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Antimicrobial Activity and Bioactive Constituents of *Alectra sessiliflora* (Vahl) Kuntze Methanol Extract

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***Alectra sessiliflora* (Vahl) Kuntze (Scrophulariaceae) is traditionally used in western Kenya in the management of microbial infections. The water, chloroform and methanol extracts of *A. sessiliflora* whole plant exhibited antimicrobial activity against a range of bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae* and *Bacillus pumilus*) and fungi (*Candida albicans, Aspergillus niger* and *Cryptococcus neoformans*). The methanol extract exhibited the highest activity with minimum inhibitory concentration (MIC) of 3.13-6.25 and 3.13-12.5 mg/ml for bacteria and fungi, respectively. Chromatographic fractionation of the methanol extract through non-polar D101 macroporous resin beads yielded three bioactive compounds: two phenolic compounds, *p*-coumaric acid and 3,4-dihydroxybenzoic acid, and a flavonoid, luteolin. The compounds exhibited appreciable activities against tested bacteria and fungi with MIC values ranging from 0.13 to 0.25 and 0.13 to 0.50 mg/ml, respectively. These constituents might be responsible either individually or collectively for the traditional use of the plant to manage bacterial and fungal ailments. The *in vitro* antimicrobial activity and isolation of bioactive compounds from this plant are being reported for the first time.**

Key words: *Alectra sessiliflora,* ethnomedicine, antibacterial and antifungal activity, bioactive constituents

INTRODUCTION

Despite the availability and use of standard antifungal and antibacterial drugs, there is still concern about the emergence of new pathogenic species, development of resistance or breakthrough infections resulting in the use of second line and subsequently expensive third-line drugs which sometimes are more toxic when administered [1-3]. The search for new molecules from plants that are useful as lead compounds for and/or as medicines that can handle these setbacks must continually be pursued as plants have the limitless ability to synthesise substances probably with different modes of action and working on target sites different from drugs in current use [4].

Plants reportedly used traditionally in rural western Kenya for antimicrobial activity are significantly used in oral thrush, mouth ulcers and skin dermatophyte infections. One such used plant is *Alectra sessiliflora* (Vahl) Kuntze (Scrophulariaceae), commonly known as the yellow witchweed, a parasitic weed in farms, grasslands and waste lands [5-6].

Traditionally, many rural communities in tropical Africa use different parts of this plant, mostly in combination with others in diverse ways, to manage a number of ailments including toothache, diarrhoea, kwashiorkor, oral thrush in children, gastrointestinal and sexually transmitted infections, wound infections, to hasten child birth, to treat scars caused by leprosy and as a yellow dye for colouring wood probably to reduce termite attack [5,7].

There are few reports on the phytochemistry and antimicrobial activity of plants in the genus *Alectra* hence the need to investigate the phytochemical and the potential antimicrobial activity of *A. sessiliflora* to authenticate the claimed traditional and continued use in western Kenya. Here, we report the *in vitro* antibacterial and antifungal activities of both the crude extracts and the isolated bioactive constituents from *A. sessiliflora* methanol extract.

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MATERIALS AND METHODS

Plant material collection and identification

The whole plant material of *A. sessiliflora* was collected from Vihiga County of western Kenya and identified by Mr. Simon G. Mathenge and voucher specimens deposited at the School of Biological Sciences Herbarium, University of Nairobi under voucher number BA/10/2004.

Solvents and reagents

Solvents for extraction and/or preparation of mobile phases or reagents were of analytical grade and were double distilled in a glass apparatus before use. All reagents used were prepared as per published or pharmacopoeal protocols.

Plant material preparation and extraction

The collected plant materials were spread out and dried at room temperature away from direct light for 10 days before being ground to a fine powder using a commercial mill. About 750 g of the powdered dried plant material was exhaustively extracted in a Soxhlet apparatus sequentially for 48 h each with petroleum ether (40-60°C), chloroform and methanol. The extract solutions obtained were each filtered and filtrate dried *in vacuo* on a rotary evaporator. To obtain the water extract, another fresh batch of the dried material (100 g) was subjected to a cold maceration, with stirring, in distilled water (500 ml) for 24 h, filtered and freeze dried. The dried crude extracts were stored at 4°C until use.

Equipment

The ultraviolet (UV) scans were run on Shimadzu ultra violet visible (UV/Vis) spectrophotometer and the infrared (IR) spectra recorded on a Shimadzu Fourier transfer infrared (FTIR) spectrophotometer using 1% KBr disks. The proton and carbon-13 nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR, respectively) spectra were recorded in MeOD, on a Bruker spectrometer operating at 500 MHz and 125 MHz, respectively, while the mass spectra were recorded at high resolution on an Agilent Technologies mass spectrometer using the electron spray ionization mass spectrometry (ESI-MS) at 70 eV. All aseptic and microbiological procedures were carried out in laminar bioflow equipment while the inhibition zone diameters were read using a Wezu electronic digital callipers.

Chromatography

Column chromatography was carried out on normal silica gel (70-230 nm mesh) and the non-polar D101 macroporous resin beads (0.3-1.25 mm particle diameter, 80-90 nm average pore size) each packed in an open chromatographic glass column. Analytical thin layer chromatography (TLC) was carried out on 0.25 mm thick silica gel 60 GF_{254} precoated on aluminium plates.

Isolation of Compounds 1, 2 and 3

About 50 g of the methanol crude residue were sonicated for 1 h in 300 ml 70% ethanol. The solution obtained was filtered and filtrate concentrated *in vacuo* to about 90 ml. The concentrate was then centrifuged and the supernatant loaded slowly (1 ml/min) onto the top of the D101 macroporous resin bed wet-packed in distilled water. Initially, the column was eluted with distilled water and subsequently, the polarity of the eluting solvent was sequentially decreased with ethanol in water at 10%, 20%, 30%, 40% and 70%. Fractions having the same TLC profiles were pooled, each concentrated *in vacuo* to about 30 ml on a rotary evaporator followed by freeze drying. About 2 g of the dried fraction eluted with 30% and 40% ethanol in water was dissolved in minimum amount of mobile phase and loaded onto a silica chromatographic column (18 g silica) eluted with chloroform-methanol (9:1, v/v) mobile phase. Fractions having the same chromatographic profiles were pooled (FA-FE) and then reduced to dryness. The dried fraction FB was dissolved in a minimum amount of methanol and kept in the refrigerator at 4°C upon which a brownish amorphous powder of *para*-coumaric acid (**1**) solidified out (28 mg, 0.007% yield). Similarly, fractions FC and FD yielded white crystals of 3,4-dihydroxybenzoic acid (**2**) (20 mg, 0.005% yield) and yellow

amorphous solids of luteolin (**3**) (4 mg, 0.001% yield), respectively.

Antimicrobial activity testing

The antimicrobial activity of the plant extracts and the isolated compounds was tested by the disk diffusion method as described by Hewitt and Vincent [8] against two Gram-positive bacteria: *Staphylococcus aureus* (NC07447) and *Bacillus pumilus* (local strain); three Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (local strain) and *Shigella dysenteriae* (local strain); as well as three fungi: *Aspergillus niger* (NCPF 2275), *Candida albicans* (NCPF 3179) and *Cryptococcus neoformans* (local strain). The microorganisms were grown optimally on tryptone soy agar at 37°C for bacteria and Sabouraud's dextrose agar at 30°C for fungi.

Reservoirs were formed in the set nutrient agar layer by cutting out with a cork borer to form 6.80 mm diameter and 3 mm depth wells into which 50 µl test solutions (extracts: 50 mg/ml, 2500 µg/well) or the negative controls (sterile distilled water or 1% dimethylsulfoxide depending on the extract solvent of reconstitution) were put. The isolated compounds and the positive controls, chloramphenicol and nystatin (for assessing antibacterial and antifungal activity, respectively), each at 1 mg/ml (50 µg/well) were similarly tested for activity. The plates were allowed a diffusion period of 1 h at 4°C before incubation for 18 h at the standardised conditions of 37°C for bacteria and 30°C for fungi. The diameter of the zones of inhibition after incubation was used as the quantitative parameter. All determinations were in triplicate.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration **(**MIC) for each plant extract or isolated compound against the test microorganisms was determined by serial two-fold dilution of extracts/isolates by a modification of the broth micro-dilution method as described by the National Committee for Clinical Laboratory Standards [9] in 24 well microtitre plates. The MIC was determined against standardised 24 hours-old broth cultures of test organisms with approximately 10^6 c.f.u./ml incubated at the optimum temperatures for 18 h. After incubation, 20 µl of a 0.2 mg/ml solution of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each row and the plate returned to the incubator for an additional one hour. Inhibition of growth was indicated by a yellowish colour in the well. The MIC of a compound was defined as the lowest concentrations of the compound that visually showed the yellow colouration compared with purple colour in control wells.

RESULTS AND DISCUSSION

Structural elucidation of isolated compounds

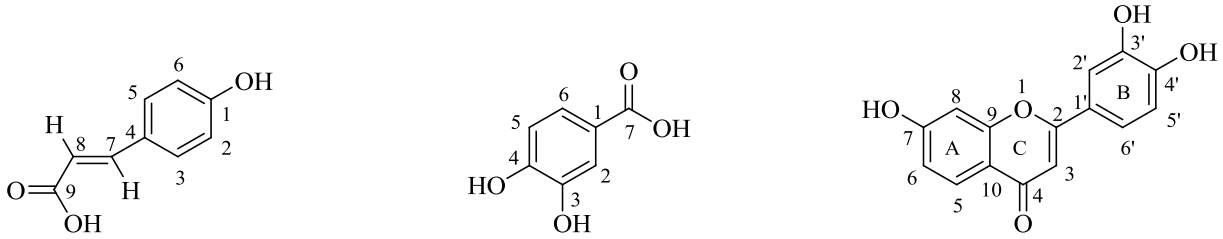
The chemical structures of the three isolated compounds (Figure 1) were elucidated from their physical, chemical and spectroscopic characteristics (m.p., UV, MS, IR and NMR) as two phenolic compounds namely *p*-coumaric acid (**1**) and 3,4-dihydroxybenzoic acid (**2**), as well as a flavonoid, luteolin (**3**) [10-12]. The obtained data matched well and is consistent with previous published data [13-15]. This is the first report of the isolation of *p*-coumaric acid, 3,4-dihydroxybenzoic acid and luteolin from *Alectra sessiliflora* (Vahl) Kuntze.

The corresponding data are as follows:
Para-coumaric acid (**1**): brownish amorphous powder; UV (MeOH, $\lambda_{max,}$ nm): 293, 296; IR (KBr, γ_{max}, cm^{-1}): 3600-2800, 1680, 1610, 1520, 1480, 1440, 960; ^{1}H-NMR (500 MHz, MeOD) δ, ppm: 6.3 (1H, H-8), 6.7 (1H, H-6), 6.8 (1H, H-2), 7.5 (1H, H-7), 7.6 (1H, H-5), 7.7 (1H, H-3); ^{13}C-NMR (125 MHz, MeOD) δ, ppm: 115.7 (C-8), 116.9 (C-6), 127.3 (C-4), 131.2 (C-3), 161.2 (C-1) and 171.0 (C-9); MS (ESI): m/z 163 (M-H), 119.

3,4-Dihydroxybenzoic acid (**2**): white needles; UV (MeOH, $\lambda_{max,}$ nm): 213, 258, 295; IR (KBr, γ_{max}, cm^{-1}): 3640-2880, 2960, 2320, 1640, 1480, 1360, 960; ^{1}H-NMR (125 MHz, MeOD) δ, ppm: 7.44 (1H, H-2), 7.42 (1H, H-6), 6.79 (1H, H-5); ^{13}C-NMR (500 MHz, MeOD) δ, ppm: 170.3 (C-

7) 151.6 (C-4), 146.1 (C-3), 123.2 (C-6), 123.4 (C-1), 117.8 (C-2), 115.8 (C-5); MS (ESI): m/z 153 (M-H), 151, 130, 123, 117, 107, 101.

Luteolin (**3**): yellow amorphous powder; UV ($\lambda_{max,}$ nm): 251, 288, 345; IR (KBr, γ_{max} cm^{-1}): 3440, 3220, 1680, 1620, 1520, 1480, 1300; ^{1}H-NMR (125 MHz, MeOD) δ, ppm: 7.4 (dd, H-2′), 6.9 (dd, H-5′), 6.5 (s, H-3), 6.4 (d, H-8), 6.2 (dd, H-6); ^{13}C-NMR (500 MHz, MeOD) δ, ppm: 183.9 (C-4), 166.4 (C-2), 166.1 (C-7), 163.3 (C-5), 159.5 (C-9), 151.0 (C-4′), 147.1 (C-3′), 123.8 (C-1′), 120.3 (C-6′), 116.8 (C-5′), 114.2 (C-2′), 105.4 (C-10), 103.9 (C-3), 100.2 (C-6), 95.0 (C-8); MS (ESI): m/z 285, 217, 199, 175, 151, 133, 107.



1 **2** **3**

Figure 1. Chemical structures of isolated compounds from *Alectra sessiliflora*: *p*-coumaric acid (1); 3,4-dihydroxybenzoic acid (2); and luteolin (3).

Antibacterial and antifungal activity

All the extracts tested (chloroform, methanol and water) showed antimicrobial activity except the chloroform extract against *E. coli* and *S. dysenteriae* (Table 1). The highest activity was exhibited by the methanol extract with *B. pumilus* (15.49 mm) being the most susceptible bacteria while *C. neoformans* was the most susceptible fungi. Among the test bacteria, *E. coli*, *P. aeruginosa* and *S. dysenteriae* exhibited least susceptibility to the extract, while *A. niger* showed least susceptibility to the extract's antifungal activity. Generally, the extracts showed broad antibacterial activity across Gram-negative (*P. aeruginosa* and *E. coli*), Gram-positive *(S. aureus* and *S. dysenteriae*), aerobic (*S. aureus*) and anaerobic (*S. dysenteriae*) bacteria. Similarly, antifungal activity was observed against both yeast (*C. albicans* and *C. neoformans*) and filamentous (*A. niger*) fungi.

The MIC range of the active extracts against the test microbes (Table 2) was 3.13-25.0 mg/ml for bacteria and 12.5-25.0 mg/ml for fungi. The observation of appreciable activity in polar extracts (water and methanol) is indicative of probable water-soluble antimicrobial constituents in the extracts. The presence of activity in the polar extract is of importance in traditional herbal medicine as traditionally, macerates or hot water decoctions are used in the preparation of the herbal medicines that would probably be rich in water soluble bioactive constituents.

The isolated compounds, *p*-coumaric acid (**1**), 3,4-dihydroxybenzoic acid (**2**), and luteolin (**3**) exhibited appreciable activities against tested bacteria and fungi with MIC of 0.13-0.25 and 0.13-0.50 mg/ml, respectively. All the bioactive compounds showed activity across all tested microorganisms. Among the bacteria tested, *B. pumilus* was the most susceptible to the compounds while *E. coli* was the least susceptible. In the antifungal activity screening, *A. niger* showed the least susceptibility while *C. neoformans* exhibited the highest susceptibility towards antifungal activity of the isolated compounds. The activity in *A. sessiliflora* methanol extract may primarily be due to the three isolated polar components, an observation that partly explains the preferred traditional use of aqueous based preparations.

Generally, the isolated compounds/isolates exhibited much higher activity compared to their parent extracts. For example, 3,4-dihydroxybenzoic acid (**2**), the most active compound isolated, tested against *S. aureus* exhibited a lower MIC, 0.13 mg/ml, compared to its parent extract MIC of 3.13 mg/ml.

Table 1: Diameters of the zones of inhibition of the crude extracts and isolated constituents of *Alectra sessiliflora* produced on the test microorganisms

Sample	Diameters of the zones of inhibition							
	S. aureus	*P. aeruginosa*	*E. coli*	*S. dysenteriae*	*B. pumilus*	*C. albicans*	*A. niger*	*C. neoformans*
Alectra sessiliflora chloroform extract	9.78	7.85	NI	NI	10.60	8.80	8.72	8.92
Alectra sessiliflora methanol extract	15.46	10.72	9.76	10.76	15.49	8.90	8.80	8.96
Alectra sessiliflora water extract	11.68	8.24	9.40	9.40	12.08	8.46	8.34	8.69
Compound **1**	8.90	8.18	8.20	8.20	8.94	8.64	8.46	8.72
Compound **2**	9.40	8.69	8.22	8.69	9.77	9.62	8.77	9.65
Compound **3**	8.71	8.63	8.16	7.87	8.90	8.41	7.20	8.56
Chloramphenicol	19.23	19.22	19.10	18.90	19.24	-	-	-
Nystatin	-	-	-	-	-	19.32	19.32	19.40

Key: NI = no inhibition; concentration of crude extract = 50 mg/ml; concentration of isolated compounds and standard drugs = 1 mg/ml.

Table 2: Minimum inhibitory concentrations of *Alectra sessiliflora* crude extracts and isolated compounds against test microorganisms

	Minimum inhibitory concentration (mg/ml)							
Sample	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. dysenteriae*	*B. pumilus*	*C. albicans*	*A. niger*	*C. neoformans*
Alectra sessiliflora chloroform extract	12.5	25.0	-	-	6.25	12.5	12.5	12.5
Alectra sessiliflora methanol extract	3.13	6.25	6.25	12.5	3.13	12.5	12.5	12.5
Alectra sessiliflora water extract	6.25	12.5	12.5	25.0	3.13	12.5	25.0	12.5
Compound **1**	0.13	0.25	0.25	0.25	0.13	0.25	0.25	0.13
Compound **2**	0.13	0.13	0.13	0.13	0.13	0.13	0.25	0.13
Compound **3**	0.25	0.25	0.25	0.25	0.50	0.25	0.50	0.13
Chloramphenicol	0.06	0.06	0.06	0.06	0.06	-	-	-
Nystatin	-	-	-	-	-	0.06	0.06	0.06

The isolated compounds have previously been reported to possess general antimicrobial activity including anticandidal activity [15]. In addition, the compounds are also reported to possess other biological activities of pharmacological importance [16-20]. Phenolic compounds have drawn interest in their exhibition of antimicrobial activity with site(s) and number of phenol groups thought to be related to their relative toxicity to microorganisms with evidence that increased hydroxylation results in increased toxicity [21-22].

CONCLUSION

It is postulated from this study that the observed antibacterial and antifungal activity of the extracts is due to the isolated phytochemical constituents either participating individually or collectively due to some synergisms with other compounds in the extract, which were either isolated or not isolated. This is the first reported *in vitro* antibacterial and antifungal activity in *A. sessiliflora* and the isolation of bioactive *p*-coumaric acid (**1**), 3,4-dihydroxybenzoic acid (**2**) and luteolin (**3**) from the methanol extract that might be responsible for the traditional use of the plant to manage microbial ailments. It is therefore possible that some of the isolated compounds in this study may be of importance in drug development by providing templates for semisynthetic derivation of superior antimicrobial compounds. Further studies need to be carried out on the three isolated bioactive compounds including evaluation of their toxicity profiles and computational studies to evaluate their potential application as drug leads for novel and useful compounds to tackle the current or any future emerging microbial infections.

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