

# A new antifungal eudesmanolide glycoside isolated from *Sphaeranthus indicus* Linn. (Family Compositae)

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## Abstract

A new antifungal eudesmanolide glycoside 11,13-dihydro-3-*O*-( $\beta$ -digitoxopyranose)-7 $\alpha$ -hydroxy eudasman-6,12-olide (**2**) in addition to known compounds **1** and **3**, has been isolated from *Sphaeranthus indicus* Linn. Its structure was determined by spectral analysis (UV, IR, 1D and 2D NMR and mass spectrum).



Keywords: *Sphaeranthus indicus*, eudesmanolide, glycoside, antifungal activity

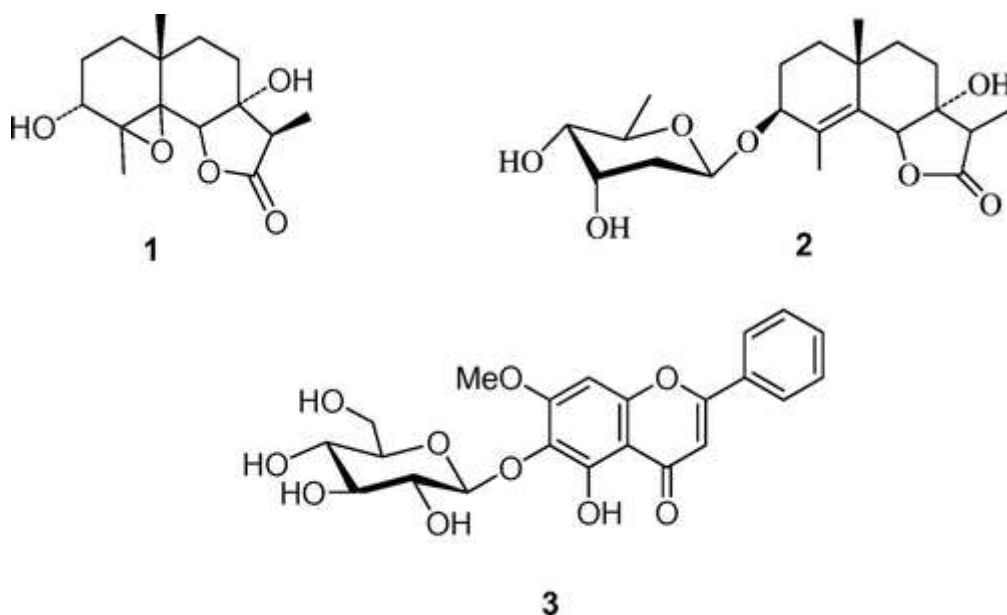
## 1. Introduction

India has been endowed with a very rich flora owing to the extreme variations in climate and geographical conditions prevalent in the country. With the advent in science, many of the crude drugs used in traditional system have been investigated scientifically (Galani et al.

2010). The therapeutic areas of infectious diseases and oncology have benefited from these numerous drug classes, able to interact with many specific targets within the cell, and indeed for many years have been central in the drug discovery and development (Mishra & Tiwari 2011; Singh et al. 2014). The plant *Sphaeranthus indicus* Linn., a herb of about 30 cm in height, with spreading branches and round purple flowers, is found all over the Indian plains as weed in the rice fields (Shekhani et al. 1990; Chatterjee & Pakrashi 2003). It is used as folk medicine to treat wide range of diseases like wound healing, anthelmintic, insanity, tuberculosis, bronchitis, spleen diseases, elephantiasis, etc. (Farzana et al. 2006; Nisha et al. 2007; Mathew et al. 2012).

Among some identified phytochemicals in *S. indicus*, methyl chavicol,  $\alpha$ -ionone, *p*-methoxy cinnamaldehyde,  $\alpha$ -terpinene, citral, geraniol, geranyl acetate,  $\beta$ -ionene, sphaerene, etc. are present as minor constituents in essential oil (Lodha 2003). Carbohydrates such as arabinose, galactose, glucose, fructose, lactose, maltose, raffinose and rhamnose have been reported from leaves of *S. indicus* (Yadava & Kumar 1998). Presence of several eudesmanoids, phenolic glycosides and sesquiterpene lactones has been reported by different scientific groups (Shekhani et al. 1990; Yadava & Kumar 1999). In present paper, we report the isolation and characterisation of a new antifungal eudesmanolide glycoside **2** (Figure 1) from the aerial parts of plant *S. indicus* using analytical techniques (IR, NMR and MS).

**Figure 1.** Structure of compounds 1–3.



## 2. Results and discussion

The IR spectrum (KBr) of compound **2** showed characteristic 5-membered  $\gamma$ -lactone absorption band at  $1780\text{ cm}^{-1}$  and also revealed the presence of OH and non-conjugated olefin function. Elemental analysis (Found: H, 8.17%; C, 63.64%; O, 28.32%/requires: H, 8.14%; C, 63.60%; O, 28.26%) in combination with a molecular ion peak at  $m/z$  396 (45%) and 21 carbon resonance signals observed in  $^{13}\text{C}$  NMR spectrum (75 MHz,  $\text{CD}_3\text{OD}$ ) of

compound **2** confirmed the molecular formula to be  $C_{21}H_{32}O_7$ . In EI-MS spectrum, the prominent peaks at  $m/z$  299, 214, 200, 165 and 158 showed a related eudesmanolide skeleton (Irwin & Geissman 1969, 1971). A base peak observed at  $m/z$  265 (100%), indicated for the compound **2** to be a eudesmanolide monoglycoside with a dideoxyhexose as a sugar unit.

In  $^1H$  NMR spectrum (300 MHz,  $CD_3OD$ ) of compound **2**, a three-proton singlet appeared at  $\delta$  1.94 was evidenced for angular methyl resonance while another singlet integrated to three protons was identified for the presence of a methyl group attached to olefinic carbon.

A three-proton doublet at  $\delta$  1.14 ( $J = 7.2$  Hz) for 13-methyl protons and a one proton quartet at  $\delta$  2.79 ( $J = 7.2$  Hz) for H-11, indicated the vicinal relation between H-11 and 13-methyl group. A down-field one proton doublet observed at  $\delta$  5.00 for H-6 showed only a long-range coupling (1.0 Hz). It's unusual down-field shifting suggested that, it is attached to an oxygen bearing carbon which is also allylic in nature. Another one proton down-field broad doublet at  $\delta$  4.12 ( $J = 2.0$  Hz) was identified for H-3. Based on biogenetic considerations, the stereochemistry at C-7 was assigned. Such an assignment has been confirmed earlier through X-ray studies (Sohoni et al. 1988). Biogenetically, C-10 methyl and C-7-C-11 bond are  $\beta$ -oriented and therefore, the hydroxy group at C-7 was considered  $\alpha$ -oriented. The multiplets two proton each observed at  $\delta$  1.95,  $\delta$  1.85,  $\delta$  1.70 and  $\delta$  1.45 were identified for four methylene resonances of aglycone moiety in compound **2**.

An anomeric one proton signal appeared at  $\delta$  4.75 ( $dd$ ,  $J = 12.1, 2.0$  Hz) was identified for dideoxyhexose moiety. The dideoxyhexose was confirmed to be  $\beta$ -digitoxopyranose (2,6-dideoxy-*ribo*-hexopyranose) on the basis of characteristic anomeric signal ( $\delta_H$  4.75;  $\delta_C$  95.5) in  $^1H$  and  $^{13}C$  NMR spectrum. Two signals one proton each observed at  $\delta$  1.81 ( $ddd$ ,  $J = 14, 3.2$  Hz) and  $\delta$  2.93 ( $ddd$ ,  $J = 14.3, 12.1, 2.5$  Hz) were identified as enantiotropic protons  $2'\alpha$  and  $\beta$ , respectively. Other signals observed at  $\delta$  4.21( $q$ ,  $J = 3.0$  Hz), 3.43 ( $dd$ ,  $J = 9.5, 3.0$  Hz), 3.83 ( $dq$ ,  $J = 9.5, 6.2$  Hz) and 1.32 (3H,  $d$ ,  $J = 6.2$  Hz) were assigned for sugar protons  $3'$ ,  $4'$ ,  $5'$  and  $6'$  in compound **2**, respectively.

The  $^{13}C$  NMR spectrum (75 MHz,  $CD_3OD$ ) of compound **2** exhibited 21 carbon resonance signals. A carbonyl carbon resonated at  $\delta$  178.2 while other oxygen functionalities resonated at  $\delta$  81.5 and  $\delta$  77.9. The location of the sugar unit was determined to be C-3 on the basis of HMBC correlations observed between H-1' of the digitoxose unit and C-3 of the aglycone moiety. Also, the multiple correlations were observed between olefin methyl to C-3 and C-5. Likewise, angular methyl protons (H-15) exhibited correlation with C-1, C-5 and C-9. Therefore, the structure of compound **2** was elucidated as 11,13-dihydro-3- $O$ -( $\beta$ -digitoxopyranose)-7 $\alpha$ -hydroxyeudesman-6,12-olide.

The hydrolysis of compound **2** with 2N  $H_2SO_4$  in 50% ethanol furnished an aglycone. The proton and  $^{13}C$  NMR shift pattern were compared and found similar to frullanolides (Bohlmann et al. 1980; Segal et al. 1984). The sugar residue was evidenced as  $\beta$ -digitoxopyranose by paper chromatographic comparison with different known sugars.

The antifungal activity of compound **2** was evaluated against pathogenic fungi by disc diffusion assay (DDA), broth microdilution assay (BMA) and percent spore germination inhibition assay (PSGIA). The results were compared using standard antifungal drug

Clotrimazole with minimum inhibitory concentrations (MICs) of 0.195–3.125 µg/mL (Table 1). Compound **2** with MIC values of 3.12, 15.60 and 15.60 µg/mL in DDA, BMA and PSGIA, respectively, showed significant activity against *Aspergillus fumigatus*, *Aspergillus flavus* and *Saccharomyces cerevisiae*.

**Table 1. MICs of compound 2 by various antifungal susceptibility assays.**

Fungi	Compound 2			Clotrimazole
	DDA	BMA	PSGIA	
<i>A. fumigatus</i>	3.12	15.60	15.60	1.562
<i>A. flavus</i>	3.12	15.60	15.60	1.562
<i>A. niger</i>	6.25	31.25	31.25	3.125
<i>C. parapsilosis</i>	12.5	62.5	62.5	1.56
<i>C. tropicalis</i>	10.25	51.25	51.25	1.56
<i>C. albicans</i>	12.5	62.5	62.5	3.125
<i>T. mentagrophytes</i>	6.25	31.25	31.25	0.390
<i>C. neoformans</i>	25	>100	>100	0.195
<i>S. schenckii</i>	12.5	62.5	62.5	1.562
<i>T. viride</i>	10.25	51.25	51.25	0.781
<i>M. gypseum</i>	25	>100	>100	1.562
<i>A. ramosa</i>	25	>100	>100	0.390
<i>P. boydii</i>	25	>100	>100	0.781
<i>S. cerevisiae</i>	3.12	15.60	15.60	1.562

Notes: Minimum inhibitory concentration (MIC), the lowest concentration of a compound that completely inhibited the visible growth against tested fungal species.

Clotrimazole (standard drug), MICs in µg/mL.

DDA, MICs in µg/disc; BMA, MICs in µg/mL; PSGIA, MICs in µg/mL.

### 3. Experimental

#### 3.1. General experimental procedures

The solvents were of pure analytical grade. Melting points were measured in open capillary tubes on a Buchi 530 apparatus and are uncorrected. Thin-layer chromatography was performed on 60 F254 silica gel, precoated on aluminium plates and revealed with either a UV lamp ( $\lambda_{\text{max}} = 254 \text{ nm}$ ) or by spraying with methanolic  $\text{H}_2\text{SO}_4$  solution and subsequent charring by heating at  $100^\circ\text{C}$ .  $^1\text{H}$  and  $^{13}\text{C}$  NMR were recorded at 300 and 75 MHz, respectively. Chemical shifts given in ppm downfield from internal TMS;  $J$  values in Hz. Mass spectra recorded using electrospray ionisation mass spectrometry (ESI–MS). Infrared spectra recorded as Nujol mulls in KBr plates. Elemental analysis was done using a C, H, N analyser and results were found to be within  $\pm 0.4\%$  of the calculated values.

### 3.2. Plant material

The plant was collected in March 2012 in a suburb of Varanasi, India. Plant identification was verified by Prof N.K. Dubey, Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi, India. A specimen sample of plant material has been preserved in lab No. 39 (Code BBM/SI/12/V), Department of Chemistry, Faculty of Science, Banaras Hindu University, Varanasi-221005, India.

### 3.3. Extraction and isolation

The dried and powdered aerial parts of *S. indicus* (3 kg) were first extracted with petroleum ether (5 L) for 24 h, and then with ethanol (5 L) for 38 h. After *in vacuo* concentration of ethanol extract (500 g), the residue was extracted with CHCl<sub>3</sub> (2 L × 3), ethyl acetate (3 L × 3) and *n*-butanol (3 L × 2), respectively. Subsequent chromatographic purification of ethyl acetate fraction resulted into isolation of **1–3** (Figure 1). Structural assessment of these compounds was effected by analyses of EI-MS and NMR spectroscopic data.

The known compounds obtained in this study, 11 $\alpha$ ,13-dihydro-3 $\alpha$ ,7 $\alpha$ -dihydroxy-4,5-epoxy-6 $\beta$ ,7-eudesmanolide **1** (Sohoni et al. 1988) and 5-hydroxy-7-methoxy-6-C-glycosylflavone **3** (Mishra et al. 2007) were identified by comparison of their physical and spectroscopic data with literature reports.

The ethyl acetate extract (30 g) was chromatographed over silica gel (60–120 mesh; 600 g), and eluted with gradient mixtures of toluene–methanol to provide five fractions (F01–05). The fraction F-05 (8 g) was further chromatographed over silica gel (234–400 mesh), and eluted with gradient mixture of CHCl<sub>3</sub>–MeOH to afford nine sub-fractions (SF1–9). The sub-fraction SF-2 eluted with CHCl<sub>3</sub>–MeOH (9:1, v/v) yielded 11 $\alpha$ ,13-dihydro-3 $\alpha$ ,7 $\alpha$ -dihydroxy-4,5-epoxy-6 $\beta$ ,7-eudesmanolide (**1**), white cubes (10 mg). Likewise, SF-5 eluted with CHCl<sub>3</sub>–MeOH (4:6, v/v) afforded compound **2**, recrystallisation from CHCl<sub>3</sub>–MeOH (1:1, v/v) into white powder, Yield = 120 mg; m.p. 262–264 °C; <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD):  $\delta$  5.00 (1H, *d*, *J* = 1.0 Hz), 4.75 (1H, *dd*, *J* = 12.1, 2.0 Hz), 4.21 (1H, *q*, *J* = 3.0 Hz), 4.12 (1H, *d*, *J* = 2.0 Hz), 3.83 (1H, *dq*, *J* = 9.5, 6.2 Hz), 3.43 (1H, *dd*, *J* = 9.5, 3.0 Hz), 2.93 (1H, *ddd*, *J* = 14.3, 12.1, 2.5 Hz), 2.79 (1H, *q*, *J* = 7.2 Hz), 1.95 (2H, *m*), 1.94 (3H, *s*), 1.85 (2H, *m*), 1.81 (1H, *ddd*, *J* = 14, 3.2 Hz), 1.70 (2H, *m*), 1.45 (2H, *m*), 1.32 (3H, *d*, *J* = 6.2 Hz), 1.14 (3H, *d*, *J* = 7.2 Hz), 1.01 (3H, *s*); <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD):  $\delta$  178.2, 139.2, 133.8, 95.5, 81.5, 77.9, 75.9, 65.2 (3), 49.2, 35.8, 35.2, 34.9, 34.7, 25.4, 24.2 (2), 18.6, 15.5, 14.8; IR (KBr)  $\nu_{\text{max}}$ : 3610, 1780, 1648, 1215, 1162, 1050, 1030, 948, 830, 765 cm<sup>-1</sup>; MS *m/z* 396 (45%), 379 (32%), 364 (47%), 265 (100%); Anal. Calcd for C<sub>21</sub>H<sub>32</sub>O<sub>7</sub>: H, 8.14%; C, 63.60%; Found: H, 8.17%; C, 63.64%.

The SF-7 eluted with CHCl<sub>3</sub>–MeOH (6:4, v/v) furnished 5-hydroxy-7-methoxy-6-C-glycosylflavone (**3**), recrystallisation from ethanol (100%) into yellow needles (30 mg, m.p. 230–232 °C) (Mishra et al. 2007).

### 3.4. Micro-organism and media

Clinical isolates of *A. fumigatus*, *A. flavus*, *A. niger*, *Candida parapsilosis*, *Candida tropicalis*, *Candida albicans*, *Candida neoformans*, *Trichophyton mentagrophytes*, *Sporothrix schenckii*, *Trichoderma viride*, *Microsporum gypseum*, *Absidia ramosa*, *Pseudallescheria boydii* and *S.*

*cerevisiae* obtained from the Microbiology Department, Vallabhbhai Patel Chest Institute, Delhi, India were used for antifungal assay. The pathogenic strains of micro-organism were cultured on Sabouraud dextrose agar plates. Spores/conidia from the fungal colonies of 72–96 h cultures were incubated at 28 °C, the conidia were counted using hemocytometer and the number was adjusted to  $1 \times 10^6$  spores/mL.

### 3.5. Antifungal susceptibility assays

#### 3.5.1. Disc diffusion assay

The radiation sterilised Petri dishes (10 cm diameter) were poured with adequate amount of Sabouraud dextrose agar (Yadav et al. 2005). One ml of conidial suspension equivalent to  $1 \times 10^6$  conidia was prepared in agar plate. The disc diffusion test was performed with different concentrations of compound **2** ranging from 50 to 1.56 µg. Medium was impregnated in a sterilised disc Whatman filter paper No. 1, 5.0 mm in diameter and the discs were placed on the surface of agar plates already impregnated with fungi. The plates were incubated for 48 h at 37 °C and examined for around the disc, and the lowest concentration that develops zone of inhibition of 6 mm diameter or more, was considered as MIC, Clotrimazole was used as positive control in the assay.

#### 3.5.2. Broth microdilution assay

For broth microdilution assay (Dabur et al. 2005), autoclaved Sabouraud dextrose broth was placed in each well of 96 well culture plates (Nunc, Nunclon). Various concentrations of compound **2** in the range of 1000.0–7.86 µg/mL were prepared in the wells by twofold dilution method. The wells were inoculated with  $1 \times 10^6$  spores in 10 µL of spore suspension. Appropriate control wells treated with Clotrimazole or without any treatment were included in the study. The plates were incubated at 28 °C and examined visually after 48–72 h of cultures for the growth of fungal mycelia. The activity was represented as –ve if visible growth was there and +ve if medium appeared clear without any growth of fungal mycelia. The lowest concentration, which inhibits the growth of fungi, was considered as the MIC of the compound.

#### 3.5.3. Percent spore germination inhibition assay

For the spore germination assay (Rajesh & Sharma 2002), various concentrations ranging from 1000.0 to 7.86 µg/mL of compound **2** in 90.0 µL of culture medium were prepared in 96 well flat bottom micro-culture plates (Nunc, Nunclon) by double dilution method. The wells were prepared in triplicates for each concentration. The concentration ranged from 1000.0 to 7.86 µg/mL. Each well was then inoculated with 10 µL of spore's suspension containing  $100 \pm 5$  spores. The plates were incubated at 28 °C for 24 h and then examined for spore germination under inverted microscope. The numbers of germinated and non-germinated spores were counted. The PSGI was calculated using following formula.

$$\text{PSGI} = 100 - \frac{\text{No. of spores germinated in treated well}}{\text{No. of spores germinated in control well}} \times 100$$

The tests were repeated at least three times. The lowest concentration that gave 97–100% inhibition of germination of conidia in the wells was considered as MIC.

## 4. Conclusion

In conclusion, we have identified a new compound, 11,13-dihydro-3-*O*-( $\beta$ -digitoxopyranose)-7 $\alpha$ -hydroxyeudesman-6,12-olide (**2**) from ethanolic extract of aerial parts of *S. indicus* exhibiting significant *in vitro* antifungal activity against pathogenic strains of fungi. Synthesis of this naturally occurring compound **2** and its analogues may provide a lead for the development of a potent antifungal drug.

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