Journal of Enzyme Inhibition and Medicinal Chemistry, August 2008; 23(4): 543–548

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Anti-inflammatory activity of a new triterpenoid saponin from *carthamus tinctorius* linn

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(Received 1 February 2007; in final form 24 August 2007)

Abstract
A new bioactive triterpenoid saponin 3β-O-[β-D-xylopyranosyl(1 → 3)-O-β-D-galactopyranosyl]-lup-12-ene-28 oic acid-28-O-α-L-rhamnopyranosyl ester compound (**A**), was isolated from the methanolic fraction of the roots of this plant by various colour reactions, chemical degradations and spectral analysis. Compound (**A**) showed anti-inflammatory activity.

Keywords: *Carthamus tinctorius* Linn, compositae, roots, triterpenoid saponin, anti-inflammatory activity

Abbreviations: *TMS, Tetramethylsilane; DMSO, dimethyl sulphoxide; DMF, Dimethyl formamide; Co-PC, Paper chromatography with authentic sample*

Introduction

Carthamus tinctorius Linn [1–3] belongs to the family Compositae which is commonly known as "Kusum" in Hindi and is found in India, China, Afghanistan, Egypt, Italy, Spain and Australia. It is said to be a laxative and is administered in a warm infusion. It is used as a substitute for saffron in measles. The leaves are diuretic, appetizer, cure urinary discharge, and good for eyes. The flowers are tonic to liver, hypnotic, diuretic, expectorant, cure inflammations, boils, ringworm and piles. The seeds are purgative, aphrodisiac, cure leucoderma, pain in chest and throat. Earlier workers [4] have reported various constituents from this plant.

The present paper deals with the isolation and structural elucidation of compound **A**, a new triterpenoid saponin 3β-O-[β-D-xylopyranosyl(1 → 3)-O-β-D-galactopyranosyl]-lup-12-ene-28oic-acid-28-O-α-L-rhamnopyranosyl ester from the roots of this plant which shows anti-inflammatory activity.

Materials and methods

Plant material

The roots of *Carthamus tinctorius* Linn. were collected locally around Sagar region and were taxonomically authenticated by the Taxonomist, Department of Botany, Dr H.S. Gour University, Sagar (M.P.) India. A voucher specimen has been deposited in the Natural Products Laboratory, Department of chemistry, Dr. H.S. Gour University, Sagar (M.P.) India.

General

All the melting points were determined by Thiele melting point apparatus and are uncorrected. The IR spectra were recorded in Perkin Elmer 1800 FTIR Spectrophotometer. ^{1}H-NMR spectra were recorded at 300 MHz on Bruker DRX 300 NMR spectrometer using TMS as internal standard and $CDCl_3$ as solvent. ^{13}C-NMR were recorded at 90 MHz using DMSO-d_6

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2008 Informa UK Ltd.
DOI: 10.1080/14756360701715422

as solvent and mass spectra on a Jeol SX-102 (FAB) mass spectrometer.

Extraction and isolation

Dried powdered roots(3 Kg) of the plant were extracted with methanol (50–60°C) in a Soxhlet extractor. The total methanolic extract was concentrated under reduced pressure to yield light brown coloured viscous mass. It gave two spots on TLC examination using chloroform: methanol: water (3:6:4) as solvent and I_2 vapours as visualizing agent, indicating it to be a mixture of two compounds **A** and **B**. These were separated by column chromatography over a silica-gel column using hexane:ethyl acetate (4:2) as eluents and further purified by preparative TLC. Compound **B** was found in very small quantity therefore it was not possible to examine it further.

Compound A. It was crystallized from methanol to yield light yellowish needles (1.85 gm), m.p.297–298°C, $C_{47}H_{76}O_{16}$ $[M]^+$ 896 (FABMS); found C; 62.86, H; 8.43, calcd for $C_{47}H_{76}O_{16}$ C; 62.90, H; 8.48%; IR ν_{max}^{KBr} cm^{-1} 3442, 1685, 1640, 1386, 1376, 1030, 1026, 990. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.62 (6H, s, H-23, H-24), 0.80 (3H, s, H-25), 0.96 (3H, s, H-26), 1.03 (3H, s, H-27), 1.17 (3H, d, J 6.9 Hz, H-29), 1.24 (3H, d, J 6.1 Hz, H-30), 5.16 (1H, t, J 3.0 Hz, H-12), 4.52 (1H, t, J 7.7 Hz, H-3), 1.29-1.83 (23 H, m, polymethylene CH_2 and CH), δ 5.33 (1H, d, J 2.1 Hz, H-1′), 4.76 (1H, s, OH-1′), 4.34 (1H, dd, J 1.3, 2.4 Hz, H-2′), 4.5 (1H, s, OH-2′), 4.01 (1H, dd, J 2.5, 8.9 Hz, H-3′), 3.9 (1H, s, OH-3′), 3.69 (1H, t, J 9.2 Hz, H-4′), 3.88 (1H, m, H-5′), 1.24 (3H, d, J 2.6 Hz, H-6′, CH_3), 6.10 (1H, d, J 7.6 Hz, H-1″), 3.81 (1H, dd, J 7.3, 8.5 Hz, H-2″), 4.15 (1H, s, OH-2″), 3.54 (1H, dd, J 8.2, 4.3 Hz, H-3″), 4.2 (1H, dd, J 4.2, 2.8 Hz, H-4″), 1.89 (1H, s, OH-4″), 4.02 (1H, d, J 6.4 Hz, H-5″), 4.23 (2H, dd, J 12.3, 4.7 Hz, H-6″), 2.03 (1H, s, OH-6″), 5.31 (1H, d, J 7.7 Hz, H-1‴), 3.90 (1H, dd, J 7.9, 8.5 Hz, H-2‴), 2.13 (1H, s, OH-2‴), 4.04 (1H, d, J 8.1 Hz, H-3‴), 2.25 (1H, s, OH-3‴), 4.06 (1H,s, H-4‴), 2.30 (1H, s, OH-4‴), 3.73 (2H, d, J 10.3, H-5‴)

MS (FABMS) $[M]^+$896; m/z 750,618, 486, 456, 439,438, 413, 412, 395, 248, 247, 220, 219, 208, 207, 205, 203, 191, 190, 189, 160, 145. Compound **A** was also characterized by ^{13}C-NMR spectrum (see Table I).

Acetylation of Compound A. Compound **A** (60 mg) was refluxed with Ac_2O and pyridine (10 mL each) at room temperature for 12 h. yielded nona acetate compound **D**, $C_{65}H_{94}O_{25}$, m.p. 303-305°C, $[M^+]$ 1274, (found C; 61.10, H; 6.9 calcd. for $C_{65}H_{94}O_{25}$, C; 61.22, H; 7.3%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.63 (6H, s, H-23, H-24), 0.82 (3H, s, H-25), 0.98 (3H, s, H-26), 1.05 (3H, s, H-27), 1.19 (3H, d, J 6.9 Hz, H-29), 1.25 (3H, d, J 6.1 Hz, H-30), 5.16 (1H, t, J 3.0 Hz, H-12), 1.28-1.81 (23 H, m, polymethylene CH_2 and CH), δ 4.52 (1H, t, J 7.7 Hz, H-3), 5.32 (1H, d, J 2.0 Hz, H-1′), 4.33 (1H, dd, J 1.2, 2.3 Hz, H-2′)), 4.78(3H, s, OAc-2′), 4.02 (1H, dd, J 2.4, 9.1 Hz, H-3′), 4.16(3H, s, OAc-3′), 3.68 (1H, t, J 9.1 Hz, H-4′), 3.11 (3H, s, OAc-4′), 3.87(1H, m, H-5′), 1.23 (3H, d, J 2.5 Hz, H-6′, CH_3), 6.11 (1H, d, J 7.5 Hz, H-1″), 3.80 (1H, dd, J 7.2, 8.4 Hz, H-2″), 4.16 (3H, s, OAc-2″), 3.53 (1H, dd, J 8.1, 4.2 Hz, H-3″), 4.18 (1H, dd, J 4.1, 2.7 Hz, H-4″), 1.90 (3H, s, OAc-4″), 4.01 (1H, d, J 6.3 Hz, H-5″), 4.22 (2H, dd, J 12.2, 4.6 Hz, H-6″), 2.05 (3H, s, OAc-6″), 5.34 (1H, d, J 8.1 Hz, H-1‴), 3.89 (1H, dd, J 7.8, 8.4 Hz, H-2‴), 2.15 (3H, s, OAc-2‴), 4.03 (1H, d, J 7.9 Hz, H-3‴), 2.26 (3H, s, OAc-3‴), 4.05 (1H, s, H-4‴), 2.31 (3H, s, OAc-4‴), 3.78 (2H, d, J 10.3 Hz, H-5‴).

Table I. ^{13}C-NMR (90 MHz, DMSO-d_6) spectrum of Compound A

Atom	δ Value	Atom	δ Value	Atom	δ Value
C-1	39.1	C-17	57.2	C-3′	77.8
C-2	26.4	C-18	47.4	C-4′	71.1
C-3	88.9	C-19	49.8	C-5′	66.8
C-4	39.5	C-20	150.9	C-6′, CH_3	17.3
C-5	55.8	C-21	30.5	C-1″	104.2
C-6	18.6	C-22	36.9	C-2″	73.8
C-7	34.6	C-23	27.8	C-3″	75.0
C-8	41.8	C-24	16.4	C-4″	69.6
C-9	48.9	C-25	16.3	C-5″	75.6
C-10	37.2	C-26	14.9	C-6″	61.2
C-11	23.5	C-27	14.8	C-1″	101.9
C-12	124.1	C-28	175.0	C-2″	71.7
C-13	141.2	C-29	112.0	C-3″	79.8
C-14	42.8	C-30	19.1	C-4″	73.1
C-15	30.2	C-1′	106.2	C-5″	69.9
C-16	32.2	C-2′	83.3		

Acid hydrolysis of Compound A. Compound **A** (650 mg) was dissolved in ethanol (20 mL) and refluxed with 10% H_2SO_4 on water bath for 10 h. The contents were concentrated and allowed to cool and the residue was treated with Et_2O. The ethereal layer was washed with water and evaporated to dryness. The residue was subjected to column chromatography over a silica-gel column using hexane-EtOAc (1:3) to give compound **C**, identified as 3β-hydroxy-lup-12-ene 28-oic acid by comparison of (m.m.p, Co-tlc) and its spectral data (IR, ^{1}H-NMR, MS, ^{13}C-NMR) with authentic sample. The aqueous hydrolysate was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination on whatman filter paper No.1 (25 × 5) using n-Butanol:Acetic Acid:Water (BAW, 4:1:5) as solvent and aniline hydrogen phthalate as spraying reagent, showed the presence of D-xylose (R_f 0.29), D-galactose (R_f 0.17), and L-rhamnose (R_f 0.35) (Co-PC).

Study of Compound C. It had m.p. 290-291°C, $C_{30}H_{48}O_3$, $[M]^+$456(FABMS); found C; 78.86; H; 10.47 calcd for $C_{30}H_{48}O_3$; C; 78.94; H; 10.52%; IR

ν_{max}^{KBr} cm^{-1} 3440, 1689,1642, 1382, 1372, 1032, 1026, 996. ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.65(6H, s, H-23, H-24), 0.75(3H, s, H-25), 0.91(3H, s, H-26), 1.03(3H, s, H-27), 1.13(3H, d, J 6.4 Hz, H-29), 1.21(3H, d, J 6.6 Hz, H-30), 4.64(1H, s, 3-OH), 5.11(1H, t, J 2.6 Hz, H-12), 4.46(1H, t, J 7.1 Hz, H-3), 1.28-1.82 (23 H, m, polymethylene CH and CH_2); MS (FABMS) $[M]^+$456; m/z 438, 413, 412, 411, 395, 248 (base peak), 247, 220, 219, 208, 207, 205, 203, 191, 190, 189, 160, 145. ^{13}C-NMR (90 MHz, DMSO-d_6), δ 39.0 (C-1), 26.1 (C-2), 88.6 (C-3), 39.4 (C-4), 55.6 (C-5), 18.2 (C-6), 34.4(C-7), 41.2 (C-8), 47.9 (C-9), 36.8 (C-10), 23.3 (C-11), 124.0 (C-12), 138.2 (C-13), 42.5 (C-14), 30.1 (C-15), 32.1 (C-16), 56.9(C-17), 47.1 (C-18), 49.6 (C-19), 150.7(C-20), 30.7 (C-21), 36.8 (C-22), 28.0 (C-23), 16.2 (C-24), 16.1(C-25), 14.7 (C-26), 14.6 (C-27), 174.9 (C-28), 110.0(C-29), 19.3(C-30).

Alkaline hydrolysis of Compound A. Compound **A** (100 mg) was dissolved in 5 mL of 2% KOH and heated at 50°C for one hour. The contents were cooled, neutralized with 5N-HCl, and extracted with n-BuOH. The extract was washed with water and concentrated under reduced pressure and recrystallized from MeOH to give compound **E**.

The water soluble portion was treated with 1% H_2SO_4 for 1 h. The reaction mixture was neutralized with $BaCO_3$ and $BaSO_4$ filtered off. The filtrate was concentrated under reduced pressure and subjected to PC examination showed the presence of L-rhamnose (R_f 0.35).

Study of Compound E. It had m.p. 296–297°C, $C_{41}H_{66}O_{12}$, $[M]^+$750 (FABMS); found C; 65.1 H; 8.79 calcd for m.f. $C_{41}H_{66}O_{12}$ C; 65.16, H; 8.82%; IR ν_{max}^{KBr} cm^{-1} 3442, 1685, 1638, 1385, 1372, 1020, 990; ^{1}H-NMR (300 MHz, $CDCl_3$), δ 0.67 (6H, s, H-23, H-24), 0.76 (3H, s, H-25), 0.95 (3H, s, H-26), 1.03 (3H, s, H-27), 1.15 (3H, d, J 6.7 Hz, H-29), 1.22 (3H, d, J 6.8 Hz, H-30), 5.12 (1H, t, J 2.4 Hz, H-12), 4.45 (1H, t, J 7.2 Hz, H-3), 1.27-1.80 (23 H, m, polymethylene CH_2 and CH), 6.11 (1H, d, J 7.9 Hz, H-1′), 3.82 (1H, dd, J 7.4, 8.6 Hz, H-2′), 4.14 (1H, s, OH-2′), 3.56 (1H, dd, J 8.3, 4.4 Hz, H-3′), 4.3 (1H, dd, J 4.3, 2.9 Hz, H-4′),1.88 (1H, s, OH-4′), 4.01 (1H, d, J 6.5 Hz, H-5′), 4.24 (2H, dd, J 12.4, 4.8 Hz, H-6′), 2.02 (1H, s, OH-6′) 5.32 (1H, d, J 7.8 Hz, H-1″), 3.91 (1H, dd, J 7.8, 8.6 Hz, H-2″), 2.13 (1H, s, OH-2″), 4.05 (1H, d, J 8.2 Hz, H-3″), 2.26 (1H, s, OH-3″), 4.07 (1H, s, H-4″), 2.29 (1H, s, OH-4″), 3.74 (1H, d, J 10.4 Hz, H-5″). ^{13}C-NMR (90 MHz, DMSO-d_6), δ 39.2 (C-1), 26.5 (C-2), 88.7 (C-3), 39.4 (C-4), 55.6 (C-5), 18.7 (C-6), 34.6(C-7), 41.9 (C-8), 48.8 (C-9), 37.4 (C-10), 23.7 (C-11), 124.2 (C-12), 139.2 (C-13), 42.9 (C-14), 30.1 (C-15), 32.4 (C-16), 57.3 (C-17), 47.6 (C-18), 49.7 (C-19), 151.0 (C-20), 30.4 (C-21), 36.8 (C-22), 27.9 (C-23), 16.3 (C-24), 16.5 (C-25), 14.8 (C-26), 14.7 (C-27), 174.8 (C-28), 112.2(C-29), 18.9(C-30), 104.4 (C-1′), 73.7 (C-2′), 75.2 (C-3′), 69.8 (C-4′), 75.7 (C-5′), 61.3 (C-6′), 101.7 (C-1″), 71.5 (C-2″), 79.6 (C-3″), 73.4 (C-4″), 69.7 (C-5″).. MS (FABMS) $[M]^+$750 m/z, 618, 486, 456, 439,438, 413, 412, 395, 248, 247, 220, 219, 208, 207, 205, 203, 191, 190, 189, 160, 145. It was identified as 3β-O-[β-D-xylopyranosyl-(1 → 3)-O-β-D-galactopyranosyl-lup-12-ene-28-oic acid.

Partial hydrolysis of Compound E. Compound **E** (250 mg) was treated with Kiliani mixture [50 mL, HCl:AcOH:H_2O (15:35:50)] and stirred at room temperature for 6 h. The reaction mixture was then treated with n-BuOH to give compound **F** (50 mg) and D-xylose (R_f 0.29). Compound **F** on further hydrolysis gave compound **C** identified as 3β-hydroxy-lup-12-ene-28-oic acid and D-galactose (R_f 0.17).

Study of Compound F. It had m.p. 300–302°C, $C_{36}H_{57}O_8$, $[M]^+$618 (FABMS) found C; 69.92 H; 9.18 calcd for $C_{36}H_{57}O_8$ C;69.96, H; 9.223%; IR ν_{max}^{KBr} cm^{-1} 3445, 1685, 1380, 1372, 1036, 1023, 990. ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.68 (6H, s, H-23, H-24), 0.75 (3H, s, H-25), 0.94 (3H, s, H-26), 1.02 (3H, s, H-27), 1.14 (3H, d, J 6.6 Hz, H-29), 1.21(3H, d, J 6.7 Hz, H-30), 5.11(1H, t, J 2.3 Hz, H-12), 4.46(1H, t, J 7.1 Hz, H-3), 1.26-1.81 (23 H, m, polymethylene CH and CH_2), 6.13 (1H, d, J 8.2 Hz, H-1′), 3.83 (1H, dd, J 7.2, 8.5 Hz, H-2′), 4.73 (1H, s, OH-2′), 3.57 (1H, dd, J 8.2, 4.4 Hz, H-3′), 2.24 (1H, s, OH-3′), 4.1 (1H, dd, J 4.4, 2.8 Hz, H-4′), 4.13 (1H, s, OH-4′), 4.02 (1H, d, J 6.4 Hz, H-5′), 4.23 (2H, dd, J 12.4, 4.7 Hz, H-6′), 1.89 (1H, s, OH-6′) MS (FABMS) $[M]^+$618; m/z, 486, 456, 439,438, 413, 412, 395, 248, 247, 220, 219, 208, 207, 205, 203, 191, 190, 189, 160, 145. It was identified as 3β-O-β-D-galactopyranosyl-lup-12-ene-28-oic acid.

Permethylation followed by hydrolysis of Compound A, E, and F: Compound **A**, **E**, **F** and (15 mg each) separately in MeI (3 mL) / Ag_2O (30 mg) / DMF (5 mL) were treated at room temperature for one day. The total reaction mixture was diluted with water and extracted with $CHCl_3$ (20 mL) to give compound **H**, **J**, **K** respectively.

Compound **H**, **J**, **K** were hydrolyzed with Kiliani mixture separately, and filtered. The filtrates of each were neutralised with $BaCO_3$ and $BaSO_4$ was filtered off. The filtrate was concentrated and examined by PC using n-BuOH:ACOH:H_2O (4:1:5) as eluents. The methylated sugars were identified as 2,3,4 tri-*O*-methyl-L-rhamnose, 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4 tri-*O*-methyl D-xylose for **H**, 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4 tri-*O*-methyl D-xylose for **J** and 2,4,6-tri-*O*-methyl-D-galactose for **K**.

Periodate oxidation of Compound A. Compound **A** (15 mg) was dissolved in MeOH and treated with (8 mL) of 1N sodium meta periodate. The contents were left for 36 h at room temperature. The liberated formic acid and consumed periodate were estimated by Jone's method.

Enzymatic hydrolysis of Compound A. Compound **A** (20 mg) was dissolved in MeOH (15 mL) and hydrolysed with an equal volume of almond emulsin enzyme in a flask fitted with air condenser. The total reaction mixture was allowed to stand at room temperature for two days and filtered and studied separately. The hydrolysate was concentrated and subjected to paper chromatography examination using B:A:W (4:1:5) as solvent system and aniline hydrogen phthalate as spraying agent gave D-xylose (R_f 0.29), followed by D-galactose (R_f 0.17) and 3β hydroxy lup-12-ene-28-oic acid 28-O-α-L-rhamnopyranosyl ester as proaglycone, suggesting the presence of β-linkage between D-xylose and D-galactose as well as between D-galactose and proaglycone. Proaglycone on further hydrolysis with enzyme takadiastase yielded L-rhamnose (R_f 0.35), and aglycone (sapogenin) which confirmed the presence of α-linkage between L-rhamnose and aglycone.

Anti-inflammatory activity of Compound A

Anti-inflammatory activity of compound A was carried out by non-immunological carrageenin-induced hind paw Oedema method in accord with accepted Institute Protocol for Animal work. Adult albino rats (145–160 gm), either male or female, were taken for the present study. The volume was measured by Plethysmograph. Initial volume of right hand hind paw of albino rats were measured by Plethysmograph without administration of test drug. Acetylsalicylic acid was used as a standard drug. Activity was carried out by measuring the change in the volume of inflammed foot produced by injection of 0.05 ml of 1% freshly prepared carrageenin suspension. Albino rats were divided into three groups, each group consisting of four rats. First group of rats were treated intraperitonially(i.p.) with 50 mg/Kg body weight of the methanol-soluble fraction of the compound. Second group was administered i.p. with 35 mg/Kg body weight of the aqueous suspension of acetylsalicylic acid and third control group was administered with the same volume of distilled water. After one hour of drug administration, the rats were injected 0.02 mL suspension of carrageenin in the right paw. The measurement of the paw volume was carried out by mercury displacement technique with the help of plethysmograph immediately before and after the carrageenin injection after 1,2 and 3 h (see Table II).

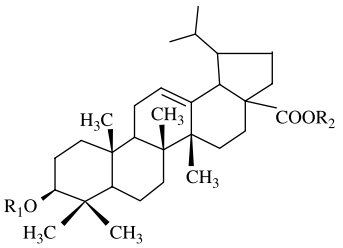
The percentage inhibition (I) of inflammation after 4 h was calculated by the following formula

$$I = 100[1 - (a - x)/(b - y)]$$

Where x = mean foot volume of rats before the administration of carrageenin injection in the test and the standard drug
a = mean foot volume of rats after the administration of carrageenin and the test drug injection in the test and the standard drug.
y = mean foot volume of rats before the administration of carrageenin injection in the control group.
b = mean foot volume of rats after the administration of carrageenin injection in the control group.

Results and discussion

The methanolic extract of the roots of the plant afforded a new compound **A**,. m.p. 297–298°C, with molecular formula $C_{47}H_{76}O_{16}$, $[M]^+$ m/z 896 (FABMS) (see formulae).



Compound	Rhamnopyranosyl	R_2
A	*xyl-(1→3)- *gal	*rham
C	H	H
D	xyl-(1→3)-gal hexa acetate	rham tri acetate
E	xyl-(1→3)-gal	H
F	gal	H
H	xyl-(1→3)-gal hexa methylate	rham tri methylate
J	xyl-(1→3)-gal hexa methylate	H
K	gal tri methylate	H

* xyl = β-D-xylopyranosyl, gal = β-D-galactopyranosyl, rham = α-L-rhamnopyranosyl

It gave positive test for triterpenoid saponin [5–8]. It also gave a yellow colour with the Liebermann-Burchard test [9] confirming it to be triterpenoid saponin. Its IR spectrum showed peaks at ν_{max}^{KBr} cm^{-1} 3445, 1685, 1640, 1386, 1376, 1030, 1026, 990. In the IR spectrum, a peak at 3445 cm^{-1}, was assigned for hydroxyl group, 1685 cm^{-1} for carboxyl group and 1640 cm^{-1} for unsaturation, 1386 cm^{-1} and

Table II. Anti-inflammatory activity of Compound A

S. No.	Test solutions applied	Dose mg/Kg (i.p.)	Volume of paw after drug administration 0 h	1 h	2 h	3 h	Total increase in paw volume	% inhibition
1.	Control group	...	0.45 ±	0.58 ±	0.71 ±	0.82 ±	0.37 ±	
2.	Treated group	60	0.45 ±	0.50 ±	0.62 ±	0.70 ±	0.25 ±	32.5%
3.	Acetylsalicylic acid	40	0.45 ±	0.62 ±	0.68 ±	0.72 ±	0.27 ±	72.6%

1376 cm^{-1} for gem-dimethyl group in the compound, The stretching bands at 1030 cm^{-1} and 1026 cm^{-1} showed that the hydroxyl group is equatorial and is present at the C-3 position [10]. In the UV spectrum, Compound **A** did not display any absorption above 200 nm. In ^{13}C-NMR spectrum, compound **A** exhibited 47 signals, 30 of which could be assigned to the aglycone and 17 signals to the sugar moieties, revealing the nature of compound A as bidesmosidic. The disaccharide sugar chain was linked to C-3 and one monosaccharide unit was attached to C-28. In the ^{13}C-NMR spectrum (see Table I), two signals at δ 88.9 and δ 141.2 assigned to C-12 and C-13. confirmed Δ^{12} oleanane skeleton. A signal at δ 175.0 suggested the presence of a –COOH group at C-28.

Fragment ion peaks found at m/z 456, $[M]^+$413, 411, 248,(base peak), 247, 220, 219, 208, 207, 205, 203, 191, 190, 189, 160, and 145 from retro-Diels-Alder fragmentation [11,12] of compound **C** which showed a characteristic feature of Δ^{12} unsaturated triterpenoids [13]. Formation of fragment ion m/z at 248 showed the presence of a hydroxyl group at C-3 position.

Fragment ion peak at m/z 203 shows loss of a carboxyl group, suggested the possibility of carboxyl group can be either at C-27 or C-28 position. Out of the two possibilities, the assignment of a carboxyl group at C-27 was ruled out. Hence the position of carboxyl group was assigned at C-28 position [14].

On acetylation of compound **A**, gave nona acetate compound **D**, showed the presence of nine hydroxy groups in the compound **A**. In the ^{1}H-NMR spectrum, a peak at δ 0.63 (6H, s, H-23, H-24), δ 0.82 (3H, s, H-25), δ 0.98 (3H, s, H-26), δ 1.05 (3H, s, H-27) were assigned to five tertiary methyl groups [15]. Two doublets appearing at δ 1.19 (3H, d, J 6.9 Hz, H-29) and at δ 1.25 (3H, d, J 6.1 Hz, H-30) showed the presence of two secondary methyl groups. A proton singlet at δ 4.65 was assigned to a hydroxyl proton. A triplet at δ 5.16 (1H, t, J 3.0 Hz, H-12) was assigned for olefinic proton. The appearance of another proton centered at δ 4.52 (1H, t, J 7.7 Hz) showed the presence of hydroxy-methine in the molecule [16]. Three signals at δ 5.31 (d, J 6.8 Hz), δ 6.10 (d, J 7.6 Hz), δ 5.34 (d, J 8.0 Hz), were assigned to anomeric protons of H-1′, H-1″, H-1‴ of L-rhamnose, D-galactose, D-xylose respectively. In the ^{1}H-NMR spectra, the coupling constants of the anomeric protons showed β-configuration for D-xylose and D-galactose and α-configuration for L-rhamnose.

Acid hydrolysis of compound **A** with 10% ethanolic H_2SO_4 gave compound **C** m.p. 286–287°C, $C_{30}H_{48}O_3$, $[M]^+$; 456 (FABMS). It responded to all the colour reactions of triterpenoids [17–20]. It was identified as 3β-hydroxy lup-12-ene-28-oic acid by comparison of its m.m.p., co-tlc, and spectral data i.e. ^{1}H-NMR, mass., ^{13}C-NMR with reported literature values [21].

The aqueous hydrolysate obtained after acid hydrolysis of the compound **A** was neutralized with $BaCO_3$ and $BaSO_4$ was filtered off. The filtrate was concentrated under reduced pressure and subjected to paper chromatography examination using nBAW (4:1:5) as solvent and aniline hydrogen phthalate as detecting agent yielded L-rhamnose (R_f 0.35), D-xylose (R_f 0.29) D-galactose(R_f 0.17) [Co-PC] [22].

Alkaline hydrolysis [23,24] of compound A with 2% KOH gave compound **E**, m.f. $C_{41}H_{66}O_{12}$, m.p. 296–297°C, $[M^+]$; 750 (FABMS) and L-rhamnose, confirming that C-1′ of L-rhamnose was attached to the C-28 carboxyl group of the compound **E**. In mass spectrum of compound **E**, characteristic ions at m/z, 618 $[M\text{-}H\text{-}132]^+$ and 456 $[M\text{-}H\text{-}132\text{-}162]^+$ were found by subsequent losses of one molecule of D-xylose followed by one molecule of D-galactose from the molecular ion, revealing D-xylose as terminal sugar and D-galactose was directly attached to –OH group at C-3 position in compound **E**, therefore it was identified as 3β-O-[β-D-xylopyranosyl-(1 → 3-O)-β-D-galactopyranosyl]-lup-12-ene-28-oic acid.

The sequence of the sugar residue in compound **E** was confirmed by its partial hydrolysis [25] with Kiliani mixture [HCl:AcOH:H_2O (15:35:50)] [26] which gave compound **F** and D-xylose. compound **F** on further hydrolysis gave compound C and D-galactose indicating D-xylose was attached with D-galactose and D-galactose was directly linked to sapogenin.

Permethylation [27] of compound **A**, followed by hydrolysis yielded permethylated sapogenin which was identified as 3β-hydroxy, lup-12-ene-28-oic acid.and and permethylated sugars were identified as, 2,3,4, tri-O-methyl-D-xylose, 2,4,6 tri-O-methyl-D-galactose and 2,3,4, tri-O-methyl-L-rhamnose [by Co-PC] suggesting that C-1‴ of D-xylose was attached with C-3″ of D-galactose, C-1″ of D-galactose was attached to C-3- OH of the sapogenin and C-1′ of L–rhamnose was attached to the carboxylic group of sapogenin at C-28. Thus the interlinkage (1 → 3) were found between D-xylose

and D-galactose. These linkages were further confirmed by its ^{13}C-NMR spectral data (see in Experimental Section). Periodate oxidation [28] of compound A confirmed that the three sugars were present in pyranose form.

Enzymatic hydrolysis [29] of compound A with enzyme almond emulsin liberated D-xylose (R_f 0.29), followed by D-galactose(R_f 0.17) and 3β hydroxyl lup-12-ene-28-oic acid-28-O-α-L-rhamnopyranosyl ester as proaglycone suggesting the presence of suggesting the presence of β-linkage between D-xylose and D-galactose as well as between D-galactose and proaglycone Proaglycone was hydrolysed with enzyme takadiastase liberated L-rhamnose (R_f 0.35) and sapogenin (aglycone) revealing the presence of α-linkage between L-rhamnose and sapogenin.

Quantitative estimation [30] of the sugars present in the hydrolysate revealed that the three sugars were present in equimolecular proportions indicating that the saponin consists of one molecule of aglycone (sapogenin), L-rhamnose, D-galactose and D-xylose.

On the basis of above evidences, the structures of compound A was established as 3β-O-[β-D-xylopyranosyl(1 → 3)-O-β-D-galactopyranosyl(1 → 4)-α-L-rhamnopyranosyl]-lup-12-ene-28-oic acid-28-O-β-D-glucopyranosyl ester.

The anti-inflammatory activity of compound A was carried out by non-immunological carrageenin Oedema method [31]. The results recorded in Table II showed that compound **A** may be potentially useful as an anti-inflammatory drug.

Acknowledgements

The authors are thankful to Director, CDRI Lucknow for recording various spectral data, elemental analysis and anti-inflammatory activity and Head Department of Chemistry, Dr. H.S. Gour University, Sagar (M.P.), for providing laboratory facilities.

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