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Original Research Article

Isolation and Characterization of Bio Active Triterpenes and Phytosterols from *Alysicarpus monilifer* L.

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A B S T R A C T

Plants produce chemical compounds as part of their normal metabolic activities. These can be split into two broad categories-primary metabolites and secondary metabolites. The functions of secondary metabolites are extremely varied. It is these secondary metabolites which can have therapeutic action in humans and they can be refined to produce potential therapeutic drugs. This study aimed to isolate and elucidate the chemical compounds that are found in *Alysicarpus monilifer* L. The whole plants were extracted using 80% methanol and the resulted hydro-alcoholic extract was fractionated using hexane, ethyl acetate and methanol. The hexane fraction was further eluted using column chromatography followed by thin layer chromatography. The structure elucidation was performed using nuclear magnetic resonance spectrometry (NMR). The detailed phytochemical investigation of hexane fraction of hydro-alcoholic extract of this plant isolated two triterpenoids and a phytosterol which were identified as 3-β-hydroxy-urs-12-en-28-oic acid, Stigmasterol respectively. The study on qualitative phytochemical screening also identified some important bioactive phytochemical principles such as steroids, triterpenoids, saponins, flavonoids, tannins, carbohydrates and glycosides in this plant which were also validated as antioxidants and biologically active phytoconstituents. The biological evaluation of the protective effect of this plant extract against hepatotoxicity was well established validates the efficacy and use of this herb in traditional systems of medicine. These bioactive triterpenes isolated from *Alysicarpus monilifer* L., can further be explored to open a new perspective for pharmaceutical applications in future.

Keywords

Alysicarpus monilifer L., Phytochemical investigation, spectrometry, Phytoconstituents, Triterpenoids, phytosterol

Introduction

Alysicarpus monilifer L. has been used in indigenous system of medicine is a widely used plant in the north coastal districts of Andhra Pradesh, India. The leaves are used to treat jaundice (Sankarnarayan, 1988). Its paste is used for coetaneous problems (Rahmatullah *et al.,* 2010). The roots are used for the treatment of leprosy and urinary troubles. The decoction of roots is prescribed for cough. The boiled leaves are used as purgative. The herb is credited with anti- pyretic, anti- periodic and expectorant properties, febrifuge and also recommended for cutanious scabies and boils and to cure pain. Eather and ethnolic extracts of leaves of *Alysicarpus veginalis* showed antiproliferation activity against tumor cells (Rathi *et al.,* 2010). The plant was also screened for its bioactivity showing hepatoprotective activity in albino rats (Manikya Kumari *et al.,* 2012). With this background of its bioactivity, the hexane

fraction of the methanolic extract was eluted to isolate and characterize the bioactive molecules present in them.

Materials and Methods

Plant material

The whole plant of the *Alysicarpus monilifer* was collected from the surroundings of Visakhapatnam, Andhra Pradesh and its identity was confirmed by the department of Botany, Andhra University, Visakhapatnam. The herbarium specimen of the plant was deposited in the department of Botany, Andhra University with the Voucher no: VPJ/DOB/AM2509.

Preparation of extracts

The shade dried plants of about 500 g were subjected to size reduction to coarse powder. The powder was then extracted with 80%methanol using Soxhlet apparatus till exhaustion for about 48 hours. Later it was concentrated under vacuum to get the residue. The percentage yield was found to be 8% (w/w). The hydro alcoholic extract was further fractionated to obtain hexane, ethyl acetate and methanol soluble fractions.

Preliminary Phytochemical Screening

In the present study, methanol extracts and their hexane, ethyl acetate, methanol soluble fractions of *Alysicarpus monilifer* whole plant were subjected to qualitative chemical tests using standard procedure to detect various phytoconstituents present in them (Wagner, 1984; Kokate, 1991).

Column Chromatography of Hexane fraction

Column chromatography was done by standard procedure. Silica gel (Qualigens), 60-120 mesh was used as absorbent for column chromatography. The column was eluted using hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol mixtures by gradient using 8g of residue, 320g of the silica gel and 100ml of each eluant. This was followed by Thin Layer Chromatography (TLC).

Results and Discussion

Preliminary phytochemical screening

The preliminary phytochemical screening showed the presence of steroids, terpenoids, saponins, flavonoids, carbohydrates and glycosides.

Structure elucidation and characterization of compound AMH – 3 & 4

AMH-3&4 were obtained as colorless or white needle shaped crystalline solid from hexane soluble fraction when eluted with 20% ethyl acetate in hexane. The compound recorded m.p. at 140^{o}C. IR spectrum showed significant bands at: 3429,2930,2852,1710, 1463 and 1381 cm^{-1};

The compound showed M^{+} at *m/z* 412, EIMS spectrum confirming the molecular formula as $C_{29}H_{48}O$. This compound showed very strong colour reaction with sulphuric acid indicating its steroidal nature.

The ^{1}H NMR spectra of AMH -3 &4 revealed a one-proton multiplet at δ 3.51, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a multiplet at δ 5.35 integrating for one-proton. The olefinic protons (H-22 and H-23) appeared as characteristics downfield signals at δ 5.15 and δ 5.05 respectively in

the ^{1}H NMR spectrum (Fig). Each of the signal was observed as doublets (J=14.4, 8.4 Hz) which indicated coupling with the neighboring olefinic and methane protons.

The spectrum further revealed signals at δ 0.70 and δ 1.01 (three-proton each) assignable to two tertiary methyl groups at C-13 and C-10.respectively. The ^{1}H NMR spectrum also showed two doublets centered at δ 0.80(J=7.4 Hz) and 0.85(J=7.4 Hz) which could be attributed to the two methyl groups at C-25. The doublet at δ 0.91 (J=6.4 Hz) was demonstrative of a methyl group at C-20. These spectral features are in close agreement to those observed for stigmasterol (Fig. 3) by Khan (1991). On this basis, the identity was confirmed as stigmasterol (Greca *et al.*, 1990).

^{13}C NMR spectrum (Fig. 1) revealed 29 carbon resonances, out of them six CH_3, nine CH_2, eleven CH and three quaternary carbons. On the basis of NM assignment and literature data, the compound was identified as stigmasterol (Pouchert and Behnke 1968 & Habib *et al.*, 2007).

Physical and structural characteristics of AMH-3, 4 & 9

Stigmasterol

Entry Name: Stigmast-5-en-3-ol

Synonym(s): 24-Ethylcholest-5-en-3-ol.

CRC Number: CBR05-M

Melting point: 140^{o}C

Molecular Formula: C_{29} H_{48} O

Molecular Weight: 414.713

Accurate Mass: 414.386165
Percentage Composition: C 83.99%; H 12.15%; O 3.86%

Partition Coefficient (Calculated): Log P 10.45(uncertain value) (calc)

Table.1 Nature of phytoconstituents present in different extracts of *Alysicarpus monilifer*

Phytoconstituent	Hydro alcohol extract (80%)	Hexane fraction	Ethyl acetate fraction	Methanol fraction
Phytosterols	+	+	+	+
Triterpenes	+	+	-	+
Saponins	+	-	-	+
Alkaloids	-	+	+	-
Flavonoids	+	+	+	+
Tannins	+	-	+	+
Carbohydrates	+	+	-	+
Glycosides	+	+	-	-

+ = Present, - = absent

Table.2 ^{1}H NMR (400 MHz) ^{13}C NMR (100 MHz) spectral data of compound AMH- 3 (Stigmasterol) and AMH-5 (Ursolic acid) in CDCl3,δ in ppm

Position	Chemical Shifts (δ) of Stigmasterol		Chemical Shifts (δ) of Ursolic acid	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	-	37.2	-	39.3
2	-	31.6	-	28.2
3	-	71.8	3.48(1H,*m*)	77.3
4	-	39.7	-	40.4
5	-	140.7	-	56.0
6	5.35(1H,s)	121.7	-	18.7
7	-	31.9	-	32.7
8	-	31.6	-	40.4
9	-	50.1	-	47.5
10	-	36.5	-	39.8
11	-	21.1	-	23.1
12	-	39.8	5.15(1H,*m*)	127.9
13	-	40.4	-	138.2
14	-	56.8	-	40.4
15	-	24.3	-	28.8
16	-	28.8	-	24.2
17	-	56.0	-	46.2
18	0.70(3H,s)	11.8	-	52.8
19	0.93(3H,s)	19.3	-	37.4
20	-	33.8	-	36.5
21	1.01(3H, d, *J*=8.0Hz	19.8	-	30.8
22	5.05(1H,m)	138.2	-	36.8
23	5.15(1H,m)	129.3	0.78(3H,s)	31.5
24	-	45.9	0.89(3H, s)	14.0
25	-	31.8	0.86(3H,s)	18.9
26	0.85(3H,d,J=8.5Hz)	19.0	0.70(3H,s)	19.0
27	0.84(3H,d,J=7.5Hz)	29.0	1.03(3H,s)	27.1
28	-	25.3	-	18.7
29	0.88(3H,d,J=8.0 MHz)	12.0	0.82(3H,d,J=6.5Hz)	21.0
30			0.91(3H,s,J=6.5 Hz)	22.5

Fig.1 ^{1}H NMR and ^{13}C NMR spectra of compound – AMH -3: Stigmasterol

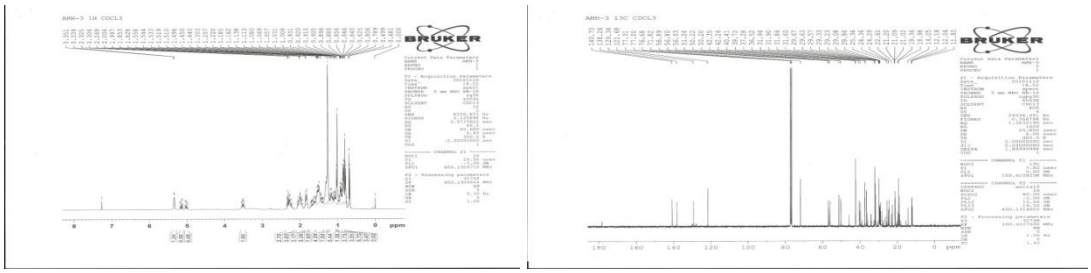


Fig.2 ^{1}H NMR and ^{13}C NMR spectra of compound AMH – 5: Ursolic acid

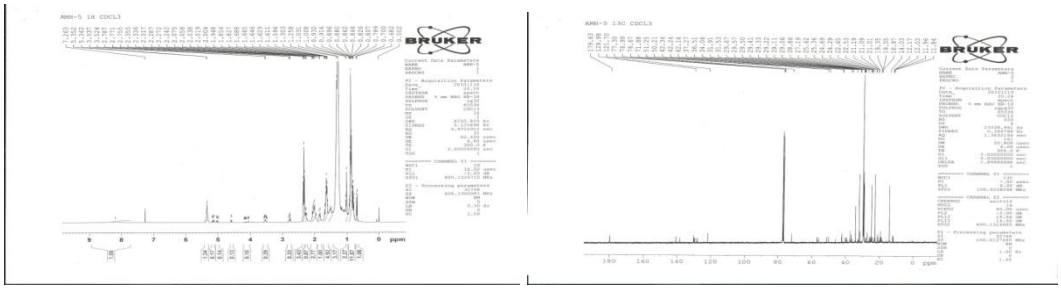


Fig.3 Structure of Stigmasterol (AMH-3&4)

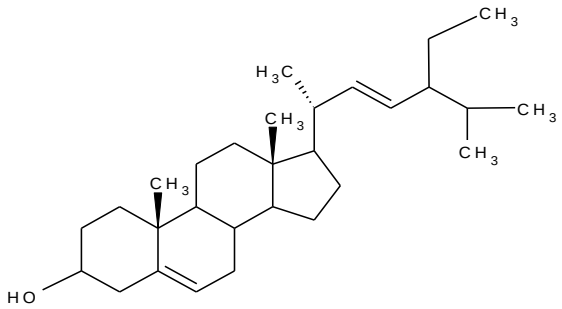
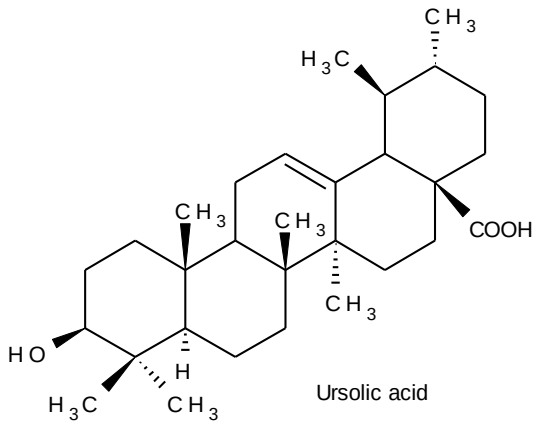


Fig.4 Structure of -Ursolic acid (AMH-5)



Structure elucidation and characterization of compound AMH–5 (Ursolic acid)

This compound was isolated from hexane fraction of the whole plant of *Alysicarpus monilifer*. The fraction was subjected to column chromatography using hexane and ethyl acetate mixture as the eluant. Twenty fractions were collected of which fractions 12-16 showed the presence of one major component together with some impurities. These fractions were washed with hexane to remove the impurities until it showed a single spot on TLC followed by recrystallisation. These fractions were combined and upon removing the solvent gave a whitish amorphous powder. It has a melting point at around 271-274°C (lit. m.p. 266-267°C.

The IR spectrum of AMH – 5(Fig.7.16c) showed bands at 3435, 2950, 1705, 1605, 1460, 1220, 1100, 900 cm^{-1}.δ 4.02 (1H, *brs*, D_2O exchangeable OH at C-3). Mass spectrum revealed MS (EIMS, 70 eV, $C_{30}H_{48}O_3$): 95, 211, 249, 319,345, 375, 391, 410, 443,445, 456 [M^+]. The ^{13}C spectrum showed seven CH_3, nine CH_2, seven CH and seven quaternary carbons. The ^1H spectrum (7.16a) of the compound showed five tertiary methyl groups at δ 0.78 (H-23), 0.70 (H-26), 0.86(H-25), 0.89 (H-24) and 1.03 (H-27) and two secondary methyl groups at δ 0.82(H-29) and 0.91 (H-30). (Mathela *et al.*, 1987 & Guvenalp *et al.*, 2006.).

The IR spectrum exhibited absorption bands at 3400 cm^{-1} (hydroxyl) and 1692 cnr1 (carbonyl). The mass spectrum showed a molecular ion peak at m/z 456 which corresponds to molecular formula $C_{30}H_{48}O_3$. The mass spectrum of the compound showed a retro-Diels Alder fragmentation of ring C resulting in the appearance of a base peak at m/z 248 a typical for an α or β-amyrin type triterpenes. The mass spectrum pattern allows the compound to be tentatively assigned as a triterpene type compound which in turn could either be α or β amyrin type compound. The two types of triterpene can be differentiated by looking at the signals of carbons-12, -13, -29 and -30. The fundamental difference between the two is that in the α-type triterpene, both the methyl groups are secondary whereas in the β-type the methyl groups are tertiary. Hence by comparing the nature of the carbon-13 values at C-29 and C-30, the compound is concluded as of α-type triterpene (Fig. 2).

The spectral data of the compound are consistent with ursolic acid with molecular formula $C_{30}H_{48}O_3$ (FAB, *m/z* 457, M^+ +1) (Mathela *et al.*, 1987 & Guvenalp *et al.*, 2006). Therefore AMH – 5 could be identified as ursolic acid (Fig. 4).

Ursolic acid is a pentacyclic triterpene acid, used in cosmetics, that is also capable of inhibiting various types of cancer cells by inhibiting the STAT3 activation pathway and human fibrosarcoma cells by reducing the expression of matrix matelloproteinase-9 by acting through the glucocorticoid receptor. It may also decrease proliferation of cancer cells and induce apoptosis (Pathak AK., *et al.*, 2007)

Ursolic acid is present in many plants, including apples, basil, bilberries, cranberries, elder flower, peppermint, rosemary, lavender, oregano, thyme, hawthorn, prunes. Apple peels contain large quantities of ursolic acid and related compounds.

Ursolic acid can serve as a starting material for synthesis of more potent bioactive derivatives, such as anti-tumor agents. It has been found to reduce muscle atrophy and to stimulate muscle growth in mice (Kunkel, S. *et al.*, 2011*)*. Ursolic acid has potential use as a cardioprotective compound (Liobikas *et al.*, 2011). Ursolic acid was found to be a weak aromatese inhibitor (IC_{50}=32 μm).

Physical and spectral characteristics of AMH-5

Entry Name: 1S, 2R, 4aS, 6aR, 6aS, 6bR, 8aR, 10S, 12aR, 14bS)-10-hydroxy-1, 2, 3, 4, 5, 6, 6a, 7, 8, 8a, 10, 11, 12, 13, 14b-tetradecahydro-1H-pice. Ursolic acid

Synonyms: 3-beta-3-hyrdoxy-urs-12-ene-28-oic-acid, 3-β-hydroxy-urs-12-en-28-oic acid, urson, prunol, and malol.

Melting point: 140^oC

Molecular Formula: C_{30} H_{48} O_3

Molecular Weight: 456

Alysicarpus monilifer, is an herb which is used in traditional medicine in the north coastal districts of Andhra Pradesh. The phytochemical screening of the plant proved to possess bioactive phytoconstituents such as phytosterols and triterpene - Ursolic acid. The compounds isolated and characterized from hexane fraction of *Alysicarpus monilifer* whole plant were β- sitosterol, Stigmasterol and Ursolic acid. These compounds were isolated for the first time from this plant. They proved to be bioactive through biological evaluation of plant extract and fractions which showed significant dose dependant hepatoprotective activity validating its use in traditional systems of medicine.

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