Natural Product Research: Formerly Natural Product Letters
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

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Published online: 18 Nov 2013.

To cite this article: U. Hawas, S. Eltomy, R. Nassif, G. El-Hossary & S. AbouZid , Natural Product Research (2013): A new antifungal labdane diterpene from the leaves of Saraca indica, Natural Product Research: Formerly Natural Product Letters, DOI: 10.1080/14786419.2013.855931

To link to this article: http://dx.doi.org/10.1080/14786419.2013.855931

Natural Product Research, 2013
http://dx.doi.org/10.1080/14786419.2013.855931

A new antifungal labdane diterpene from the leaves of *Saraca indica*

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(*Received 18 August 2013; final version received 17 September 2013*)

A new labdane diterpene, along with 10 known sterols and flavonoids, was isolated from the hydroalcoholic extract of the leaves of *Saraca indica*. The chemical structure of the new compound was identified as 6,9-epoxy marrubiinic acid on the basis of spectroscopic analyses including two-dimensional NMR. The antimicrobial potential of the new compound was evaluated against Gram-positive and Gram-negative bacteria as well as fungi. It showed a significant antifungal activity against *Geotrichum candidum* with MIC 0.48 μg/mL. It also showed potential cytotoxicity against human cancer cell lines with IC_{50} ranging from 1.07 to 1.29 μg/well.

Keywords: *Saraca indica*; labdane diterpene; epoxy marrubiinic acid; antifungal; cytotoxicity

1. Introduction

Saraca indica L. (Fabaceae), commonly known as Ashoka, is an evergreen tree with numerous spreading and drooping glabrous branches. The bark of this plant is traditionally used as astringent, demulcent, emollient and stomachic. The leaves are useful in stomachalgia, and the flowers are used in syphilis, inflammation, dysentery, haemorrhoids and scabies in children (Kashyapa & Chand 2006). The aerial part is known for its hypothermic, central nervous system depressant and diuretic activities (Kokate et al. 2007). This plant has shown many pharmacological properties such as antidiabetic (Preethi et al. 2010), oxytocic (Satyavati et al. 1970), antiulcer (Maruthappan & Shree 2010), antimicrobial (Sainath et al. 2009; Hawas et al. 2012) and anthelmintic (Sharma et al. 2011) activities. Previous phytochemical studies on the leaves of *S. indica* have led to the isolation of quercetin, quercetin 3-*O*-α-rhamnoside, kaempferol 3-*O*-α-rhamnoside, amyrin, ceryl alcohol and β-sitosterol (Darwish & Khalifa 1992; Sadhu et al. 2007; Pradhan et al. 2009).

In this report, phytochemical investigation on the leaves of *S. indica* was carried out. A new labdane diterpene (**1**) and 10 known compounds were isolated. The structure of the isolated new compound was evaluated based on spectroscopic data. Its antimicrobial potential and cytotoxicity were evaluated.

2. Results and discussion

Compound **1** was isolated from the chloroform-soluble fraction of the extract as a colourless oil. This fraction also afforded β-sitosterol (**2**), stigmasterol (**3**) and campesterol (**4**) (Conolly &

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Hill 1992; Habib et al. 2007). Kaempferol (**5**), quercetin (**6**), myricetin (**7**), quercetin 3-*O*-α-rhamnoside (**8**), myricetin 3-*O*-α-rhamnoside (**9**), kaempferol 3-*O*-α-rhamnoside (**10**) and catechin (**11**) were isolated from the ethyl acetate-soluble fraction of the extract (Aguiar et al. 2005; Ye & Huang 2006; Zheng et al. 2008). These known compounds (**2**–**10**) were identified based on their chromatographic and spectroscopic properties.

The complete assignment of ^{1}H and ^{13}C NMR signals was achieved by a combination of COSY, NOESY, HSQC and HMBC experiments (Supplementary material). The analysis of ^{13}C NMR spectrum ($CDCl_3$) revealed the presence of 20 carbon signals assigned to three methyls (δ_C 16.6, 22.3 and 23.0 ppm), six methylenes (δ_C 18.2, 21.0, 28.6, 28.4, 31.5 and 35.2 ppm), six methines (δ_C 32.4, 44.9, 76.3, 110.8, 138.6 and 143.1 ppm) and four quaternary carbons (δ_C 39.7, 43.8, 76.8 and 125.1 ppm). The presence of the carboxylic group was confirmed by the signal of the quaternary carbon at δ_C 184 ppm. The molecular weight of compound **1** was established by ESI MS in negative and positive mode as *m/z* 331 $[M - H]^-$ and 355 $[M + N\alpha]^+$, respectively. The mass peak at 331 in negative mode of ESI MS/MS which afforded the peak at 287 (100%) indicated the presence of a carboxylic group. The EI MS of compound **1** showed fragment peaks at *m/z* 81 and 95 indicating the presence of a β-monosubstituted furan ring. This was confirmed by the ^{1}H NMR spectrum ($CDCl_3$) that showed typical signals of a β-monosubstituted furan ring at δ_H 6.24 (H-14), 7.33 (H-15) and 7.21 (H-16). The proton NMR spectrum exhibited the deshielded methylene protons H-12 (δ_H 2.52 ppm) and the remaining protons of the methylenes of the two cyclohexane rings of the labdane skeleton between δ_H 0.94 and 2.21 ppm. All of these data agreed with the presence of a furanolabdane diterpene, and the structure is very similar to marrubiinic acid (Meyre-Silva et al. 2005). The COSY spectrum displayed cross-peaks between the two adjacent methines H-5 (δ_H 2.21 ppm) and H-6 (δ_H 4.71 ppm) and correlation between methine H-8 (δ_H 2.11 ppm) and H_3-17 (δ_H 0.94 ppm) as well as all expected COSY correlations between all neighbouring methine and methylene protons. HR ESI MS gave the molecular formula $C_{20}H_{28}O_4$, suggesting seven double bond equivalents in diterpene molecule. The index of hydrogen and oxygen deficiency in the molecular formula of compound **1** compared with the molecular formula of marrubiinic acid ($C_{20}H_{30}O_5$) with only six double bond equivalents indicated that compound **1** is marrubiinic acid losing a molecule of water and forming an epoxide ring between oxygenated carbons C-6 and C-9. This was confirmed by the ^{13}C NMR and HMQC spectra of **1**, which exhibited a close similarity to the carbons of marrubiinic acid. There was, however, a downfield shift ($\Delta\delta_C = 11.3$ and 0.9 ppm) of C-6 and C-9 carbon resonances, respectively, of compound **1** compared with the carbon resonances of marrubiinic acid.

The structure of compound **1** was finally confirmed by the HMBC experiment (Figure S1). The correlation of methine proton H-8 (δ_H 2.11) with the carbons C-6 (δ_C 76.3), C-9 (δ_C 76.8), C-10 (δ_C 39.7) and CH_3-17 (δ_C 16.6) confirmed the position of epoxy ring. The correlation of methylene protons H_2-11 (δ_H 1.88 and 1.69) and H_2-12 (δ_H 2.52) with the carbons of C-13 (δ_C 125.1), C-14 (δ_C 110.8) and C-16 (δ_C 138.6) of furan ring and C-8 (δ_C 32.4), C-9 (δ_C 76.8) and C-10 (δ_C 39.7) of the two cyclohexane rings of the labdane skeleton confirmed the furanolabdane diterpene structure. The presence of NOE cross-peaks between H_3-18 and H_2-11, H_3-17 and H_3-20, as expected, clearly established the relative stereochemistry of the chiral centres as shown in the formula. Thus, compound **1** was identified as 6,9-epoxy marrubiinic acid as a new natural product isolated for the first time in nature (Figure 1).

The antimicrobial potential of the new compound was evaluated against Gram-positive and Gram-negative bacteria as well as fungi (Table 1). The results were obtained using *Escherichia coli* (RCMBCC 000103), *Pseudomonas aeruginosa* (RCMBCC 000102), *Klebsiella pneumonia* (RCMBCC 0010093), *Bacillus subtilis* (RCMBCC 000101), *Staphylococcus aureus* (RCMBCC 000106) and *Streptococcus pyogenes* (RCMBCC 010015), as test bacterial strains. The fungal indicators were *Aspergillus fumigatus* (RCMB 002003), *Aspergillus clavatus* (RCMB 05096), *Penicillium italicum* (RCMB 001003), *Geotrichum candidum* (RCMB 05096) and *Candida*

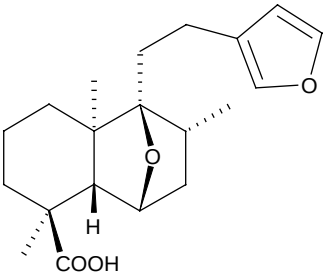

Figure 1. Chemical structure of compound **1**.

albicans (RCMB 005003). The results indicated that compound **1** has a powerful antifungal activity against *G. candidum* and *P. italicum* with MIC values of 0.48 and 3.9 μg/ml, respectively, compared with amphotericin B.

The cytotoxic effect of compound **1** was demonstrated against five human cancer cell lines (HEPG2: human liver cancer; HELA: human cervix cancer; MCF7: human breast cancer; HCT116: human colon cancer and HEP2: human larynx cancer) as well as normal cell line (HFB4: human normal melanocytes) using sulphorhodamine B assay. As shown in Figure S2, the new compound exhibited potent cytotoxicity with IC_{50} ranging from 1.07 to 1.92 μg/well against all tested human tumour cell lines.

3. Experimental

3.1. *General*

UV: Shimadzu double beam recording spectrophotometer model UV-150-02; ^{1}H NMR spectra: Joel 500 (500 MHz); ^{13}C NMR spectra: Joel 500 (125 MHz); MS (Joel mass spectrometer, 70 eV), National Research Centre, Cairo, Egypt. Column chromatography (CC), silica gel 60, 0.040–0.063 mm (E. Merck, Darmstadt, Germany), polyamide 6, 50–160 μm (Ridel de Häen AG, Seelze, Hannover, Germany) and Sephadex LH-20 (Pharmacia Fine Chemical, Uppsala, Sweden) were used. Thin layer chromatography analysis was carried out using silica gel 60 F_{254}

Table 1. Antimicrobial activity of compound **1**.

	Minimum inhibitory concentration (μg/mL)	
Tested microorganism	Compound **1**	Drug reference
Indicator fungi		Amphotericin B
A. fumigatus (RCMB 002003)	7.81	0.97
P. italicum (RCMB 001003)	3.9	7.81
C. albicans (RCMB 005003)	62.5	0.48
G. candidum (RCMB 05096)	0.48	0.48
A. clavatus (RCMB 05096)	3.9	1.95
Indicator bacteria (Gram positive)		Ampicillin
S. aureus (RCMBCC 000106)	15.63	0.12
B. subtilis (RCMBCC 000101)	1.95	0.03
S. pyogenes (RCMBCC 010015)	3.9	0.24
Indicator bacteria (Gram negative)		Gentamicin
P. aeruginosa (RCMBCC 000102)	250	31.25
E. coli (RCMBCC 000103)	15.63	7.81
K. pneumonia (RCMBCC 0010093)	0.48	0.24

plates (Merck, Darmstadt, Germany). Paper chromatography (1 and 3 mm) was visualised under UV light at 240 and 366 nm and sprayed with acidic vanillin reagent (Aldrich, Steinheim, Germany).

3.2. *Plant material*

The leaves of *S. indica* were collected on April 2009 from El-Orman garden (Giza, Egypt) and identified by Prof. Abdel Megeed Ali, Plant Taxonomy Department, Agriculture Research Centre, Giza, Egypt. Voucher specimen (No. E00317653) was deposited in the herbarium of the Agriculture Research Centre, Giza, Egypt.

3.3. *Extraction and isolation*

The air-dried powdered leaves of *S. indica* (1 kg) were extracted with 70% aqueous methanol (×2) at room temperature. The extracts were combined and concentrated under reduced pressure to give 66 g residue. The crude extract was dissolved in water and successively partitioned against *n*-hexane, $CHCl_3$ and ethyl acetate. The chloroform fraction was evaporated to dryness (10 g) and chromatographed on silica gel (800 g) using *n*-hexane and mixtures of *n*-hexane–EtOAc and EtOAc–MeOH as eluents. Fractions were purified with successive CC of silica gel and Sephadex LH-20 giving **1** (7 mg), **2** (12 mg), **3** (40 mg) and **4** (25 mg) compounds. The ethyl acetate fraction was evaporated to dryness (40 g), dissolved in water and subjected to a polyamide CC (200 g). The column was eluted with water containing increasing amounts of MeOH. Fractions showing similar paper chromatography profiles in BAW and 15% acetic acid were pooled to provide combined fractions. These fractions were re-chromatographed on preparative paper chromatography (3 mm) using 15% acetic acid as eluent. The separated bands were scraped off and eluted with 70% methanol and further purified on Sephadex LH-20 column using MeOH as eluent to get compounds **5** (6 mg), **6** (35 mg), **7** (35 mg), **8** (6 mg), **9** (6 mg) and **10** (6 mg).

3.3.1. *6,9-Epoxy marrubiinic acid (**1**)*

Colourless oil; $[\alpha]_D^{25}$: +41 (*c* 0.1, $CHCl_3$); ESI MS (neg. mode) *m/z*: 331 $[M - H]^-$, 663 $[2M - H]^-$; ESI MS/MS (neg. mode) 287 $[M - COOH]^-$; ESI MS (pos. mode) 687 $[2M + Na]^+$, 355 $[M + Na]^+$; HR ESI MS (pos. mode) 355.18798, calc. for ($C_{20}H_{28}O_4Na$: 355.1885); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 1.69 (1H, m, H-1a), 1.4 (1H, m, H-1b), 1.68 (2H, m, H-2), 2.12 (1H, m, H-3a), 1.26 (1H, m, H-3b), 2.21 (1H, d, $J = 4.5$ Hz, H-5), 4.71 (1H, q, H-6), 2.12 (1H, m, H-7a), 1.65 (1H, m, H-7b), 2.11 (1H, m, H-8), 1.88 (1H, m, H-11a), 1.69 (1H, m, H-11b), 2.52 (2H, m, H-12), 6.24 (1H, d, $J = 1.2$ Hz, H-14), 7.33 (1H, d, $J = 1.2$ Hz, H-15), 7.21 (1H, s, H-16), 0.94 (3H, d, $J = 6$ Hz, H-17), 1.25 (3H, s, H-18), 1.00 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 31.5 (C-1), 18.2 (C-2), 28.4 (C-3), 43.8 (C-4), 44.9 (C-5), 76.3 (C-6), 28.6 (C-7), 32.4 (C-8), 76.8 (C-9), 39.7 (C-10), 35.2 (C-11), 21.0 (C-12), 125.1 (C-13), 110.8 (C-14), 143.1 (C-15), 138.6 (C-16), 16.6 (C-17), 23.0 (C-18), 184.0 (C-19), 22.3 (C-20).

3.4. *Antimicrobial assay*

The antimicrobial spectrum and activity of compound (**1**) were determined by the agar plate diffusion assay (Bauer et al. 1966). Antifungal (Amphotericin B) and antibacterial (Ampicillin and Gentamicin) were used as drug references. Ten micro litres of the compound were applied to sterile paper disc (Whatman) of 6-mm diameter, placed on the surface of indicator test plate and incubated at the temperature that permitted the optimal growth of the test organisms. For determination of antibacterial and antifungal activities, indicator bacteria were grown overnight in LB medium, yeasts were grown in YPG medium (10 g/L yeast extract, 10 g/L peptone and

100 g/L glucose) for 24 h and fungi were grown in potato dextrose agar for 3–4 days. The MIC of compound (**1**) was expressed as μg/mL by the serial dilution method.

3.5. *Cytotoxic activity*

Cytotoxic activity for compound **1** was measured according to a reported method (Shehan & Storing 1990). The cells were seeded in 96-well microtitre plates at a concentration of $5 \times 10^4 - 10^5$ cell/well in a fresh medium and left to attach to the plates for 24 h. The cells were incubated with the appropriate concentration ranges of the drug, completed to total of 200 μL/well using fresh medium, and incubation was continued for 24, 48, 72 h. Control cells were treated with vehicle. After these, cells were fixed with 50 μL cold 50% trichloroacetic acid for 1 h at 4°C. Wells were washed five times with distilled water and stained for 30 min at room temperature with 50 μL 0.4% sulphorohodamine B dissolved in 1% acetic acid, and then four times with 1% acetic acid. The plates were air-dried, and the dye was solubilised with 100 μL/well of 10 mM Tris base (pH 10.5) for 5 min on an orbital shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter Tech. S960, Warminster, PA, USA). The percentage of cell survival was calculated as follows: survival fraction = OD (treated cells)/OD (control cells).

4. Conclusion

In conclusion, a new labdane diterpene was isolated from the hydroalcoholic extract of the leaves of *S. indica*. The structure of the isolated compound was identified as 6,9-epoxy marrubiinic acid based on spectroscopic analysis. The new compound showed potential antifungal and cytotoxic activities.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1 and S2.

References

Aguiar RM, David JP, David JM. 2005. Unusual naphthoquinones, catechin and triterpene from *Byrsonima microphylla*. Phytochemistry. 66:2388–2392.

Bauer AW, Kirby WM, Sherris C, Turck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 45:493–496.

Conolly JD, Hill RA. 1992. Dictionary of terpenoids. New York: Chapman and Hall. p. 4506.

Darwish FM, Khalifa AA. 1992. Phytochemical study of the constituents of *Saraca Indica* Linn. Bull Fac Sci Assiut Univ. 45:21–25.

Habib MR, Nikkon F, Rahman M, Haque ME, Karim MR. 2007. Isolation of stigmasterol and β-sitosterol from methanolic extract of root. Pak J Biol Sci. 10:4174–4176.

Hawas UW, Eltomy SA, AbouZid S, El-Hossary GA, Nassif RM. 2012. Lipid content and antimicrobial activity of some Egyptian Fabaceae (Leguminosae) plants. J Med Plants Res. 6(44):5604–5608.

Kashyapa K, Chand R. 2006. The useful plants of India. CSIR, New Delhi: National Institute of Science Communication and information Resources.

Kokate CK, Purohit AP, Gokhale SB. 2007. Pharmacognosy. 36th ed. Pune: Nirali, Prakashan.

Maruthappan V, Shree KS. 2010. Antiulcer activity of aqueous suspension of *Saraca indica* flower against gastric ulcers in albino rats. J Pharm Res. 3:17–20.

Meyre-Silva C, Yunes RA, Schlemper V, Campos-Buzzi F, Cechinel-Filho V. 2005. Analgesic potential of marrubiin derivatives, a bioactive diterpene present in *Marrubium vulgare* (Lamiaceae). Farmaco. 60:321–326.

Pradhan P, Joseph L, Gupta V, Chulet R, Arya H, Verma R, Bajpai A. 2009. A review on Ashoka. J Chem Pharm Res. 1:62–71.

Preethi F, Fernandes J, Pricilla K. 2010. Hypoglycaemic activity of *Saraca indica* Linn barks. J Pharm Res. 3:491–493.

Sadhu SK, Khatun A, Phattanawasin P, Ohtsuki T, Ishibashi M. 2007. Lignan glycosides and flavonoids from *Saraca asoca* with antioxidant activity. J Nat Med. 61:480–482.

Sainath RS, Prathiba J, Malathi R. 2009. Antimicrobial properties of the stem bark of *Saraca indica*. Eur Rev Med Pharmacol Sci. 13:371–374.

Satyavati GV, Prasad DN, Sen SP. 1970. Oxytocic activity of a pure phenolic glycoside from *Saraca indica* Linn (Ashoka): a short communication. Indian J Med Res. 58:660–663.

Sharma A, Gupta S, Sachan S, Mishra A, Banarji A. 2011. Anthelmintic activity of the leaf of *Saraca indica* Linn. Asian J Pharm Life Sci. 1:391–395.

Shehan P, Storing R. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst. 82:1107–1112.

Ye G, Huang C. 2006. Flavonoids of *Limonium aureum*. Chem Nat Compd. 42:232–243.

Zheng Z, Cheng K, Chao J, Wu J, Wang M. 2008. Tyrosinase inhibitors from paper mulberry (*Broussonetia papyrifera*). Food Chem. 106:529–535.