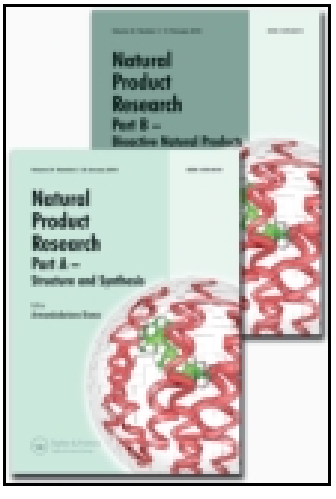

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Two antifungal active triterpenoid saponins from the seeds of Lathyrus plants

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Two antifungal active triterpenoid saponins from the seeds of *Lathyrus* plants

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Two novel triterpenoid glycosides have been isolated from butanolic seeds extract of two varieties of *Lathyrus* plants, i.e. *Lathyrus ratan* and *Lathyrus aphaca*. Their structures were elucidated as 3-O-[β-D-glucuronopyranosyl-(1 → 4)-α-L-arabinopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-olean–11,13 (18)–dien–28-oic acid (**1**) and 3-O-{β–D–xylopyranosyl–(1 → 2)–β-D-glcopyranosyl–(1 → 4)–[β-D-glucopyranosyl–(1 → 2)]–β-D-xylopyranosyl}–2, 16α-dihydroxy–4-hydroxymethyl urs-12-en-28-oic acid (**2**) on the basis of spectral evidences, i.e. FTIR, ^{1}H-NMR, ^{13}C-NMR, ESI-MS and FAB-MS data. The isolated saponins were tested for their antifungal activity. Compound **1** showed maximum inhibition against *Colletotrichum dematium* (77.8%), whereas compound **2** showed maximum inhibition against *Alternaria alternata* (53.9%).

Keywords: *Lathyrus ratan*; *Lathyrus aphaca*; Triterpenoid saponin; Caesalpiniaceae; antifungal activity

1. Introduction

Lathyrus is a large genera with 190 species and subspecies being recognised. The plants are commonly found in Central India (Allkins, Macfarfarlane, & White, 1983). *Lathyrus ratan* is a hybrid variety of grass pea developed by scientists at Plant Breeding Department, JNKVV, Jabalpur. Despite its tolerance to drought, grasspea is not affected by excessive rainfall; it can be grown on land subject to flooding and therefore is a popular crop in subsistence farming (Sinha, 1980). Its seeds are consumed as pulses as it contains a high amount of free L-homoarginine, which may act as a precursor of lysine in human nutrition (Qureshi, Pilbeam, Evans, & Bell, 1977). Oil obtained from the seeds of *L. ratan* is cathartic (Ambasta, Ramchandran, & Kashgapa, 1986), and it contains sterols, cholesterol, 24-epicholesterol and isoavenasterol and triterpene alcohols (Rotter, Marquarelt, Low, & Briggs, 1990).

Lathyrus aphaca (Jangli matar) is a common weed of wheat fields and is commonly used as fodder. The ripen seeds produce narcotic effect and are considered to be the possible cause of Lathyrism (Anonymous, 2000). The flower contains

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flavonol glycosides, mainly larycitrin and syringetin-3-O-rutinoside-7-O-β-D-glucopyraoside accompanied by a low level of kempherol, quercetine, isorhamnetin analogous and the respective 3-O-rutinosides (Markham & Hammett, 1994). The proximate principles of *L. aphaca* and certain other leguminous seeds have already been determined by the authors (Dubey, Khan, & Srivastava, 2008; Dubey, N.K. Saxena, N. Saxena, & Srivastava, 2005).

In this study, two new triterpenoid glycosides were isolated from butanolic seeds extract of the two plants. Biological screening of both the compounds showed significant antifungal activity.

2. Results and discussion

The *n*-BuOH soluble fractions of methanolic seeds extract of *Lathyrus* plants were separated by column chromatography on silica gel. *Lathyrus ratan* extract afforded compound **1** whereas compound **2** was obtained from *L. aphaca* seeds extract. Both the compounds gave positive result for Libermann–Burchard test for triterpenoid saponins. The nature of compounds was further confirmed by spectroscopic data.

Compound **1,** isolated from *L. ratan* seeds, yielded aglycone along with three sugar moieties on acid hydrolysis. The aglycone sapogenin was identified as oleanolic acid by Co-TLC analysis using authentic sample and comparing its NMR data (^{1}H and ^{13}C) with literature (Taketa, Schlager, Guillaume, Gosmann, & Schenkel, 2000). The sugar components in the hydrolysate were identified to be D-glucuronic acid and L-arabinose in 1:2 ratio (Sun, Ligne, & Huystee, 1997). The ESI–MS of compound **1** showed molecular ion peak $[M+2H]^+$ at m/z 896, suggesting the molecular formula to be $C_{46}H_{70}O_{17}$. The fragments at m/z 718, 586 and 454 attributed to the loss of terminal glucuronic acid unit followed by the loss of two arabinose. The result of ESI–MS confirms the sequence of sugar units in **1**. The presence of glucuronic acid as terminal sugar was confirmed by partial hydrolysis of compound **1** on TLC in HCl atmosphere followed by Co-TLC with an authentic sample and HPLC chromatogram (Miyase, Shiokawa, Zhang, & Ueno, 1996).

In ^{1}H-NMR spectrum of compound **1**, the doublets at δ_H 4.42 (1H d, $J = 6.5$ Hz), 5.73 (1H d, $J = 5.3$ Hz) and 5.71 (1H d, $J = 3.51$ Hz) were assigned to three anomeric protons of the sugars (Zhao et al., 1999). The proton noise decoupled ^{13}C-NMR spectrum of **1** display 46 carbon resonance peaks. The number of hydrogen attached to each carbon atoms were determined by DEPT technique (Doddrell, Pegg, & Bendel, 1982). The presence of three anomeric carbon signals at δ_c 101.34, 101.54 and 105.32 ppm in ^{13}C-NMR spectrum further confirms the presence of trisaccharide moiety in **1** (Nikaido, Koike, Mitsunaga, & Saeki, 1999). The configuration of glucuronic acid was determined to be β and that of arabinose to be α on the basis of chemical shift and coupling constant of these signals when were compared with the reported value (Rios, Berenice, & Guadarrama, 2004).

The inter-glycosidation assignment was further confirmed by the chemical shift of glycosylated carbon atom. The C-2 of Ara-I was observed at δc 84.76 whereas C-4 signal of Ara-II at δc 82.36 reveals the deshielding of carbon by 4 and 6 ppm for these carbon resonance; hence C-2 in Ara-I and C-4 in Ara-II were concluded to be the glycosidation site. The trisaccharide moiety in **1** was linked at C-3 of aglycone as C-3 showed significant downfield shift (δc 89.20) in ^{13}C-NMR spectra

indicating the glycosidation position (Abdel-khader et al., 2000), which was further confirmed by acid and enzymatic hydrolysis. On the basis of above-mentioned evidences, the structure of **1** was determined as 3-O-[β-D-glucuronopyranosyl-(1 → 4)-α-L-arabinopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-olean–11,13(18)–dien–28-oic acid (Figure 1).

Compound **2**, obtained from the butanolic extract of *L. aphaca* seeds, on acid hydrolysis yields aglycone sapogenins that was identified to be ursolic acid by Co-TLC with authentic sample and comparing its NMR data with the literature (Grishkovets, Sobolev, Shashkov, & Chirva, 2000; Weiss & Seebacher, 2002).

The aqueous hydrolysate containing sugar moieties were identified to be D-glucose and D-xylose in 1:1 ratio, by comparing HPLC chromatogram of sample with standard. The molecular formula of compound **2** was established to be $C_{52}H_{84}O_{24}$ by positive FAB-MS, which exhibited pseudo molecular ion peak $[M+3H]^+$ at m/z 1095 indicating the molecular mass to be 1092. The sequence of sugar moiety in compound **2** was confirmed by fragment ion peaks at m/z 932, 796, 634 and 506. The partial hydrolysis followed by Co-TLC with authentic sample and HPLC chromatogram showed xylose to be the terminal sugar.

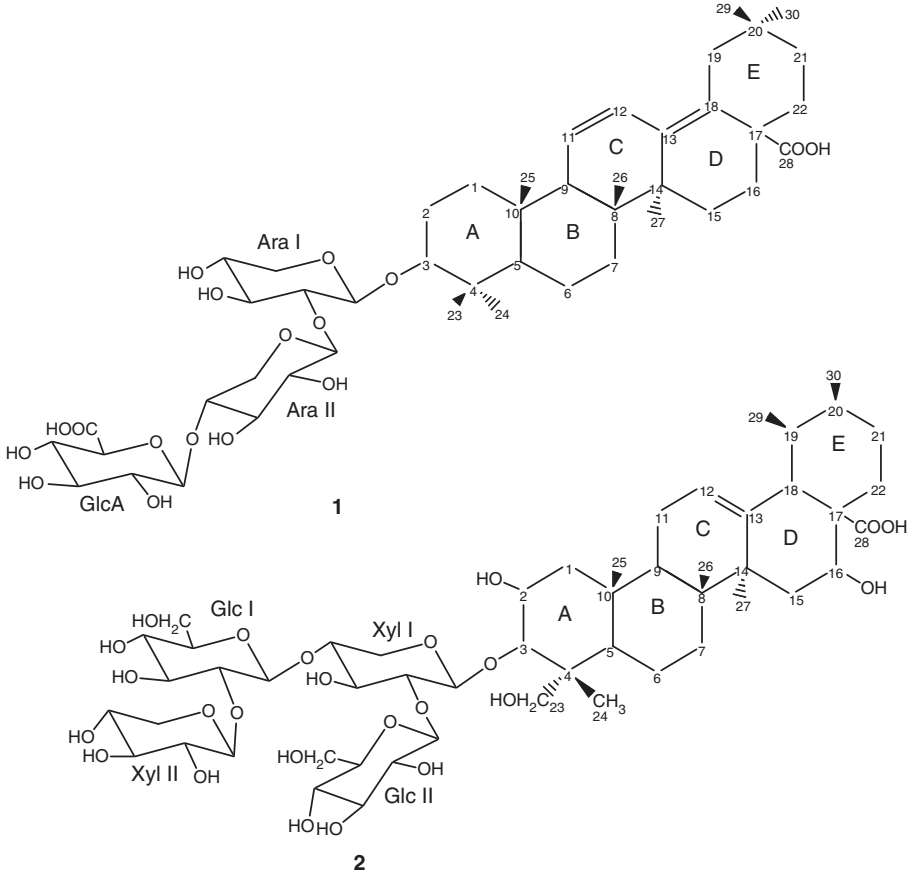

Figure 1. Structure of triterpenoid saponins **1** and **2**.

The ^{1}H-NMR spectrum of **2** showed doublets at δ_H 6.09 (1H d, $J = 7.6$ Hz), 6.10 (1H d, $J = 4.32$ Hz), 5.79 (1H d, $J = 3.42$ Hz) and 5.71 (1H d, $J = 5.312$ Hz), which were assigned to four anomeric protons of the sugars (Tamai et al., 1989). The proton noise decoupled ^{13}C-NMR spectrum of **2** displayed 52 carbon resonance peaks. The presence of five quaternary carbon atoms, 26×CH, 12×CH_2, 6×CH_3 and three sp^2 (CH=, C= and C=O) hybrid carbon atoms were determined by DEPT technique. The four anomeric carbon signals at δc 104.78, 105.14, 106.31 and 106.10 ppm further confirmed the presence of tetrasaccharide moiety in **2**. The chemical shift and coupling constant of these signals suggested the β anomeric configuration for all sugar moieties (Kovacik et al., 1995).

The chemical shift values of glycosylated carbon atoms in sugar moiety reveals the peak at δc 81.21, 79.14 and 82.46 ppm. The downfield shift of carbon signal suggested that C-2 and C-4 in Xyl-I, and C-2 in Glu-I were concluded to be the glycosidation site. The tetrasaccharide moiety in **2** was linked at C-3 of aglycone as C-3 showed significant downfield shift (δc 84.12) in ^{13}C-NMR spectra indicating the glycosidation position. The structure of **2** was elucidated as 3-O-{β-D-xylopyranosyl–(1 → 2)–β-D-glucopyranosyl–(1 → 4) –[β-D-glucopyranosyl–(1 → 2)]–β-D-xylopyranosyl}–2,16α-dihydroxy–4-hydroxymethyl urs-12-en-28-oic acid (**2**) (Figure 1).

The isolated compounds were tested for their antifungal activity against human and plant pathogenic fungi. The compounds showed maximum inhibition at 1000 μg mL^{-1}. At this concentration, compound **1** shows maximum inhibition against *Colletotrichum dematium* (77.8%) and minimum against *Curvularia lunata* (27.8%). Similarly, compound **2** showed maximum inhibition of 53.9% against *Atlernaria alternata* and minimum against *Aspergillus flavus* (29.5%) (Table 1).

Table 1. *In vitro* fungicidal bioassay (% inhibition) of compounds **1** and **2**.

Name of fungus	Concentration (μg mL^{-1})				Positive control (*S*)
	100	250	500	1000	
Aspergillus flavus (FGCC-133)					
1	6.8	11.3	21.3	33.5	98
2	–	–	16.4	29.5	98
Alternaria alternata (FGCC-418)					
1	22.5	36.5	47.5	59.4	100
2	25.2	35.6	44.8	53.9	100
Collatotrichum dematium (FGCC-165)					
1	22.5	36.1	37.7	77.8	100
2	17.6	35.5	42.1	50.5	100
Fusarium roseum (FGCC-500)					
1	14.5	24.5	29.4	36.7	100
2	–	10.1	21.5	29.6	100
Curvularia lunata (FGCC-280)					
1	4.6	10.5	12.7	27.8	100
2	4.9	15.1	19.8	35.9	100

Notes: The results are mean of triplicates reading. The mean were compared by the analysis of variance (ANOVA) with 5% significance level. S = Fluconazole (20 μg mL^{-1}), Negative control (10% MeOH) = Shows no inhibition.

3. Experimental

3.1. *General methods*

Melting points were measured on an MAC model melting point apparatus and were uncorrected. Optical rotations were measured on Rudolf Autopol III polarimeter. HPLC of sugars was done on Shimadzu 10 A Liquid Chromatography system using EL-SD Detector. UV spectra were measured on Unicam Thremospectronic UV-500 model Double Beam spectrophotometer in MeOH solution. ^{1}H-NMR and ^{13}C-NMR were recorded on Bruker DRX 300 model operating at 300 and 75 MHz (CD_3OD or $CDCl_3$). All the NMR spectra were recorded using TMS as internal standard. IR spectra were recorded on a Shimadzu 8400 S spectrophotometer having a range of 4000–450 cm^{-1} and sample was prepared in powered KBr in a ratio 1:3. The ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. FAB-MS was recorded on a Jeol SX 102/DA-6000 spectrometer using argon as FAB gas and accelerating voltage of 10 KV with nitro benzyl alcohol as matrix. Column chromatography was carried out on silica gel (B.D.H.; 60–120 mesh), TLC and preparative TLC on 20×20 cm plates coated with 2-mm thick silica gel (Merk; F_{254}). Spots were visualised by 10% ethanolic H_2SO_4, followed by heating at 110°C. Paper chromatography of sugars was performed on Whatman no.1 paper using descending mode in *n*-BuOH:AcOH:H_2O (BAW 4:1:5) and developed with aniline hydrogen phthalate.

3.2. *Plant material*

The seeds of plant *L. ratan* (Commercial variety) and *L. aphaca* (Wild variety) were collected from Plant Breeding Department, and wheat fields of Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur, in April 2005. The seeds of *L. aphaca* were further identified by Head, National Research Centre for Weed Sciences, Jabalpur, and a voucher specimen (NRCWS- LA121) was deposited in the herbarium of this institute.

3.3. *Extraction and isolation*

The air-dried and powdered seeds (1 Kg each) were extracted with petroleum ether (60–80°C) for 12–14 h.The defatted seeds powder was then extracted with MeOH for 18–20 h and combined extract was concentrated in *vacuum*. The resulting dark yellow residue (150 g) was suspended in water. The aqueous methanolic extract was then fractionated successively with *n*-Hexane, $CHCl_3$ and *n*-BuOH to get a total of four fractions. The bioactive *n*-BuOH fraction (20 g) was subjected to column chromatography on silica gel (100 g, 60–120 mesh) using $CHCl_3$–MeOH–H_2O (v:v = 70 : 25 : 5–50 : 45 : 5) with a 5 mL each as gradient eluent to give 48 fractions and was monitored by TLC. The fractions 25–36 showing same R_f on TLC were pooled together and recolumn chromatographed on silica gel with $CHCl_3$: MeOH (60 : 40–50 : 50), followed by preparative TLC in EtOAc: MeOH: H2O (13:8:2) to yield saponin **1** and **2**.

3.3.1. *Oleanolic acid (**1**)*

A dark yellow amorphous powder; m.p. 190–194°C; $[\alpha]_D^{25} = +20.8$ (MeOH; $c = 1.05$); UV (MeOH) λ_{max}: 286.4 and 341.6 nm; IR (KBr)ν max (cm^{-1}): 3687, 1714, 1589, 1499,1360; ^{1}H-NMR (CD_3OD, 300 MHz) δ_H: 0.974, 0.961,0.950, 0.931,0.912, 0.882, 0.873 (3H, s, 7×Me), 4.715 (1H, d, $J = 5.6$ Hz, H-3), 5.364 (1H, *br s*, H-11), 5.105 (1H, *br s*, H-12); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ_c: 38.87 (C-1), 26.21(C-2), 89.20 (C-3), 40.27 (C-4), 48.52 (C-5), 14.57 (C-6), 33.16 (C-7), 40.41 (C-8), 52.21 (C-9), 35.12 (C-10), 124.37 (C-11), 127.93 (C-12), 132.61(C-13), 42.23 (C-14), 28.89(C-15), 24.13(C-16), 47.12 (C-17), 138.01 (C-18), 46.30 (C-19), 30.84 (C-20), 34.96 (C-21), 32.77 (C-22), 23.72 (C-23), 17.50 (C-24), 19.70 (C-25), 18.25 (C-26), 25.07 (C-27), 182.90 (C-28), 23.83 (C-29), 16.25 (C-30); ESI-MS m/z 896 $[M + 2H]^+$, 895 $[M + H]^+$, 718, 586, 454, 246, 208,201 and 190.

3.3.2. *Ursolic acid (**2**)*

A light yellow amorphous powder; m.p. 208–211°C; $[\alpha]_D^{25} = -13.6$ (MeOH; $c = 0.95$); UV (MeOH) λ_{max}: 224 and 295 nm; IR (KBr)ν max (cm^{-1}): 3751, 1677, 1571, 1427,1360; ^{1}H-NMR (CD_3OD, 300 MHz) δ_H: 0.925, 0.892, 0.932, 0.914, 0.964, 0.944 (3 H, *s*, 6×Me), 3.86 (1H, *d*, $J = 9.81$ Hz, H-2), 4.37 (1H, *d*, $J = 7.21$ Hz, H-3), 5.21 (1H, *t*-like, H-12), 3.67 (1H, *br s*, $J = 7.56$ Hz, H-16), 3.52 (1H, d, $J = 9.03$ Hz, H-16); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ_c: 45.02 (C-1), 71.03 (C-2), 84.12 (C-3), 44.50 (C-4), 53.20 (C-5), 18.50 (C-6), 31.90 (C-7), 41.23 (C-8), 47.32 (C-9), 37.31 (C-10), 23.46 (C-11), 124.01 (C-12), 141.02 (C-13), 43.01 (C-14), 29.69 (C-15), 65.13 (C-16), 48.91 (C-17), 52.60 (C-18), 39.51 (C-19), 40.21 (C-20), 30.90 (C-21), 37.01 (C-22), 63.95 (C-23), 16.42 (C-24), 17.25 (C-25), 18.01 (C-26), 24.02 (C-27), 179.05 (C-28), 18.21 (C-29), 21.08 (C-30); FAB-MS m/z 1095 $[M + 3H]^+$ 932, 796, 634, 506, 490, 296 and 265.

3.3.3. *Acid hydrolysis*

Saponins **1** and **2** (25 mg each) were refluxed with 10% H_2SO_4 on a boiling water bath for 4 h, respectively. The hydrolysed contents were then neutralised with $BaCO_3$ solution. The neutralised portions were extracted with EtOAc. The EtOAc fraction when dried afforded the aglycone moiety, whereas the aqueous fraction contains hydrolysed sugars.

3.3.4. *Identification of sugar moieties of **1** and **2***

The neutralised aqueous layer separated after the removal of sapogenin was filtered and concentrated under reduced pressure. The residue obtained was compared with standard sugar on TLC and paper chromatography (BAW 4:1:5) indicating them to be D-glucuronic acid and L-arabinose in compound **1** and D-xylose and D-glucose in compound **2**.

3.4. *Premethylation of* 1 *and* 2

A solution of **1** and **2** (15 mg) in DMSO was treated with NaH (0.2 g) and CH_3I (5 mL) at room temperature for 6 h. The usual work up of the reaction mixture

yielded a residue, which was purified by preparative-TLC in *n*-hexane-EtOAc (1:1). Hydrolysis of premethylated **1** and **2** was performed by refluxing with 10 mL of 5% methanolic HCl. Paper chromatography of the neutralised and concentrated hydrolysate in benzene:acetone (3:1) showed the presence of 3,4-di-O-methyl-L-arabinose, 2,3-di-O-methyl-L-arabinose, 2,3,4-tri-O-methyl-D-glucuronic acid in compound **1**, and 3-O-methyl-D-xylose, 3,4,6-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose in compound **2** (paper chromatography).

4. Antifungal activity

The antifungal activity was performed on five human and plant pathogenic fungi by 'Poison food technique' (Grover & Moore, 1962). The maximum inhibition by compound **1** was recorded in *C. dematium* (77.8%) and minimum in *C. lunata* (27.8%), whereas for compound **2** maximum inhibition was shown against *A. alternata* (53.9%) and minimum against *A. flavus* (29.5%) at a concentration of $1000\,\mu g\,mL^{-1}$. The crude extract was found to be more effective than the pure compound.

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