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In vitro antimicrobial activity of triterpenoid saponin from *Tephrosia purpurea* seeds extract

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ABSTRACT

A novel oleanen type triterpenoid glycoside has been isolated from the butanolic extract of the seeds of *Tephrosia purpurea*. Its structure was elucidated as 3-O-{β-D-glucopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-β-D-xylopyranosyl}-2,16-dihydroxy-23,29-dihydroxymethylolean-11,13(18)-diene-28-oic acid on the basis of spectral evidences, i.e. FT-IR, ^{1}H NMR, ^{13}C NMR and FAB-MS data. The isolated saponin was tested for its antimicrobial activity. Significant results were obtained by evaluating the antibacterial activity by "Disc diffusion method" and antifungal activity by "Spore dilution method". Maximum inhibition was recorded in gram positive bacterium-*Streptococcus pneumoniae*, while complete inhibition on the growth of fungus-*Alternaria alternata* was observed at a concentration of 200 µg/mL. The potency of the extract was quantitatively assessed by determining the minimum inhibitory concentration values against selected bacteria. The minimum inhibitory concentration values were in agreement with antibacterial results where minimum value was recorded to be 23 µg/mL for *Streptococcus pneumoniae*.

1. Introduction

Chemical diversity in natural products is an immensely rich source of new pharmaceuticals [1]. These diverse natural compounds are secondary metabolites that are found to inhibit the growth of microbes *in vitro* [2]. The anti-microbial activities of natural extracts in many instances can be attributed to the presence of terpenoid saponin [3-5]. These terpenes are known to be active against a broad range of micro-organisms, including gram-positive, gram-negative bacteria and fungi [6], and are widely reported in plant system having pharmaceutical potential. Among these medicinal herbs, *Tephrosia purpurea* L. (Fabaceae, Subfamily-Paplionaceae) is a medicinal weed well known for its pharmaceutical potential. The plant is a small herb about 1 m in height and is popularly known as Sarponkha (wild indigo) [7]. It is widely distributed in Central India and also found in wild habitats, waste lands and along the road side.

The plant is known to contain bioactive compounds like-pongamol, β-sitosterol, ursolic acid, spinosterol, α-tetratriacontane, ratenone, tephrosine, betutinic acid, 12 a-hydroxyl retanone and dimethyl glabranin. In addition, epoxy flavonone-5,7-dimethyl-8-(2,3-epoxy-3-methylbutyl)-2-phenyl-2,3-dihydro-4-*H*-1-benzopyran-4-one have also been isolated from the plant [8].

Previous studies showed that plant juice is used for curing rheumatism, hyperacidity and hypoglycemic activity [9,10]. In diabetic rabbits, the extract exerted 60-70% hypoglycemic effect as compared to tolbutamide [11,12]. Aqueous extract of the roots possesses anti-hepatotoxic property. Maximum hepatoprotective activity (52.4%) at a dose of 200 mg/kg body weight was exhibited when CCl_4 extract was administered to rats [13,14]. The ethanolic extract of aerial parts of *T. purpurea* possesses potential for healing wounds and is used in the form of an ointment [15].

Keeping in view the above reports the present research work was carried out for the bioassay directed isolation studies on the seeds of this plant. The isolated molecule was characterized and its antimicrobial activity is reported hereby for the first time.

2. Experimental

2.1. Instrumentation

Melting points were determined on a MAC model melting point apparatus. Optical rotations were measured on Rudolf Autopol III polarimeter. UV spectra were recorded on Thremospectronic UV 100 model spectrophotometer in MeOH solution. ^{1}H NMR and ^{13}C NMR were recorded on Bruker DRX 300 model operating at 300 MHz and 75 MHz (CD_3OD or $CDCl_3$). All the NMR spectra were recorded using TMS as internal standard. IR spectra (KBr disc) were recorded on a Perkin Elmer spectron RXI spectrophotometer having a range of 4000-450 cm^{-1}. 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), Thin layer chromatography (TLC) and preparative TLC on 20x20 cm plates coated with 2 mm thick silica gel (Merck; F_{254}). Spots were visualized using 10% H_2SO_4, followed by heating at 110 ^{o}C. Paper chromatography of sugars was performed on whatman no.1 paper using descending mode in *n*-BuOH:AcOH:H_2O (4:1:5) and developed with aniline hydrogen phthalate.

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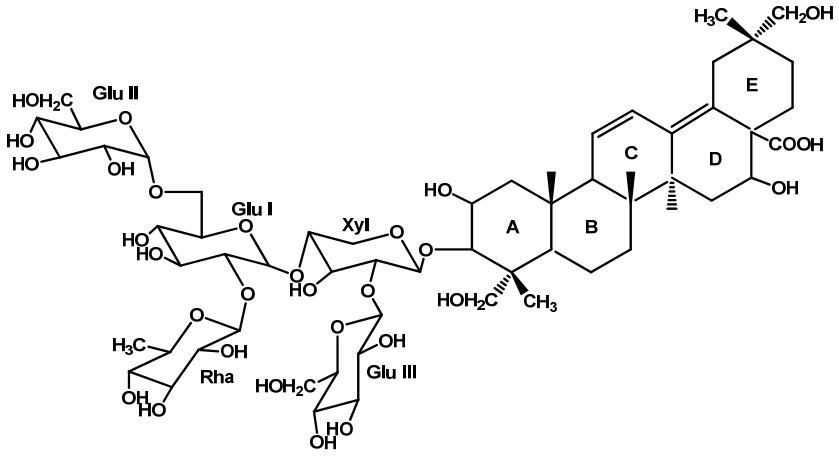

Figure 1. Structure of triterpenoid saponin **I**.

2.2. Plant material

The seeds of *Tephrosia purpurea* were collected from Rani Durgawati University campus, Jabalpur, Madhya Pradesh, India. The seeds were identified and a voucher specimen was deposited in the herbarium of the Department of Biosciences, Rani Durgawati University.

2.3. Extraction and isolation

The air dried and powdered seeds (1 Kg) 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. The combined extract was concentrated in vacuum and 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:v; 70:25:5 to 50:45:5) with 5 mL each as gradient eluent to give 48 fractions. Each fraction was monitored by TLC. The fractions 25-36 showing the same R_f on TLC were pooled together and repeated column chromatograph on silica gel with $CHCl_3$: MeOH (60:40 to 50:50), followed by preparative TLC in EtOAc:MeOH:H_2O (13:8:2) to yield saponin **1** (Figure 1).

2.4. Acid hydrolysis 1

Saponin **1** (25 mg) was refluxed with 10% H_2SO_4 on a boiling water bath for 4 h. The usual work of the reaction mixture afforded sapogenin **2.** M.p.: 215 °C. $[\alpha]_D$ +21.5 [MeOH; *c* 1.36]. FAB-MS (m/z): 518 $[M]^+$, 501, 278, 240, 233,215, 208, 190, 183.

2.5. Identification of sugar moiety of 1

The aqueous layer separated after the removal of sapogenin was neutralized with barium carbonate, filtered and concentrated under reduced pressure. The residue obtained was compared with standard sugar on TLC and paper chromatography (*n*-BuOH:AcOH:H_2O, 4:1:5) indicating the sugars to be D-glucose, L-rhamnose and D-xylose.

2.6. Premethylation of 1

A solution of **1** (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 yields a residue, which was purified by prep-TLC in *n*-hexane:EtOAc (1:1). Hydrolysis of premethylated **1** was performed by refluxing with 10 mL of 3% methanolic HCl. Paper chromatography of the neutralized and concentrated hydrolysate in benzene:acetone (3:1) showed the presence of 2,3,4,6-tetra-O-methyl-D-glucose, 3-O-methyl-D-xylose, 2,3,4-tri-O-methyl-L-rhamnose, 3,4-di-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose (paper chromatography).

2.7. Antimicrobial activity

The antimicrobial activity was assessed as per the method of NCCLS. Five bacteria viz. *Bacillus subtilis* (MTCC-1789), *Escherichia coli* (MTCC-443), *Staphylococus aureus* (MTCC-737), *Klebsiella pneumoniae*(MTCC-2405), *Streptococcus species* (Obtained from Chandrakar Pathology Laboratory) and five fungi viz. *Alternaria alternata* (FGCC-418), *Fusarium roseum* (FGCC-500), *Colletotrichum dematium* (FGCC-165), *Curvularia lunata* (FGCC-280), *Aspergillus flavus* (FGCC-133)that are known to be pathogenic to plants and humans [16,17], were used for the assay.

The antibacterial activity was performed by 'Disc diffusion method' [18]. In this method the filter paper disc (6 mm in diameter) were individually impregnated with 50 µL of the extract of desired concentration and placed on agar plates, which had previously been inoculated with the tested micro-organism. The Petri-plates were kept at 4 °C for 2 h and then incubated at 37±1 °C for 24 h. The diameters of the inhibition zone were measured in mm by means of a transparent ruler. Similar method was used for reference antibiotic gentamicin sulphate. The antibacterial activity of the extract and their potency were quantitatively assessed by determining the minimum inhibitory concentration (MIC) values [19]. The MIC values were determined by 'Well Assay Method'. Four wells of 6 mm diameter were bored on the agar plats and each well was loaded with 50 µL of the extract of desired concentrations. The concentration range of isolated saponin was selected on the basis of the results of antibacterial activity. The range of concentration taken was from 45 µg/mL to a lower dilution of 10 µg/mL. The Petri-plats were kept at room temperature for 1 h and then incubated at 37±1 °C for 24 h. The diameters of the inhibition zone were measured in mm by means of a transparent ruler.

Similarly, antifungal activity was measured by 'Spore dilution method' [20]. Different dilutions of isolated saponin i.e. 100, 200, 300, 400 and 500 µg/mL were employed and fluconazole was used as reference antifungal. A loopful of fungal spores was taken from 7 days old fungal culture and was suspended in 10 mL of distilled water. This solution was

subjected to 3 fold dilution to obtained 10^{-3} dilution. This dilution contains $1x10^4$ cfu/mL as observed in haemocytometer. 1 mL of spore suspension and 1 mL of solution of desired concentration was added in the 18 mL of Potato Dextrose Agar (PDA) media and was poured in sterilized Petri-plats. The media was allowed to solidify for an hour. The plates were then incubated at 28±1 °C for 72 h, and thereafter number of colonies was counted. For control 1 mL of distilled water was added in place of tested solution. The experiment was run in triplicates.

3. Results and discussion

The methanolic extract of dried seeds powdered was partitioned with *n*-hexane, chloroform, *n*-butanol and water. The butanol layer was repeatedly column chromatographed over silica gel to give saponin **1**. Saponin **1** (M.p.: 223 °C, $[\alpha]_D$ +12.6 [MeOH; *c* 1.11]) was a light yellow amorphous powder that showed positive liebermann-burchard test for triterpene. Its UV spectrum contained absorption maxima at 281.6 and 389.1 nm, while the IR spectrum exhibits peaks at 2910 (C-H *str*.), 1666 (C=O *str*.) 1515 (C=C *str*.) and 1282 (C-O) cm^{-1}. A broad band at 3234 cm^{-1} indicates its glycosidic nature.

Saponin **1** on acid hydrolysis yields sapogenin **2** (M.p.: 215 °C) as the aglycone along with sugar moiety. Sapogenin **2** was identified as oleanolic acid by Co-TLC analysis using an authentic sample and comparing its NMR data (^{13}C and ^{1}H) with the data reported in literature [21,22]. The sugar components in the hydrolysate were identified as D-glucose, D-xylose and L-rhamnose in the ratio 3:1:1, indicating **1** to be a sapogenin pentaglycoside. The ratio of sugar was established by comparing with the high-performance liquid chromatography (HPLC) chromatogram of the standard. The position of FAB-MS showed a molecular ion peak at m/z 1306 $[M+Na]^+$ indicating a molecular mass of 1282 which is in good agreement with the molecular formula $C_{59}H_{94}O_{30}$. The fragment at m/z 1136 is consistent with the loss of a terminal rhamnose unit from the molecular ion, whereas the fragment ion peak at m/z 1120 indicates the loss of terminal glucose unit(III) as $[M-162)]^+$. The peaks at 973 $[M-(162+147)]^+$, 810 [M-(162+147+162)], 649 [M-(162+147+162+162)] and 518 [M-(162+147+162+162 +132]$^+$ were attributed to the loss of glucose II, rhamnose, glucose I and xylose units respectively. The results obtained by FAB-MS indicated the sugar sequence in **1.** The presence of glucose and rhamnose as the terminal sugar was confirmed by detection on partial hydrolysis of saponin **l** on TLC in HCl atmosphere [23]. The presence of glucose and rhamnose in the hydrolysate was confirmed by Co-TLC with authentic sample and by HPLC chromatogram.

The ^{1}H NMR spectrum of saponin **1** showed the singlet of five tertiary methyl group (δ 0.987, 0.920, 0.962.0.943 and 1.003 ppm), two olefinic proton (δ 5.13 and 5.216 ppm) and five anomeric protons at 5.901 (d, *J* = 8.21 Hz, 1H), 6.885 (d, *J*=7.3 Hz, 1H), 6.166 (d, *J*=8.01 Hz, 1H), 6.175 (d, *J*=8.14 Hz, 1H) and 6.909 (d, *J*=8.08 Hz, 1H) ppm. The proton noise decoupled ^{13}C NMR spectrum of **l** displayed 59-carbon resonance peaks. The number of attached hydrogen to each carbon was determined by DEPT technique, which suggested the presence of 6 quaternary carbon atom, 29xCH, 13xCH_2, 6xCH_3 and 5 sp^2 hybrid carbon atom (for aglycone CH=, CH=, C=, C= and C=O) (Table 1). The presence of five-anomeric carbon signal at δ 104.91, 105.32, 105.45, 104.8 and 101.9 ppm were in accordance with the presence of pentasaccharide moiety in **l**. On the basis of analysis of DEPT spectrum the molecular formula of **l** could be assigned as $C_{59}H_{94}O_{30}$. A comparison of ^{13}C NMR spectral data of the aglycone moiety of **l** with those of aglycone of triterpene further confirmed its identity [24,25]. The inter glycosidation assignment were further confirmed by the chemical shift of glycosylated carbon atom- δ 80.23, 78.01, 82.15 and 74.31 ppm. The C-2 and C-4 signals of xylose were observed at δ 80.23 and 78.01 ppm, whereas C-2 and C-6 signal of glucose I at δ 82.15 and 74.31 ppm revealed the deshielding of carbon by 4 and 6 ppm for these carbon resonance; hence C-2 and C-4 in xylose and C-2 and C-6 in glucose were concluded to be the glycosidation site. The chemical shift and coupling constant of these signals suggest the β-anomeric configuration for all sugar moieties when compared with the reported values. The pentasaccharide moiety in **l** was linked at C-3 of the aglycone as C-3 showed a significant downfield shift (δ 85.31 ppm) in ^{13}C NMR spectra indicating the glycosidation position [26]. Further the glycoside was hydrolyzed with 10% sulphuric acid, which is a specific reagent for hydrolyzing only β-glycosidic linkage without attacking other sugar ester linkages. Thus sugars are attached to aglycone moiety through beta-glycosidic linkage. The ^{13}C NMR spectral data of aglycone was in good agreement with those of ^{13}C NMR data of saponin **l** and other related saponin. On the basis of above spectroscopic evidences, saponin **1** is 3-O-{β-D- glucopyranosyl- (1→6)- [α - L - rhamnopyranosyl - (1→2)] - β - D - glucopyranosyl - (1→4) - [β-D- glucopyranosyl - (1→2)]- β-D-xylopyranosyl} -2, 16-dihydroxy - 23, 29 - dihydroxymethylolean - 11, 13 (18) - diene - 28- oic acid (Figure 1).

Table 1. ^{13}C NMR chemical shifts and DEPT data of saponin 1.

Carbon	Chemical shift	DEPT	Carbon	Chemical shift	DEPT
1	46.37	CH_2	Xyl 1	104.8	CH
2	72.90	CH	2	80.23	CH
3	85.31	CH	3	71.12	CH
4	43.01	C	4	78.01	CH
5	50.00	CH	5	65.23	CH_2
6	21.23	CH_2	Glu I 1	105.32	CH
7	31.73	CH_2	2	82.15	CH
8	39.01	C	3	73.90	CH
9	49.79	CH	4	71.82	CH
10	35.91	C	5	74.15	CH
11	122.98	CH	6	74.31	CH_2
12	127.97	CH	Glu II 1	105.45	CH
13	146.93	C	2	72.12	CH
14	42.31	C	3	73.01	CH
15	27.31	CH_2	4	76.10	CH
16	62.54	CH	5	73.23	CH
17	47.81	C	6	61.21	CH_2
18	147.14	C	Glu III 1	104.91	CH
19	47.21	CH_2	2	71.02	CH
20	31.51	C	3	74.12	CH
21	33.90	CH_2	4	70.13	CH
22	31.56	CH_2	5	77.56	CH
23	63.81	CH_2	6	64.21	CH_2
24	18.12	CH_3	Rha 1	101.9	CH
25	19.10	CH_3	2	73.14	CH
26	20.51	CH_3	3	76.89	CH
27	24.51	CH_3	4	75.14	CH
28	181.22	COOH	5	69.10	CH
29	61.25	CH_2	6	18.90	CH_3
30	20.23	CH_3			

The isolated compound was tested for its antimicrobial activity against human and plant pathogenic bacteria and fungi. Maximum zone of inhibition was obtained in case of *Streptococcus pneumoniae,* whereas minimum zone of inhibition was observed against *Klebsiella pneumoniae* (Table 2). The *T.purpurea* seeds extract shows MIC values below 50 µg/mL, therefore representing a good activity against the selected bacteria. However, the MIC value recorded was maximum for *B.subtilis* and *K.pneumoniae*- therefore the extract was least effective against these bacteria. The lowest value was observed for *Streptococcus pneumoniae*, which shows that the seeds extract was more effective against this bacterium (Table 2). Among fungi complete inhibition on the growth of fungus - *Alternaria alternata* was observed at a concentration of 200 µg/mL (Table 3).

Table 2. Antibacterial activity and Minimum Inhibitory Concentration values of saponin **1** obtained from butanolic seeds extract of *T. purpurea.*

S. No.	Name of Bacteria	Zone of Inhibition (in mm)[b]		MIC (µg/mL)
		Saponin 1 (50 µg/mL)	Comparison antibiotic.[c]	
1	*Klebsiella pneumoniae* (MTCC-2405)	9.3±0.84	38±0.02	42±0.11
2	*Escherichia coli* (MTCC-443)	11.6±0.17	38±0.50	35±0.09
3	*Staphylococcus aureus* (MTCC-737)	11.3±0.033	28±0.02	36±0.07
4	*Streptococcus pneumonia*[a]	16.1±0.46	40±0.11	23±0.05
5	*Bacillus subtilis* (MTCC-1789)	9.8±0.177	33±0.04	43±0.02
CD[d] at 5%		2.45		

[a] Obtained from Chandraker Pathology Laboratory, Jabalpur.
[b] Zone of inhibition includes diameter of disc.
[c] Gentamicin sulphate (40 µg/mL) used as a comparison antibiotic.
[d] CD: Critical Difference.
* Values are the mean of triplicate readings; Mean±S.E.M (Standard error of the mean); The effect of saponin on different bacteria is different. At 40 µg/mL concentration, some of the bacteria do not show inhibition at all. So a higher concentration is selected to maintain uniformity. The concentration of gentamicin sulphate take is 40 µg/mL, where a well-defined ZI's is seen. This concentration is used for all the bacteria that are studied in our laboratory and is well confirmed from the review of literature.

Table 3. *In vitro* antifungal activity[a] of butanolic seeds extract of *T. purpurea**.

Butanolic extract (µg/mL)	*Alternaria alternata*	*Fusarium roseum*	*Colletotrichium dematium*	*Curvularia lunata*	*Aspergillus fumigatus*
50	32	36	78	83	6
100	18	34	59	69	59
200	Complete inhibition	30	08	38	28
300	NT	06	301	17	02
400	NT	Complete inhibition	Complete inhibition	Complete inhibition	Complete inhibition
500	NT	NT	NT	NT	NT
Fluconazole (10 µg/mL)	15	18	12	16	15
CD at 5%	1.985	1.334	1.75	2.39	1.75

* NT: Not tested; CD: Critical Difference; Values are the mean of triplicates.

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