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Antifungal anthraquinones from *Saprosma fragrans*

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Abstract—A new 3,4-dihydroxy-1-methoxy anthraquinone-2-corboxaldehyde (**1**) together with a known anthraquinone, damnacanthal (**2**), were isolated from the chloroform fraction of the aerial part (whole plant without root) of *Saprosma fragrans*. The isolated anthraquinones (**1**) and (**2**) were found to exhibit antifungal activity against *Trichophyton mentagrophytes* and *Sporitrichum schenckii*. Their structures were established by chemical and spectral analysis.
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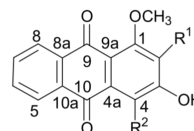
The clinical relevance of fungal diseases increases enormously due to the increasing of the immunocompromised host in the second half of the 20th century including individuals infected with HIV, transplant recipients and patients with cancer.[1,2] The fungal threat will continue to increase, as shown by the occurrence of aspergillosis in severe acute respiratory syndrome (SARS)[3] and by the inclusion of *Coccidioides immitis* as a potent agent of bioterrorism.[4] The crude mortality from opportunistic fungal infections still exceeds 50% in most human studies and has been reported to be as high as 95% in bone marrow transplant recipients infected with *Aspergillus* sp.[5] The adequate treatment of mycotic infections is difficult since fungi are eukaryotic organisms with a structure and metabolism that is similar to those of eukaryotic host. Furthermore, long-term treatment with commonly used antifungals, such as amphotericin-B, has toxic effects: ketoconazole, fluconazole and clotrimazole are limited in their spectrum and efficacy and use may result in strain resistance.[6,7] For this reason, there is a need for new active molecules which can serve as lead for further development in antifungal chemotherapy.

During our continuing efforts to identify antifungal leads from plant sources, *Saprosma fragrans*[8] was collected from Kerala (Silent Valley), India, on the western slope at the alt. 2000 ft. and identified by our Botany division of CDRI, Lucknow.

A preliminary biological screening of the ethanolic extract of the aerial part (whole plant without root) of this plant exhibited significant antifungal activity which was found to be localized in chloroform fraction. Further chromatographic purification leads to isolation of one novel 3,4-dihydroxy-1-methoxy anthraquinone-2-carboxaldehyde (**1**) along with a known anthraquinone (**2**). In this paper, we describe the isolation, structure elucidation and antifungal activity of 3,4-dihydroxy-1-methoxy anthraquinone-2-carboxaldehyde (**1**) and previously known anthraquinone, damnacanthal (**2**).



Compound	R^1	R^2
1	CHO	OH
1R	CH_2OH	OH
2	CHO	H

Air-dried plant material (10 kg) was pulverized and extracted with 95% ethanol (4× 15 L) for 24 h at room temperature. The whole extract was concentrated under reduced pressure by using rotatory evaporator at 50 °C and finally dried under vacuo to get solid mass coded as D002 (315 g). The crude ethanolic extract (145 g)

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was macerated with *n*-hexane to get *n*-hexane-soluble fraction (F003, 2.5 g). The insoluble residue was partitioned between chloroform and water to give chloroform-soluble fraction (F004, 40 g) and aqueous portion. The aqueous portion was then partitioned with *n*-butanol to give *n*-butanol-(F005, 63 g) and water-soluble fraction (F006, 39 g). All these fractions were submitted for evaluation of antifungal activity against human pathogenic fungi. The chloroform fraction (F004) showed significant antifungal activity. The fraction F004 (15 g) was subjected to column chromatography by using *n*-hexane containing increasing amount of acetone as an eluent to give a fraction F007 *n*-hexane–acetone (9:1 v/v) in which activity was localized. The fraction F007 (7 g) was further subjected to repeated column chromatography by using benzene and increasing amount of methanol as an eluent to give two compounds **1** and **2**. The compound **2** was identified as 3-hydroxy-1-methoxy anthraquinone-2-carboxaldehyde by comparison of their spectral data with those reported in the literature[9–11] and the structure of the newly isolated compound **1** was elucidated as 3,4-dihydroxy-1-methoxy anthraquinone-2-carboxaldehyde by their detailed spectral and chemical analysis. Compound **1** was obtained as yellowish amorphous powder and crystallized in methanol acetone mixture, mp 273–275 °C. In IR spectrum of the compound a strong absorption band for hydroxyl group was observed at 3400 cm^{-1}, other characteristic bands appeared at 1670, 1587 cm^{-1} due to anthraquinone nucleus[9,12,13] and at 1652 cm^{-1} and 1640 cm^{-1} due to the two chelated carbonyl groups. $ESMS^{(+ve)}$ displayed $[M+H]^+$ peak at 299 corresponding to the molecular formula $C_{16}H_{10}O_6$ and other fragment peaks at *m*/*z* 271 and 267 were observed after loss of CO and OCH_3 groups from the molecular ion. The ^1H NMR spectrum of the compound (Table 1) showed signal at δ 10.45 (1H, s) for aldehydic proton and two 1H singlets at δ 12.07 and 13.06 due to chelated hydroxyl protons. A multiplate at δ 7.77–7.84 (2H, m), signal at δ 8.17 (1H, d, J = 7.9 Hz), 8.24 (1H, d, J = 7.9 Hz) for the aromatic proton of the type 'AA′, BB″' confirmed the presence of the four aromatic proton.[13–15] Other signal in the ^1H NMR spectrum at δ 4.10 was assignable to the OCH_3 group. The aldehyde group of the compound **1** was reduced to CH_2OH with methanolic sodium borohydride to give compound **1R**.[16] The ^1H NMR spectrum of the compound **1R** having a 1H singlet at δ 13.06 due to the hydroxy group chelated with one of the carbonyl group of the anthraquinone nucleus revealing the position of one hydroxy group at C-4 of the anthraquinone nucleus. The signal at δ 12.07 which disappeared on reduction of compound **1** to compound **1R** thus belonged to the hydroxyl group chelated to the aldehyde group. It must be located at the carbon ortho to the aldehydic group. Moreover, the methoxy proton signal appeared somewhat down field (δ 4.10) from its normal position δ (3.50) indicating that methoxy group must be flanked by anthraquinone carbonyl and aldehydic groups. The ^{13}C NMR spectrum of the compound (Table 1) showed a total 16 carbons. Their multiplicity assignment was made by DEPT experiment. It revealed the presence of 1 methyl, 5 methine and 10 quaternary carbons. Out of these signals at δ 127.5, 134.25, 134.35 and 127.5 assigned to four aromatic methines, signals at δ 180.4 and 182.8 for two carbonyl groups of anthraquinone nucleus,[12] signal at δ 195 for aldehydic carbon, signals at δ 164.0, 164.5 and 164.09 to oxygenated aromatic carbons, signal at δ 119.0 to the aromatic quaternary carbon bearing aldehydic group and remaining signals at 117.0, 109.0, 134.0 and 133.0 to the aromatic quaternary carbons and the signal at δ 65.6 is due to methoxy carbon. All these spectral data suggested compound **1** to be 3,4-dihydroxy-1-methoxy anthraquinone-2-carboxaldehyde.

Table 1. ^1H and ^{13}C data of compound **1** in $CDCl_3$ (200 MHz)

Position	δ_H (*J* in Hz)	δ_C
1	—	164.0
2	—	119
3	—	164.5
4	—	164.09
5	8.24 (1H, d, J = 7.9 Hz)	127.5
6	7.77–7.84 (1H, m)	134.25
7	7.77–7.84 (1H, m)	134.35
8	8.17 (1H, d, J = 7.9 Hz)	127.5
9	—	180.4
10	—	182.8
9a	—	117
4a	—	109
8a	—	134
10a	—	133
CHO	10.45 (1H, s)	195
OCH_3	4.10 (3H, s)	65.6
OH	13.06 (1H, s)	—
OH	12.07 (1H, s)	—

The isolated anthraquinones exhibited prominent antifungal activity as compared to the activity profile of the reported anthraquinones.[17–19] Anthraquinone (**1**) exhibited antifungal activity with MIC values of 12.5 μg/ml against *Trichophyton mentagrophytes* and 25 μg/ml against *S. schenckii*. The MIC values observed were 1.56 μg/ml against *T. mentagrophytes* and 6.25 μg/ml against *Sporothrix schenckii* for anthraquinone **2**. The isolated compounds had better activity than ethanolic extract. The clotrimazole served as a standard antifungal drug having MIC value of 0.39 μg/ml against *T. mentagrophytes* and 1.56 μg/ml against *S. schenckii* in the same assay. Bioassay was performed by 2-fold serial dilution method in Sabouraud's dextrose broth. The spore suspension of 10^3 and 10^4 spores/ml was used for this purpose. The tests were performed at 28 °C and inhibition in growth of fungi, that is, MICs was recorded by visual observation after 24–72 h incubation.[20,21] The details in vitro activity profile of the ethanolic extract, fractions and the compounds **1** and **2** against five human pathogenic fungi are given in Table 2.

In conclusion, we have identified a novel naturally occurring anthraquinone along with a known one displaying promising antifungal activity in vitro. Further, synthesis of these naturally occurring anthraquinones will help in the study of SAR. This may provide a lead molecule in developing a potent antifungal drug.

Table 2. In vitro antifungal activity (minimum inhibitory concentration μg/ml) of ethanolic extract (D002), fractions (F003-007) and compounds **1** and **2** against human fungal pathogens

Code no.	Minimum inhibitory concentration (MIC) in μg/ml				
	Candida albicans	*Cryptococcus neoformans*	*Sporothrix schenckii*	*Trichophyton mentagrophytes*	*Aspergillus fumigatus*
D002	250	125	500	62.5	NA
F003	500	125	NA	62.5	500
F004	125	62.5	250	15.6	250
F005	250	125	NA	62.5	500
F006	250	125	NA	62.5	500
F007	62.5	62.5	62.5	5.6	125
Compound **1**	50	50	25	12.5	>100
Compound **2**	50	12.5	6.25	1.56	50
Clotrimazole	3.1	0.09	1.56	0.39	1.56

NA, Inactive at the tested concentration between 1.56–500 μg/ml.

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16. Reduction of compound **1**. The compound **1** (5 mg) was dissolved in ethanol (2 ml) and 5 mg $NaBH_4$ was added to the solution then stirred the reaction mixture at room temperature for 3 h and maintained the pH-5 of the reaction mixture with acetic acid and evaporated to dryness, then 2 ml water was added and extracted with chloroform. The chloroform layer gave the reduced product (**1R**). ^{1}H NMR ($CDCl_3$, 200 MHz) δ ppm: 13.06 (1H, s), 7.75–7.82 (2H, m), 8.13 (1H, d, $J = 7.9$ Hz), 8.23 (1H, d, $J = 7.9$ Hz), 4.0 (3H, s, OCH_3), 4.15 (2H, s, CH_2OH).
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