ORIGINAL ARTICLE

In vitro antimicrobial activities of anthrones from the leaf latex of *Aloe sinana* Reynolds

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Background: *Aloe sinana* Reynolds is endemic to Ethiopia where its leaf latex is traditionally used in and around the town of Debre Sina and other central highlands of the country for the treatment of various illnesses, including wound, snake bite and malaria. However, despite its use in traditional medicine, to date, there appears to have been no chemical or biological studies published on this plant. **Aim:** The aim of this study was to investigate the leaf latex of *A. sinana* for its antibacterial and antifungal activities, and to isolate and characterise the compounds that are responsible for the antimicrobial effect of the latex. **Materials and Methods:** The latex was extracted with methanol. Isolation of compounds was achieved by repeated preparative TLC, and spectroscopic techniques including ^{1}H, ^{13}C-NMR and MS were used for characterisation of the isolated compounds. Antimicrobial activity tests were performed against 21 bacterial and 4 fungal pathogens using the disc diffusion method. **Results and Conclusion:** Three compounds isolated from the leaf latex were identified as the anthrones, aloin, aloinoside and microdontin. Among the isolated compounds, aloinoside and microdontin were found to possess comparable activity (MIC 5 µg/ml) with that of ciprofloxacin against several Gram-negative bacterial strains and *Staphylococcus aureus*. Additionally, microdontin showed potent and comparable activity with the standard antifungal drug griseofulvin against *Penicillum* spp. These findings support the traditional uses of the plant for the treatment of various infections and wound.

Key words: *Aloe sinana*, anthrones, antibacterial, antifungal, disc diffusion, latex

INTRODUCTION

Infectious diseases remain key agents of the debilitating poverty afflicting so much of the world today. Each year, these diseases kill almost 9 million people, many of them are children under five, and they also cause enormous burdens through life-long disability.[1] The major contributing factor for these is the increase of drug resistance in human pathogenic microorganisms due to the indiscriminate use of antibiotics commonly used in the treatment of infectious diseases.[2] This has led to an urgent global call for new antimicrobial drugs, particularly from natural resources.

Medicinal plants represent a rich source of antimicrobial agents.[3] Many of the plant materials used in traditional medicines are readily available in rural areas at relatively cheaper prices than modern medicines.[4] Over 80% of the Ethiopian population depends on traditional medicine for their healthcare need.[5] Medicinal properties of plants are normally dependent on the presence of certain phytochemical principles such as alkaloids, anthraquinones, cardiac glycosides, saponins, tannins and polyphenols, which are the bioactive bases responsible for the antimicrobial property.[4]

The genus *Aloe* comprises about 600 species, most of which are native to South Africa, the Saudi Arabian Peninsula, and to many islands of the western Indian Ocean, including Madagascar.[6] Ethiopia has 40 species of *Aloe*, of these 20 species are endemic.[7] *Aloe sinana* Reynolds locally known as "Rate" is one of those endemic plants that grow on basaltic slope in Wollo and Debre Sina, North-eastern of Shewa floristic regions.[8] Local people use the leaves of this plant as a wound-healing agent, insecticide and for the treatment of snake bite and malaria.[9]

In the present study the leaf latex of *A. sinana* and three anthrones, namely microdontin, aloin and aloinoside isolated thereof have been investigated for their antimicrobial activities based on the ethnomedicinal application of the plant. To the best of our knowledge, no phytochemical or biological activity studies have been carried out on the leaf latex of *A. sinana*.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.126812

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Received: 04-10-2013; **Accepted:** 06-12-2013

MATERIALS AND METHODS

Plant Material

The leaf latex of *A. sinana* was collected in November 2011 from Debre Sina, Debub Wollo Zone in Amhara Region, about 150 km northwest of Addis Ababa. The authenticity of the plant material was confirmed by Professor Sebsebe Demissew, the National Herbarium, Department of Biology, College of Natural Sciences, Addis Ababa University, where herbarium specimen was deposited (collection number GM 02).

Bacterial Strains

Antimicrobial activity was determined against the following Gram-positive bacterial strains: *Bacillus pumillus* 82, *B. subtilis* ATCC 6633 and *S. aureus* ML 267 and Gram-negative bacterial strains: *Escherichia coli* K99, *E. coli* K88, *E. coli* CD/99/1, *E. coli* LT37, *E. coli* 306, *E. coli* 872, *E. coli* ROW 7/12, *E. coli* 3:37C, *Salmonella enterica* TD 01, *S. typhi* Ty2, *Shigella boydii* D13629, *S. dysentery* 8, *S. flexneri* Type 6, *S. soneii* 1, *Vibrio cholerae* 85, *V. cholerae* 293, *V. cholerae* 1313 and *V. cholerae* 1315. All the bacterial strains were procured from the Department of Technology, Jadavpur University; Central Drugs Laboratory, Kolkata and the Institute of Microbial Technology, Chandigarh, India. The stains were first checked for purity on the basis of standard microbiological, cultural and biochemical tests and then used for their sensitivity towards the test samples.

Fungal Strains

Antifungal activity testing was carried out on the following fungal pathogens: *Aspargillus niger* ATCC 6275, *Candida albicans* ATCC 10231, *Penicillum funiclosum* NCTC 287 and *P. notatum* ATCC 11625. All the fungal strains were procured from Central Drugs Laboratory, Kolkata, India.

Experimental Animals

Healthy male Swiss albino mice (weighing 20-30 g and age of 8-12 weeks) were obtained from the animal house of the Ethiopian Health and Nutrition Research Institute, and School of Pharmacy, Addis Ababa University. Animals were housed in polypropylene cages (6-10 animals per cage), maintained under standard condition (12 h light and 12 h dark cycle; 25 - 30°C) and allowed free access to pellet diet and water *ad libitum*. After randomised grouping and before initiation of the experiment, animals were acclimatised to the laboratory conditions. All procedures complied with the Guide for the Care and Use of Laboratory Animals[10] and approved by the Institutional Review Board of the School of Pharmacy, Addis Ababa University.

Spectroscopic Analysis

UV spectra were recorded on Shimadzu UV 1800 spectrophotometer (200-400 nm) at room temperature. IR spectra were determined on a Perkin-Elmer BX (400-4000 cm^{-1}) in KBr pellets. ESI-MS were run on Ultimate 3000 LC-MS. The measurement was carried out by an electrospray ionisation method with negative mode. The source voltage and temperature were fixed at 3 kV and 250°C. NMR spectra were recorded on Bruker Avance DMX 400 FT-NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature using deuterated methanol (CD_3OD). Signals were referred to an internal standard, tetramethylsilane (TMS).

Collection of Latex

Latex was collected by cutting the leaves transversally near the base and inclining it on a stainless tray. The water was allowed to evaporate upon leaving the latex in open air for 2 days, which yielded a reddish dark powder.

Isolation of Compounds

The latex was dissolved in methanol and applied directly to preparative thin layer chromatography (PTLC) (20 × 20 cm) over silica gel of 0.5-mm thickness. A mixture of chloroform and methanol (4:1) was used for isolation of compounds using PTLC. The isolated compounds were further purified by repeated 0.25-mm thick chromatographic plates. The bands were scraped off, washed with methanol and ethyl acetate (1:1) and filtered. Three yellow amorphous compounds with an R_f values of 0.15, 0.36 and 0.57 were isolated.

Aloin [Figure 1]: Yellow amorphous powder; 28.4% (w/w); TLC: R_f = 0.36; UV: λ_{max} (methanol) 208, 299, and 357 nm; IR ν cm^{-1} 3447, 1631 and 1618; -VE- ESI – MS *m/z*: 417 $[M - H]^-$ indicating a relative molecular weight (M_r) of 418 ($C_{21}H_{22}O_9$). ^{1}H and ^{13}C NMR [Table 1].

Aloinoside [Figure 1]: Yellow amorphous solid; 14.1% (w/w); TLC: R_f = 0.15; UV: λ_{max} (methanol) 211, 222, 298 and 357 nm; IR ν cm^{-1} 3394, 1634, 1618, 1451, 1288 and 1076; -VE- ESI – MS *m/z*: 563 $[M - H]^-$ indicating a relative molecular weight (M_r) of 564 ($C_{27}H_{32}O_{13}$). ^{1}H and ^{13}C NMR [Table 1].

Microdontin [Figure 1]: Yellow powder; 15.0% (w/w); TLC: R_f = 0.57; UV: λ_{max} (methanol) 302 and 311 nm; IR: ν cm^{-1} 3415, 1709, 1297, 1168, 1603 and 1453; -VE- ESI – MS *m/z*: 563 $[M - H]^-$, indicating a relative molecular weight (M_r) of 564 ($C_{30}H_{28}O_{11}$). ^{1}H and ^{13}C NMR [Table 1].

Acute Oral Toxicity Test

Female Swiss albino mice were used for acute oral toxicity study. Oral toxicity study was conducted as per the internationally accepted protocol drawn under OECD guidelines 425.[11] For each test sample, 10 mice were used and randomly divided into two groups of five mice per cage. Before oral administration of a single dose of the

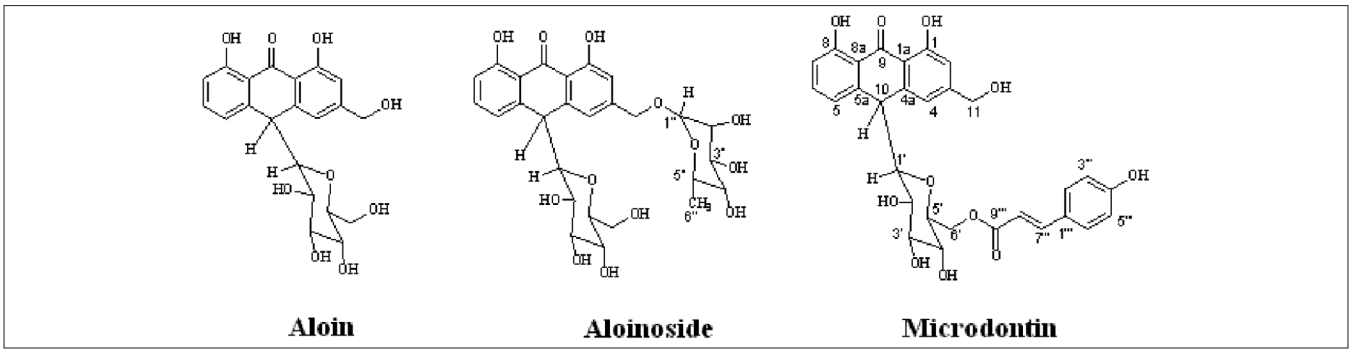

Figure 1: Structures of compounds isolated from the leaf latex of *Aloe sinana*

Table 1: ^{1}H and ^{13}C NMR spectral data of aloin, aloinoside and microdontin isolated from the latex of *Aloe sinana*

	Aloin (δ, ppm)		Aloinoside (δ, ppm)		Microdontin (δ, ppm)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	11.97	161.95	12.19	160.5	12.01	163.78
2	6.88	113.02	6.8	113.7	6.91	114.21
3	-	150.11	-	145.9	-	152.43
4	7.04	117.75	7.0	117.7	7.28	118.8
5	7.06	118.59	6.9	118.5	7.09	121.1
6	7.5	135.66	7.4	136.1	7.5	137.1
7	6.87	115.42	7.1	115.3	6.91	117.3
8	11.96	161.49	11.9	161.3	12.17	163.67
9	-	194.1	-	195.2	-	194.8
10	4.58	44.48	4.6	45.3	4.55-4.59	46.5
1a	-	117.75	-	114.6	-	116.68
4a	-	141.84	-	141.7	-	143.81
5a	-	145.15	-	146.4	-	143.53
8a	-	117.23	-	116.9	-	117.4
11	4.67	63.12	4.8	67.7	4.69-4.74	64.5
Glu-1'	3.41	85.21	3.3	77.9	3.92	85.18
2'	3.02	70.56	3.0	70.4	4.36	72.9
3'	3.28	78.56	3.0	79.6	4.05	78.3
4'	2.91	70.45	2.9	70.0	3.11	71.5
5'	2.93	80.26	2.7	85.1	3.33	81.8
6'a	3.36	61.82	3.4	61.85	3.67	63.15
6'b	3.56		-		3.87	
Rha-1''	-	-	4.9	100.7	-	-
2''	-	-	3.9	70.6	-	-
3''	-	-	3.9	72.1	-	-
4''	-	-	4.0	68.8	-	-
5''	-	-	4.5	85.0	-	-
6''	-	-	1.2	17.8	-	-
1'''	-	-	-	-	-	127.24
2'''	-	-	-	-	7.36	131.31
3'''	-	-	-	-	6.82	116.81
4'''	-	-	-	-	-	161.25
5'''	-	-	-	-	6.82	116.81
6'''	-	-	-	-	7.36	131.31
7'''	-	-	-	-	7.24	146.81
8'''	-	-	-	-	5.92	114.67
9'''	-	-	-	-	-	167.73

NMR – Nuclear magnetic resonance

test samples, the mice were deprived from food for 3 h. Doses of 2,000 and 5,000 mg/kg of the test samples were given using oral gavage to mice of Group I and Group II, respectively. The samples were dissolved in distilled water or in a mixture of distilled water: ethanol: tween-80 in the ratio of 90:3:7.

The mice were observed continuously for the first 30 min after administration of the test sample; intermittently for 4 h, over a period of 24 h and for 14 days. Gross behavioural changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality and other signs of toxicity manifestation have been noted, if any.[12]

Antibacterial Activity Test

The zone of inhibition produced by the test samples was determined and compared with that of ciprofloxacin by a disc diffusion method.[13] Two sets of dilution of 200 µg/ml, each of the test samples dissolved in dimethyl sulphoxide (DMSO) and ciprofloxacin (dissolved in sterile distilled water) were prepared in sterile McCartney bottles. Serial nutrient agar plates were prepared and incubated at 37°C for 24 h to check for any sort of contamination. Sterile filter paper discs (Whatman no. 1) of 6-mm diameter were soaked in a stock solution (200 µg/ml) of test samples and placed in appropriate position on the surface of the flooded plate seeded with 24-h-old culture grown on nutrient broth, marked as quadrant at the back of the Petri dishes. The Petri dishes were then incubated at 37°C for 24 h and the diameter of zone of inhibition were measured in millimetres. A similar procedure was adopted for the pure ciprofloxacin and zone of inhibition was compared accordingly. DMSO was used as a negative control, while ciprofloxacin was employed as a positive control.

Antifungal Activity Test

The antifungal potential of the test samples (2000 µg/ml) was evaluated by disc diffusion method (as described for the determination of antibacterial activity) against the fungal pathogens on Saborauds dextrose media. The antifungal agent, griseofulvin was used as a reference standard.

Determination of Minimum Inhibitory Concentration

MICs of the latex and isolated compounds were determined by the method described by Hecht *et al*.[13] Briefly, nutrient agar and Saborauds dextrose agar were used for bacterial and fungal growth, respectively. Broth containing varying concentrations of the test samples ranging from 5 to 800 µg/ml for antibacterial activity testing, and 50 – 2000 µg/ml for evaluation of antifungal activity were prepared. DMSO was used to dissolve the latex and the isolated compounds. A sterility control was also carried out (growth control contained nutrient broth plus DMSO, without antimicrobial substances). Each test and growth control well was incubated at 37°C for bacteria and 25°C for fungi.

RESULTS AND DISCUSSION

Acute Toxicity

No mortality was observed in mice upon oral administration of the latex of *A. sinana* and isolated compounds, up to the highest dose of 5000 mg/kg, signifying that the oral LD_{50} of the test substances is greater than 5000 mg/kg. However, minor signs of toxicity such as temporary hair erection and diarrhea were observed in two of the experimental animals. Toxicity is the main concern of indigenous therapeutic preparations.[14] The fact that changes in general behaviour, effect on body weight and mortality, which are critical for the evaluation of adverse effects, were not evident on the test animals is a good evidence for the absence of toxicity. This fulfills the criteria set by OECD guideline[11] for lack of acute toxicity. Therefore, the latex and compounds can be considered safe in mice as they did not show signs of acute toxicity within 24 h at dose levels up to 5000 mg/kg body weight.

Antibacterial Activity

The *in vitro* antibacterial activity of the latex and anthrones isolated from *A. sinana* against 21 strains was assessed by using the disk diffusion method. The latex exhibited potent inhibitory effect against the tested bacterial pathogens at a concentration of 200 µg/ml [Table 2]. The Gram-negative bacteria including all strains of *E. coli*, *S. typhi*, *Shigella* spp. and *V. cholerae* were found to be the most inhibited bacterial pathogens by the latex. The latex also showed strong

Table 2: Zone of inhibition and minimum inhibitory concentrations of latex and compounds isolated from *Aloe sinana*

Bacterial strain	Zone of inhibition in mm (200 µg/ml)[a]					MIC (µg/ml)			
	Latex[b]	Microdontin[b]	Aloin[b]	Aloinoside[b]	Cipro	Latex	Microdontn	Aloin	Aloinoside
Bacillus pumilus 82	12.0 (63.2)	9.5 (50)	10.0 (52.6)	6.0 (31.58)	19.0	50	200	200	NA
B. subtilis ATCC 6633	11.0 (61.1)	9.5 (52.8)	10.0 (55.6)	6.0 (33.3)	18.0	50	200	200	NA
Escherichia coli CD/99/1	15.5 (91.2)	17.0 (100)	15.5 (91.2)	15.5 (91.2)	17.0	10	5	10	10
E. coli K88	15.0 (88.2)	15.5 (91.2)	15.0 (88.2)	15.0 (88.2)	17.0	10	5	10	10
E. coli K99	15.5 (96.9)	16.0 (100)	15.5 (96.9)	15.5 (96.9)	16.0	10	5	10	10
E. coli LT37	14.5 (90.6)	15.0 (93.8)	14.5 (90.6)	14.5 (90.6)	16.0	10	5	10	10
E. coli ROW 7/12	14.5 (87.9)	14.5 (87.9)	14.5 (87.9)	14.5 (87.9)	16.5	10	5	10	10
E. coli 3:37C	14.5 (93.5)	14.5 (93.5)	14.5 (93.5)	14.5 (93.5)	15.5	10	5	10	10
E. coli 306	15.0 (90.9)	15.5 (93.9)	15.0 (90.9)	15.0 (90.9)	16.5	10	5	10	10
E. coli 872	14.5 (90.6)	15.0 (93.8)	14.5 (90.6)	14.5 (90.6)	16.0	10	5	10	10
Salmonella typhi Ty2	14.0 (87.5)	14.0 (87.5)	14.0 (87.5)	14.0 (87.5)	16.0	10	10	10	10
S. enterica TD 01	12.5 (65.8)	12.5 (65.8)	12.5 (65.8)	12.5 (65.8)	19.0	25	100	50	25
Shigella boydii D13629	17.5 (87.5)	16.5 (82.5)	16.5 (82.5)	17.5 (87.5)	20.0	5	10	10	5
S. dysentery 8	15.5 (73.8)	15.5 (73.8)	15.5 (73.8)	15.5 (73.8)	21.0	5	10	10	5
S. flexneri Type 6	17.5 (85.4)	16.5 (80.5)	16.5 (80.5)	17.5 (85.4)	20.5	5	10	10	5
S. soneii 1	17.0 (87.2)	16.0 (82.1)	16.0 (82.1)	17.0 (87.2)	19.5	5	10	10	5
Staphylococcus aureus ML267	14.5 (80.6)	17.0 (94.4)	14.5 (80.6)	16.5 (91.7)	18.0	25	5	25	5
Vibrio cholerae 85	14.5 (80.6)	16.5 (91.7)	14.5 (80.6)	14.5 (80.6)	18.0	10	5	10	10
V. cholerae 293	15.0 (85.7)	16.0 (91.4)	15.0 (85.7)	15.0 (85.7)	17.5	10	5	10	10
V. cholerae 1313	15.5 (91.2)	16.5 (97.1)	15.5 (91.2)	15.5 (91.2)	17.0	10	5	10	10
V. cholerae 1315	15.0 (83.3)	17 (94.4)	15.0 (83.3)	15.0 (83.3)	18.0	10	5	10	10
Fungal strain	**Zone of inhibition in mm (2000 µg/ml)[a]**								
	Latex[c]	Microdontin[c]	Aloin[c]	Aloinoside[c]	Gris	Latex	Microdontin	Aloin	Aloinoside
Aspergillus niger ATCC 6275	11.0 (73.3)	13.0 (86.7)	12.5 (83.3)	11.5 (76.7)	15.0	800	800	800	1000
Candida albicans ATCC 10231	11.5 (71.9)	14.0 (87.5)	13.5 (84.4)	12.5 (78.1)	16.0	800	800	800	800
Penicillum funiculosum NCTC 287	9.5 (67.9)	13.5 (96.4)	12.0 (85.7)	11.0 (78.6)	14.0	1500	400	800	1000
P. notatum ATCC 11625	9.5 (70.4)	13.0 (96.3)	12.0 (88.9)	11.0 (81.5)	13.5	1500	400	800	1000

[a]Zones of inhibition measured including 6 mm diameter of the disc (*n*=3). [b]Figures in parenthesis indicates % activity of the test samples compared with that of ciprofloxacin. [c]Figures in parenthesis indicate % activity of the test samples compared with that of griseofulvin . Cipro – Ciprofloxacin; Gris – Griseofulvin; NA – No activity

antibacterial activity against the Gram-positive bacteria, *S. aureus*. In general, the activity of the latex was comparable to that of the standard ciprofloxacin on most of tested bacterial pathogens.

Owing to the promising antibacterial effects of the latex, further phytochemical investigation was carried out, which led to the isolation of three anthrones identified as microdontin, aloin and aloinoside by comparing their UV, IR, ESI-MS, ^{1}NMR and ^{13}C NMR data [Table 1] with those reported in the literature.[15,16]

The isolated compounds showed broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. As shown in Table 2, strong activity was observed against the different strains of *E. coli*, *S. typhi* Typ 2, *Shigella*, *S. aureus* and *V. cholerae*, which was comparable to ciprofloxacin. The effect of all the isolated compounds was rather moderate against *S. enterica* TD 01 and weak against *Bacillus* species. According to Asamenew *et al*.[17] aloin which was isolated from *A. harlana* also showed activity against *E. coli*, *S. typii* Ty2, *Shigella* and *V. cholera* bacterial strains. *B. pumilus* and *B. subtillis* were found to be the most resistant bacterial strains to aloinoside, whereas microdontin and aloin showed weak activity against these bacterial strains with MIC values of 200 µg/ml. All strains of *V. cholerae* employed in this study were susceptible to aloinoside (MIC = 10 µg/ml). Moreover, *E. coli* which can cause a spectrum of disease ranging from diarrhea to the life threatening disease called hemolytic uremic syndrome (HUS),[18] was also the most inhibited bacterial pathogene by aloinoside with MIC value of 10 µg/ml. To the best of our knowledge, there has been no report about the antimicrobial activity of microdontin and aloinoside prior to our study. Therefore, the present results suggest the potential of these compounds as antimicrobial agents.

Literature survey indicates that, anthraquinones isolated from the exudates of *A. vera* possess antibacterial activity. According to Wang *et al*.[19] the presence of functional groups such as carboxyl, hydroxyl and hydroxylmethyl on phenyl ring of anthraquinones could improve the antimicrobial activity. These compounds exert their action by increasing membrane permeability of the bacterial cell wall and cause leakage of intracellular contents and lead to cell death.[20,21] Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes. Therefore, one possible mechanism of action of the isolated compounds on the employed bacterial strains might be their specific properties on microorganism's cell wall integrity or by some other means yet to be determined.

The widely spread of resistance to antibiotics is promoting resurgence in the search of antimicrobial agents for the treatment of diseases caused by bacteria.[22] Therefore, the *in vitro* activity of the test samples on the above disease causing bacterial strains is highly significant. However, *in vivo* antibacterial and toxicity studies have to be carried out before the compounds are considered for use in the fight against bacterial infection.

Antifungal Activity

The latex and the isolated compounds of *A. sinana* also showed variation in the level of activity against the four human pathogenic fungal strains tested [Table 2]. The latex exhibited weak activity against *A. niger* and *C. albicans* with MIC value of 800 µg/ml, while even less activity was observed against the two *Penicillum* species namely, *P. funiculosum* and *P. notatum* with MIC = 1500 µg/ml. *P. funiculosum* and *P. notatum* were found to be susceptible to microdontin, while *A. niger* and *C. albicans* were weakly susceptible to this compound. Aloin showed weak activity against all the fungal strains tested (MIC = 800 µg/ml). This compound, which was previously isolated from *A. ferox* has been reported to be active against *C. albicans*.[23] Aloinoside showed weak activity against *C. albicans*, while *A. niger*, *P. funiculosum* and *P. notatum* were even less sensitive to this compound.

CONCLUSIONS

The reputed antimicrobial effect of the leaf latex of *A. sinana* may be attributed in whole or in part due to the synergetic effects of the anthrones that occur in the plant. The genuine broad antimicrobial activity of the latex and its compounds along with the safety profile observed in the present study could make *A. sinana* a readily available natural antimicrobial agent, and also provide scientific support for the ethnomedicinal use of the plant. Needless to say that further *in vivo* experiments have to be carried out in order to evaluate the possible therapeutic value of the plant.

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How to cite this article: Minale G, Bisrat D, Asres K, Mazumder A. *In vitro* antimicrobial activities of anthrones from the leaf latex of *Aloe sinana* Reynolds. Int J Green Pharm 2014;8:7-12.

Source of Support: Nil, **Conflict of Interest:** None declared.