, δ1.735 and1.286, δ 0.969 and 1.449 were due to unequivalent methylene protons at C-2, C-1, C-6 and C-22, respectively. This was supported by cross correlations between cach methylene protons signals in COSY spectrum. The ^{1}Hand ^{13}C-NMR spectral data of compound **SA3/01/02** indicates that it belongs to was identified as Fredelin. The identification of friedelane was confirmed by comparison of the reported spectral data with compound.

Compound SA3/01/03

The compound **SA3/01/03** (Tables 1, 2, 3) was obtained as white crystalline powder MP- 293-294°C. The molecular formula of compound **SA3/01/03** was shown to be $C_{30}H_{52}O$ based on the positive ion LC-MS [*m/z* 427(M+H)$^+$] analysis. The IR spectrum showed bands at 3500 brs hydroxyl and 2944 2894(CH Symmetric and asymmetric Stretch), Ar-C-H stretch at 1452 and 1348 cm^{-1}. The ^{1}Hand ^{13}C-NMR spectrum of **SA3/01/03** indicated the presence of 30 carbon resonances. The DEPT spectrum indicated the presence of six quaternary carbons five CH carbons eleven CH_2 carbons and eight CH_3 carbons one of the carbons resonated at δ72.8 and indicative of presence of hydroxyl group. The ^{1}H NMR spectrum (CDCl3, 400 MHz) gave a peak at δ 3.73 which corresponds to H-3.In the HMBC spectrum C-1 showed correlations with H-3 and H-10. H-3 resonated at δ 3.73, dd*J* = 10.8, 4.6 Hz, the HMBC correlations and this chemical shift value allowed us to place the hydroxyl functionality at this position (C-3). H-10 resonated at δ 0.86, dd J=12.0, 2.0Hz the multiplicity, dd was due to a coupling of this proton with H-1 proton. C-3 (δ- 72.20 showed correlations with H-2 and H-23. These correlations and the chemical shift value supported to phase the hydroxyl functionality at C-3 and methyl a group (C-23) at C-4.

Further, in the 1D proton NMR spectrum, the methyl group at C-23 split in to a doublets, which was due to the coupling of these methyl protons with H-4 and fits the characteristic of this methyl group (C-23) at C-$. C-4 showed correlation with H-2, H-23 and H-24 and the quaternary carbon C-3 showed correlations with H-1, H-3,H-6 and H-24. These correlations supported the attachment of C-23 methyl group at C-4 and C-24 methyl group at C-5. The methyl protons at H-26 showed correlations with C-12, C-13, C-14 and C-18. These correlations allowed us to place the C-26 methyl group at C-15 and C-27 methyl a group at C-13, respectively. The methyl protons at H-28 showed correlations with C-16, C-17,C-18 and C-22. These correlations allowed us to place C-28 methyl group at C-17. Finally, C-29 showed correlations with H-19 and H-30; H30 showed correlations with H-19, H-21 and H-29. C-20 showed correlations with H-19 H-21, H-29 and H-30; C-21 showed correlations with H-22, H-29 and H-30 all these correlations supported the attachment of both C-29 and C-30 methyl groups to the same carbon at C-20 and the compound is an oleane type triterpenoid. The β orientation of the hydroxyl group at C-3 was confirmed by comparing the proton coupling between H-3 and H-4 and H-2 confirms the stereo-chemistry. These data had good agreements with the reported data of 3β –Friedelinol.

Compound SA3/01/04

The compound **SA3/01/04** (Tables 1, 2, 3) was obtained as colorless crystals. The MP was found to be 148-154°C. The molecular formula of compound **SA3/01/04** was shown to be $C_{29}H_{48}O$ based on the positive ion LC-MS [*m/z* 413$(M+H)^+$ 435$(M+Na)^+$] analysis. The IR spectrum compound **SA3/01/04** showed bands at brs3428 and 3301 (hydroxyl), 2934, 2863 (C-H stretch), 1646.47(olefinic), 1467.55, 1373.38 and 1054.90 cm^{-1}. The ^{1}H[400MHz,$CDCl_3$] and ^{13}C-NMR [100MHz,$CDCl_3$] spectrum of **SA3/01/04** mixture showed integration of proton signals at δ 5.356,H-6, 5.155 H-22, 5.018 H-23 and 3.522 H-3 of stigmasterol. In ^{1}H NMR spectrum the olefinic proton signal appeared at and six methyls (δ 0.83-1.08). The ^{1}Hand ^{13}C-NMR spectral data of compound **SA3/01/04** indicates that it belongs to monohydroxy sterol group and was identified as stigmasterol based on its identical ^{1}Hand ^{13}CNMR data and physical parameters. ^{1}HNMR ($CDCl_3$, 400MHz) of ST: 1HNMR has given signals at δ 3.2(1H, m, H 3), 5.26 (1H, m, H 6), 5.19(1H, m, H 23), 4.68(1H, m, H 22), 3.638(1H, m, H 3), 2.38(1H, m, H 20), 1.8 2.0 (5H, m) ppm. Other peaks are observed at ä 0.76 0.89 (m, 9H), 0.91 1.05 (m, 5H), 1.35 1.42 (m, 4H), 0.69 0.73 (m, 3H), 1.8 2.00 (m, 5H), 1.07 1.13 (m, 3H), 1.35 1.6 (m, 9H) ppm. ^{13}CNMR ($CDCl_3$, 100MHz) of ST: 13CNMR has given signal at 150.98,145.2 (C 5), 139.8 (C 22), 121.7, 118.89(C 6), 79.03 (C 3), 55.3(C 14), 55.18(C 17), 50.45 (C 9), 48.3 (C 9), 40.8 (C 20), 40.1(C 12),39.2 (C 13), 38.9 (C 4), 38.6 (C 12), 37.18 (C 1), 37.12 (C 10), 36.3 (C 8), 35.59(C 20), 34.29 (C-22), 34.24 (C-7), 32.66 (C-8), 29.86 (C-25), 29.71 (C-16), 28.41 (C-2), 28.1 (C-15), 27.4 (C-28), 26.1 (C-11,26), 21.6 (C-27), 19.32 (C-19), 17.71 (C-21), 15.6 (C-18, 29).

Table 1: Structure of isolated compounds

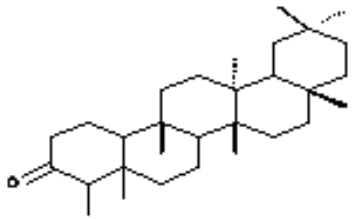
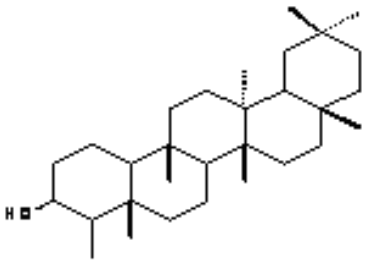
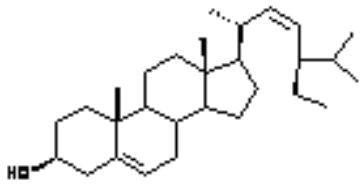
Compound code	Chemical structure	Common names
SA3/01/02		Friedelane
SA3/01/03		3β -Friedelinol
SA3/01/04		Stigmasterol

Table 2: Physical characteristics of isolated compounds from *Syzygium alternifolium*

Compound code	Physical state	Melting Range	R_f(cm)
SA3/01/02	White crystalline powder	258-260°C	0.71 (solvent system-hexane: ethyl acetate) (8:2)
SA3/01/03	White crystalline powder	293-294 °C	0.28 (solvent system-hexane: ethyl acetate) (8:2)
SA3/01/04	Colorless crystals	148-154 °C	0.5 (solvent system-hexane: ethyl acetate) (8:2)

Table 3: Spectral characteristics of isolated compounds from *Syzygium alternifolium*

Compound code	IR	^{1}H NMR	^{13}C NMR	mass
SA3/01/02	2914.53, 2949.91(C-H str) 1727.23,1458, 1367.68, 1246.49 (ester)	δ 0.80-1.02 (CH_3) δ 1.67 (CH_2) δ 4.66,4.56(CH)(brs)	δ 151.0 (C)(C-20) δ 109.3 (C)(C-29) δ 80.6 (C)(C-3) δ 13.97, 22.8, 25.5, 29, 29.8, 31.4, 34.8,173.63 (long chain ester)	663.6 (M-H)
SA3/01/03	2914 (C-H symstr) 2849.91 (C-H asymstr) 1727,1458, 1367.68,1246.49, 992 (ester)	δ5.181,tJ=2.8Hz (CH_2) 4.503 tJ=8Hz (CH_4) 0.80-1.254 (CH_3) 2.074 (acetyl CH_3)	145.38 (C)(C-13)	467.8 (M-H) 121.67 (C)(C-12)
SA3/01/04	3428, 3301(OH) 2934,2863(C-H str) 1646.47,1467.55, 1373.38, 1054.9 (olefinic)	ä 0.83- 1.08 (CH_3)		413 $(M+H)^+$ 435$(M+Na)^+$

Conclusion

The preliminary phytochemical studies identified the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, volatile oils, tannins, proteins and carbohydrates in that methanolic extract. Whereas, further phytochemical investigation led to isolate three pure compounds from active hexane fraction. A total of three compounds were isolated from active hexane fraction. The isolated compounds were characterized by TLC, IR, Mass and NMR. The three compounds were identified to be friedelane (Ardiles *et al*., 2012, David Dako, 2009 and Hishashi, *et al*., 1999), friedelinol and stigmasterol. These compounds were identified as triterpenoids and sterol and by spectroscopical data, respectively.

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