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ANTIMICROBIAL CONSTITUENTS FROM THE LEAF LATEX OF *ALOE PULCHERRIMA* GILBERT & SEBSEBE

Tekleab Teka[1], Daniel Bisrat[1], Avijit Mazumder[2], Kaleab Asres[*1]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

[2]Depatrement of Pharmaceutical Technology, Noida Institute of Engineering and Technology, Institutional Area, Noida, India.

ABSTRACT

The loss of effectiveness of chemotherapy constitutes the greatest threat to the control of microbial diseases due to rapidly evolving crisis of antibiotic resistance and this has contributed to the rise of patient morbidity. In Ethiopian traditional medicine, the endemic plant *Aloe pulcherrima* Gilbert & Sebsebe is used for the treatment of various infectious diseases. In our continuing search for antimicrobial agents from plants, we have investigated the leaf latex of this plant. The results revealed that the latex possesses varying degrees of antimicrobial activity when tested against 21 bacterial and 4 fungal strains using the disk diffusion method. Further analysis of the latex using preparative thin layer chromatography (PTLC) resulted in the isolation of two major compounds identified as 7-hydroxyaloin (**1**) and nataloin (**2**) on the basis of spectroscopic methods including HR-ESIMS, ^{1}H and ^{13}C-NMR spectral data. The latex and isolated compounds showed comparable activity with that of ciprofloxacin against most of the tested bacteria but highest effect was observed against the different *Vibrio cholerae* strains tested (MIC: 10 µg/ml). In general, the activity of the test substances on the fungal pathogens tested was relatively weaker with the exception of nataloin which showed comparable activity (MIC: 400 µg/ml) with that of the griseofulvin against *Candida albicans* and *Penicillium* spp. The present findings support the traditional use of the plant for the treatment of infectious diseases.

Key words: 7-Hydroxyaloin, *Aloe pulcherrima*, Antimicrobial activity, Disk diffusion, Nataloin.

INTRODUCTION

Diseases due to pathogenic bacteria and fungi represent a critical problem to human health and they are one of the main causes of morbidity and mortality worldwide (Shahzad *et al*., 2009). The challenge associated with fighting these diseases has become an increasingly complex one, because of the fast development of resistance to the classic antibiotics; the evolution of multiple drug resistant human pathogenic microorganisms and the changing nature of the infections observed in the elderly and other immune-compromised patients (Saify *et al*., 2005; Shahzad *et al*., 2009).

Corresponding Author

Kaleab Asres
Email: kasres@gmail.com

This has driven the search for new sources of antimicrobial substances mainly from medicinal plants. Plants constitute a potentially useful resource for new and safe drugs for the treatment of microbial infections and other diseases (Moshi *et al*., 2009). A substantial number of new antibiotics introduced on the market are obtained from natural or semisynthetic resources (Thiruppathi *et al*., 2010). The genus *Aloe* is the largest genus in the family Asphodelaceae, which is represented by 600 species and subspecies (Cousins SR and Witkowski, 2012; Kawai *et al*., 1993). Aloes are native to sub-Saharan Africa, the Saudi Arabian Peninsula, and to many islands of the western Indian Ocean, including Madagascar (Cousins and Witkowski, 2012; Ndhlala *et al*., 2009; Wabuyele and Kyalo, 2008). About 46 species of *Aloe* are known so far in Ethiopia and Eritrea with a

high proportion of endemics (Demissew *et al*., 2011). *Aloe pulcherrima* is one of the endemic *Aloe* species of Ethiopia which is traditionally used for the treatment of various infectious diseases in central and northern part of Ethiopia, such as Wollo, Gojam and Shewa around Debrelibanos Monastery (Teka, 2013).

In the present study, the leaf latex of *A. pulcherrima* and two major constituents, namely 7-hydroxyaloin (**1**) and nataloin (**2**), isolated thereof have been investigated for their antimicrobial activities using the disk diffusion method.

MATERIALS AND METHODS

Plant material

The latex of *A. pulcherrima* was collected in October 2011 from Debrelibanos Monastry around Amanuel church, 100 km Northwest of Addis Ababa, Central Ethiopia. The identity of the plant was authenticated by Professor Sebsebe Demissew, at the National Herbarium, Department of Biology, Addis Ababa University, where a voucher specimen was deposited (collection number TT001).

Bacterial strains

In vitro antimicrobial activity was determined against the following Gram-positive bacterial strains: *Bacillus pumilus* 82, *B. subtilis* ATCC 6633 and *Staphylococcus aureus* ML 267. The Gram-negative bacterial strains used were: *Escherichia coli* K99, *E. coli* K88, *E. coli* 306, *E. coli* LT37, *E. coli* 872, *E. coli* ROW 7/12, *E. coli* 3:37C, *E. coli* CD/99/1, *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 Institute of Microbial Technology, Chandigarh, India. The strains 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 tests were carried out on the following fungal pathogens: *Aspergillus niger* ATCC 6275, *Candida albicans* ATCC 10231, *Penicillium funiculosum* (NCTC 287 and *P. notatum* ATCC 11625. All the fungal strains were procured from the Central Drugs Laboratory, Kolkata, India.

Extraction of the latex

Latex was collected by cutting the leaves transversally near the base and arranging the leaves concentrically around a depression in the soil, which was covered with a plastic sheet. It was then left in open air for 3 days to allow evaporation of water, which yielded a dark purple powder.

Spectroscopic analysis

NMR spectra were recorded on Bruker Avance DMX400 FT-NMR Spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature using deuterated methanol. Signals were referred to an internal standard tetramethylsilane (TMS). Chemical shifts are reported in δ units and coupling constants (*J*) in Hz. HR-ESIMS were recorded on LTQ-XL mass spectrometer (Thermo Scientific); Electrospray ionization (ESI) negative mode; capillary temp 330 °C; source temp. 250 °C; sheath gas flow 50 arb, auxiliary gas 10 arb; source voltage 3 kV; capillary voltage -16V; normalized collision energy 35%; 4 scan events starting with full scan 50-1500 amu. Scan events 2-4 were dependent scans taking the most intensive ion from the precursor spectrum.

Isolation of compounds

The latex was initially dissolved in methanol and directly applied to preparative thin layer chromatographic (PTLC) plates over silica gel (Merck, G 6; 20 x 20 cm). The chromatograms were then developed in a solvent system of chloroform and methanol mixture (4:1), and visualized under UV light of 254 and 366 nm.

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. Two yellow amorphous compounds **1** and **2** with R_f values of 0.34 and 0.54 ($CHCl_3$/MeOH; 4:1) were isolated.

7-Hydroxyaloin (1) A pale yellow amorphous substance; –VE HRESI-MS m/z: 433.11357 $[M-H]^-$ (calc. for $C_{21}H_{22}O_{10}$: 433.113475 $[M-H]^-$); UV λ_{max} nm: 225, 257, 275, 305, 355. ^{1}H-NMR (DMSO) δ:2.90-3.62 (6H, m, H-2'-H-6'), 3.15 (1H, brs, H-1'), 4.51 (1H, brs, H-10), 4.66 (2H, brs, H_2-15), 6.88 (1H, brs, H-2), 7.04 (1H, brs, H-4), 7.06 (1H, d, J =7.6 Hz, H-6), 7.34 (1H, d, J =7.6 Hz, H-5), 7.95 (1H, brs, 7-OH), 11.96 (1H, brs, 8-OH), 11.97 (1H, brs, 1-OH). ^{13}C-NMR (DMSO) δ:43.83 (C-10), 61.85 (C-6'), 63.12 (C-15), 70.44 (C-4'), 70.54 (C-2'), 78.57 (C-1'), 80.06 (C-3'), 85.14 (C-5'), 112.67 (C-4), 116.24 (C-14), 116.36 (C-5), 117.56 (C-11), 119.40 (C-6), 120.14 (C-2), 131.23 (C-12), 142.60 (C-13), 144.35 (C-7), 145.14 (C-8), 150.88 (C-3), 161.72 (C-1), 194.59 (C-9).

Nataloin (2) Yellow amorphous powder; –VE HRESI-MS m/z: 417.11879 $[M-H]^-$ (calc. for $C_{21}H_{22}O_9$: 417.118560 $[M-H]^-$; ^{1}H-NMR (DMSO) δ:2.38 (3H, s, H_3-15), 2.91-3.57 (6H, m, H-2'-H-6'), 3.27 (1H, brs, H-1'), 4.46 (1H, brs, H-10), 6.68 (1H, brs, H-4), 6.87 (1H, brs, H-2), 6.92 (1H, d, J =8.0 Hz, H-6), 7.04 (1H, d, J =8.0 Hz, H-5), 7.91 (1H, brs, 7-OH), 11.91 (1H, brs, 8-OH), 11.92

(1H, brs, 1-OH). ^{13}C-NMR (DMSO): δ:20.80(C-15), 43.63(C-10), 61.86(C-6'), 70.48(C-4'), 70.62(C-2'), 78.58(C-1'), 80.08(C-3'), 85.26(C-5'), 115.16(C-4), 117.50(C-14), 117.56(C-5), 119.34(C-11), 119.62(C-2), 120.01(C-6), 131.18(C-12), 144.32(C-13), 145.95(C-7), 147.69(C-8), 149.75(C-3), 161.58 (C-1), 194.50(C-9).

***In vitro* antibacterial activity test**

Inhibition zone produced by the test samples was determined and compared with that of ciprofloxacin by a disc diffusion method as described by Mitchell & Carter (2000). Two sets of dilution of 200 µg/ml each of the test samples dissolved in dimethyl sulfoxide (DMSO), and ciprofloxacin (dissolved in sterile distilled water) were prepared in sterile McCartney bottles. Any contamination was checked by preparing serial nutrient agar plates and incubating at 37°C for 24 h. Sterile filter paper discs (Whatman no. 1) of 6 mm diameter were soaked in stock solution (200 µg/ml) of test samples and placed in an appropriate position on the surface of the flooded plate seeded with 24 h old culture grown on nutrient broth, marked as quadrant on the back of the Petri dishes. The Petri dishes were then incubated at 37°C for 24 h and the diameters of the zones of inhibition were measured in mm. A similar procedure was adopted for pure ciprofloxacin and the corresponding zone of inhibition was compared accordingly. DMSO was used as a negative control.

***In vitro* antifungal activity test**

The antifungal potential of the test samples (2000 µg/ml) was evaluated by using the disc diffusion method (as described for the determination of antibacterial activity) on Saborauds dextrose media. The Petri dishes were incubated at room temperature for 3 days and the diameter of zone of inhibition was measured in mm. Griseofulvin was used as a reference standard.

Determination of minimum inhibitory concentrations (MICs)

MICs of the test substances were determined using the method described by Hecht *et al*. (2007). Nutrient agar and Saborauds dextrose agar were used for bacterial and fungal growth, respectively. Broth was prepared containing different concentrations of the test samples ranging from 5 to 800 µg/ml for antibacterial activity testing, and 50 to 2000 µg/ml for antifungal activity testing. DMSO was used to dissolve the test substances. A sterility control, growth control containing nutrient broth plus DMSO, without antimicrobial substances was also carried out. Each test and growth control well was incubated at 37 °C (for bacteria) and 25 °C (for fungi).

RESULTS AND DISCUSSION

As per our on-going search for new antimicrobial agents from plants the leaf latex of *A. pulcherrima* was screened for its antimicrobial activity against 21 bacterial and 4 fungal strains using the disc diffusion method. The latex displayed a potent inhibitory effect against the majority of the tested bacterial pathogens at a concentration of 200 µg/ml (Table 1). The Gram negative bacterial strains particularly *E. coli* and *V. cholera* were found to be highly susceptible to the latex. The activity of the latex against these strains was comparable to that of standard drug ciprofloxacin. The remaining Gram negative bacteria including *Salmonella* and *Shigella* spp. have also shown good sensitivity to the latex. The latex exhibited the lowest MIC value (10 µg/ml) against *V. cholera* strains, while the value against most of the other Gram negative bacteria was 25 µg/ml. Interestingly, the latex showed potent inhibitory effect against the resistant Gram positive bacterial strain *S. aureus*, while its effect against the two *Bacillus* strains was rather moderate. Previous reports indicate that the leaf latex of some *Aloe* spp possesses significant antibacterial activities against both Gram positive and Gram negative bacterial species (Asamenew *et al*., 2011).

Owing to its promising activity, the latex was further subjected to PTLC, which led to the isolation of two major anthrones identified as 7-hydroxyaloin (**1**) nataloin (**2**) (Fig. 1). The spectral data (UV, IR, MS, ^{1}H and ^{13}C NMR) of the isolated compounds were consistent with those reported in the literature (Dagne and Alemu, 1991).

The isolated compounds showed broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Their activity was potent particularly against *E. coli* and *V. cholerae* strains, which was comparable to that of ciprofloxacin. It is interesting to note that the zones of inhibition displayed by nataloin on two strains of *E. coli* were equal to that of the standard drug. The effect of nataloin against the resistant Gram positive bacterium, *S. aureus* was also very high. In general, the activity patterns of the latex and isolated compounds appear to be similar in that they are more active against the Gram negative bacteria rather than the Gram positive ones, indicating that the antibacterial effect of the latex is largely due to the presence of these compounds.

Generally, Gram negative bacteria are more resistant to antimicrobial agents compared with the Gram positive bacteria as they are covered with a phospholipid membrane carrying the structural lipopolysaccharide component that makes their cell wall impermeable to antimicrobial substances (Dahl *et al*., 1989; Patrone and Stein, 2007). However, it is not uncommon for natural products to be more potent against Gram-negative bacteria (Mazumder *et al*., 2004). A possible explanation for this could be that the isolated anthrones may cause

dissolution of the fatty layer of the Gram negative microbes or drug resistant plasmids maybe present in the tested Gram positive strains but not in the Gram negative bacteria.

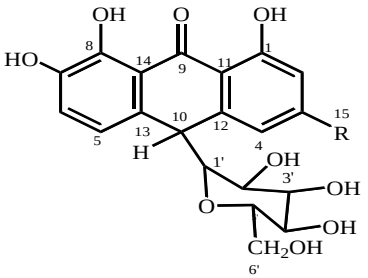
The potent action of the test substances against the Gram negative bacteria such as *Shigella* spp. and *V. choleae* is highly significant since these are among the most dangerous bacterial strains which are associated with diseases that frequently cause severe diarrhea in both man and animals. For example, in its severe form cholera can result in hypovolemia which may lead to shock and death if untreated and similarly, shigellossis or bacillary dysentery caused by *Shigella* spp. can be life threatening particularly in populations characterized by poverty (Balows and Hausler, 1981). The fact that the test substances showed comparable *in vitro* activity with that of ciprofloxacin against the above bacterial strains cannot be overemphasized when it is well known that currently ciprofloxacin is the drug of choice for travelers' diarrhoea and shigellosis. Despite being a normal inhabitant of the human gastrointestinal tract, *E. coli* is one of the most prominent bacteria frequently reported to have developed multi-drug resistance to many of the antibiotics currently available in the market (Alonso *et al*., 2001; Sader *et al*., 2002). Thus, the activity observed against this pathogenic microorganism is equally significant.

The latex and the isolated compounds also showed antifungal effect with nataloin displaying the highest activity (MIC = 400 µg/ml) against *C. albicans* and the two *Penicillium* spp. tested. Previous studies have shown that aloin isolated from *A. ferox* possesses activity against *C. albicans* (Kambizi and Afolayan, 2008).

Table 1. Zones of inhibition and minimum inhibitory concentrations (MICs) of the latex and compounds isolated from the leaf latex of *Aloe pulcherrima*

Bacterial strain	Zone of inhibition in mm (200 µg/ml)				MIC (µg/ml)		
	Latex[a]	(1)[a]	(2)[a]	Cipro	Latex	(1)	(2)
Bacillus pumilus (82)	10.5 (55.30)	10.0 (52.6)	9.5 (50.0)	19.0	100	100	100
B. subtilis ATCC 6633	10.0 (55.6)	10.0 (55.6)	9.5 (52.8)	18.0	100	100	100
Escherichia coli (K99)	15.0 (93.8)	15.5 (96.9)	16.0 (100)	16.0	25	25	25
E.coli K88	14.0 (82.4)	15.0 (88.2)	15.5 (91.2)	17.0	25	25	25
E. coli 306	15.0 (90.9)	15.0 (90.9)	15.5 (93.9)	16.5	25	25	25
E. coli LT37	14.5 (90.6)	14.5 (90.6)	15.0 (93.8)	16.0	25	25	25
E. coli 872	15.0 (93.8)	14.5 (90.6)	15.0 (93.8)	16.0	25	25	25
E. coli ROW 7/12	15.0 (90.9)	14.5 (87.9)	14.5 (87.9)	16.5	25	25	25
E. coli 3:37C	14.5 (93.5)	14.5 (93.5)	14.5 (93.5)	15.5	25	25	25
E. coli CD/99/1	15.0 (88.2)	15.5 (91.2)	17.0 (100)	17.0	25	25	25
Salmonella. enterica TD 01	12.0 (63.2)	14.0 (73.7)	12.5 (65.9)	19.0	100	100	100
S. typhi Ty2	15.0 (93.8)	14.0 (87.5)	14.0 (87.5)	16.0	25	25	25
S. boydii D13629	16.0 (80.0)	16.(80.5)	16.5 (80.5)	20.5	25	25	25
Shigella dysentery 8	16.5 (78.6)	15.5 (77.5)	15.5 (77.5)	20.0	25	25	25
S. flexneri Type 6	16.0 (78.0)	16.5 (84.6)	16.5 (84.6)	19.5	25	25	25
S. soneii 1	16.0 (82.1)	16.0 (76.2)	16.0 (76.2)	21.0	25	25	25
Staphylococcus aureus ML 267	14.5 (80.6)	14.5 (80.6)	17.0 (94.4)	18.0	25	25	25
Vibrio cholerae 1313	16.0 (94.1)	15.5 (91.2)	16.5 (97.1)	17.0	10	10	10
V. cholerae 293	15.5 (88.6)	15.0 (85.7)	16.0 (91.4)	17.5	10	10	10
V. cholerae 1315	15.5 (86.1)	15.0 (83.3)	17.0 (94.4)	18.0	10	10	10
V. cholerae 85	15.5 (86)	14.5 (80.6)	16.5 (91.7)	18.0	10	10	10
Fungal strains	**Latex[b]**	**(1)[b]**	**(2)[b]**	**Gris.**	**Latex**	**(1)**	**(2)**
Aspergillus niger ATCC 6275	12.5 (83.3)	13.0 (86.7)	11.5 (76.7)	15.0	1500	800	1000
Candida albicans ATCC 10231	13.0 (81.3)	14.0 (87.5)	14.5 (90.6)	16.0	800	400	400
Penicillium funiculosum NCTC 287	12.0 (85.7)	13.5 (96.4)	13.5 (96.4)	14.0	1500	400	400
P. notatum ATCC 11625	12.0 (88.9)	13.0 (96.3)	13.0 (96.3)	13.5	1500	400	400

[a]Figures in parenthesis indicate % activity of the test sample compared with that of ciprofloxacin; [b]Figures in parenthesis indicate % activity of the test sample compared with that of griseofulvin; (**1**): 7-Hydroxyaloin; (**2**): Nataloin; Cipro: ciprofloxacin; Gris: griseofulvin.



1 R = CH_2OH
2 R = CH_3

Figure 1. Structures of 7-hydroxyaloin (1) and nataloin (2)

CONCLUSION

The present study revealed that the latex and isolated compounds obtained from *A. pulcherrima* possess promising antimicrobial activity particularly against Gram negative bacteria. The strong antimicrobial activity of the latex and isolated compounds correlate well with the use of this plant in traditional medicine for the treatment of wound, infectious and inflammatory diseases.

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