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Bioactive Constituents of *Conyza floribunda*

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Abstract: The study describes the antibacterial and antifungal effects of both CH_2Cl_2 and MeOH crude extracts, collected fractions and pure isolates of *Conzya floribunda*. The antimicrobial activity tests were carried out using agar diffusion method. *In vitro* tests using CH_2Cl_2 and MeOH extracts of *C. floribunda* showed anti-bacterial activities against *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherischia coli* and antifungal activities against *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypsium*. The antibacterial and antifungal principles from *C. floribunda* were found to be (24S)-ethylcholesta-5, 22E, 25-dien-3-O-β-glucoside and cyasterone from methanol extract and 3-oxofriedooleanane and betullinic acid associated with CH_2Cl_2 extract. The results of the present study indicate that the plant could be a useful remedy for some of the disease conditions caused by the tested bacteria and fungi and the isolated compounds could be good candidates as phytotherapeutic agents some bacterial and fungal infections.

Key words: *Conyza floribunda*, asteraceae, active principles, antimicrobial activity, bioactive constituents, Kenya

INTRODUCTION

The genus *Conyza* (Asteraceae) comprises of about 50 species which are mainly found in tropical and subtropical regions (Agnew and Agnew, 1994; Kokwako, 1976). In Kenya, the genus is represented by 24 species, distributed country wide and one such species is *Conyza floribunda*. The plant grows up to 3 m tall when fully mature and is common in wet regions along the road sides, gardens and in disturbed soils within altitudes of 400-2000 m above sea level (Zdero *et al*., 1990). It is traditionally used for a variety of pharmacological applications including treatment of smallpox, chickenpox, soar throat, ringworm and other skin related diseases, toothache and to stop bleeding from injuries (Pandey *et al*., 1984).

Previous phytochemical studies on the plant are scarce. However, studies on other related species have lead to the isolation of secondary metabolites, some of which have been reported to exhibit biological activities including anti-inflammatory (Bohlmann and Wagner, 1982; Ahmed *et al*., 1992; Mata *et al*., 1997), antitumor (Calzada *et al*., 2001; Xu *et al*., 1998) and antioxidants (Picciaroni *et al*., 2000; De las Heras *et al*., 1998). In the present study, we report the bioassay guided fractionation of CH_2Cl_2 and MeOH extracts of the whole plant using agar well diffusion method.

MATERIALS AND METHODS

Instrumental analysis: The UV spectra were run on PYE UNICAM SP8-150 UV/Vis spectrophotometer. IR data were obtained on Perkins-Elmer 600 FTIR series using acetonitrile and KBr pellet.

The NMR data were measured in $CDCl_3$ and $CDCl_3$-DMSO-d_6 on a Brucker NMR Ultrashied TM operating at 500 and 125 MHz, respectively. The MS data were obtained on a MAT 8200 A Varian Bremen instrument.

Plant material: Authenticated *Conyza floribunda* whole plant was collected at Maseno University Botanical garden in June 2005 and a voucher specimen deposited at the National Museum of Kenya (Voucher deposit number: 2005/06/01/SAO/CHEMMK). The whole plant was air-dried under shade in the open and reduced to a powder using a Wiley mill.

Preparation of plant extracts: Dry powdered plant material (2 kg) was sequentially extracted with CH_2Cl_2 (3 L) and MeOH (3 L) by percolation for 1 week each time, with occasional shaking, thereafter filtered and then concentrated *in vacuo* to afford 65 and 105 g of extracts, respectively.

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Isolation and identification of compounds from CH_2Cl_2 extract: Approximately, 60 g of the extract was dissolved in small amount of CH_2Cl_2 and adsorbed onto silica gel for column chromatography. Fractionation of the extract using gradient of n-hexane-ethyl acetate and MeOH afforded 300 fractions (20 mL each) whose composition were monitored by TLC using solvent systems n-hexane-EtOAc (9:1; 4:1; 2:1) and CH_2Cl_2-MeOH (9:1 and 4:1), respectively. Fractions showing similar TLC profiles were combined resulting into four pools (I-IV).

Pool I (fractions 1-90, 7 g) contained mainly fatty acid and waxes and was discarded. Fractions 91-170 constituted pool II (15 g) and was found to contain two major spots which were further purified using medium pressure chromatography (pressure ≈ 1 bar), eluting with n-hexane-ethyl acetate (9:1 and 4:1) to give stigmasta-5, 22-dien-3-acetate (4, 175 mg) and 3-acetoxyfriedooleanane (9, 95 mg) (Opiyo *et al*., 2009). Pool III (fractions 171-250, 10 g) upon repeated fractionation using n-hexane-ethyl acetate (4:1 and 3:1) yielded spinasta-7, 22-dien-3-ol (6, 75 mg), 3-oxofriedooleanane (7, 55 mg) and 3-hydrox-friedooleanane (8, 165 mg) (Anjaneyulu and Narayana-Rao, 1980). Pool IV (6.5 g) gave stigmasta-5, 22-dien-3-ol (5, 100 mg) and betulinic acid (10, 85 mg) (Opiyo *et al*., 2009).

Isolation and identification of MeOH extract constituents: The extract (75 g) was pre-adsorbed onto silica gel and chromatographed with CH_2Cl_2-MeOH gradient to pure MeOH affording 120 fractions of 50 mL each. The composition of the fractions were monitored by TLC using CH_2Cl_2-MeOH (4:1, 3:2 and 1:1) and those that exhibited similar TLC profiles were combined to constitute two major pools (V and VI). Fractions 10-50 (pool V, 12 g) was further purified by chromatography using CH_2Cl_2-MeOH (9:1) followed by the same solvent system in the ratio 4:1 to give kaempferol (14, 85 mg), cyasterone (3, 78 mg), quercetin (12, 105 mg), myricetin (13, 55 mg), 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (2, 45 mg) and quercetin 3-O-β-glucoside (11, 105 mg) (Opiyo *et al*., 2009; Manguro *et al*., 2004).

The remaining fractions (pool VI, 3 g) contained two major compounds which could not be obtained in pure form and were purified by preparative HPLC using acetonitrile-H_2O (35:65): mobile flow rate 10 mL min^{-1}; injecting 10 μL each time to afford pure 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (1''>4') rhamnoside (1) (t_r = 39 min, 93 mg) (Anjaneyulu and Narayana-Rao, 1980).

Antimicrobial assay

Test microorganisms: Three bacteria and three fungi, all locally isolated microorganisms (LIO) were obtained from New Nyanza General Hospital in Kisumu, Kenya. The bacterial pathogens were *Streptococcus pneumoniae*, *Staphylococcus aureus* (gram positive) and *Escherichia coli* (gram negative) while the fungal pathogens were *Candida albicans* (yeast fungus), *Trichophyton mentagrophytes* and *Microsporium gypseum* (filamentous fungi). The microorganisms were chosen on the basis of ethnobotanical information available on the plant.

Antibacterial screening: Antibacterial activity of crude extracts and pooled fractions was done using agar well diffusion method (Duguid *et al*., 1998; Greenspan and Greenspan, 1997). The bacterial isolates were first grown on a nutrient broth (Oxoid) for 24 h before use. The inoculum suspensions were standardized to 10^7-10^8 CFU mL^{-1}. About 200 microliter of the standard cell suspensions was spread uniformly using a sterile glass spreader on a nutrient agar (Oxiod). Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Approximately 100 μL of crude extracts (at 500 μg mL^{-1}) and pooled fractions at concentrations of 200 μg mL^{-1} were separately introduced into the wells in the culture plates previously seeded with the test organisms, allowed to stand at room temperature for about 1 h and then incubated at 37°C for 24 h. Controls were set up in parallel using Dimethylsulfoxide (DMSO) that was used to reconstitute the extracts. The plates were observed for zones of inhibition after 48 h. The effects of the extracts and pooled fractions were compared with those of chlorophenicol, ofloxacin and streptomycin at a concentration of 10 μg mL^{-1} each.

Antifungal screening: The antifungal tests were done according to the known methods (Irobi *et al*., 1996). The fungal isolates were allowed to grow on a Sabouraud Dextrose Agar (SDA) (Oxoid) at 25°C until they sporulated. The fungal spores were standardized before use and 100 microliter of the standardized fungal suspension was evenly spread on the SDA (oxoid) using a glass spreader.

Wells were then bored into the agar media using a sterile 6 mm cork borer and filled with solutions of crude extracts and pooled fractions at concentrations of 500 and 200 μg mL^{-1}, respectively. The plates were allowed to stand for 1 h for proper diffusion of the extracts and pooled fractions into the media. The plates were incubated at 25°C for 72 h and later observed for zones of inhibition. Controls were set up in parallel using DMSO.

The effect of the extracts on the fungal isolates was compared with fluconazole, cinamizole and amphotericin B at a concentration of 10 μg mL^{-1} each.

Minimum Inhibitory Concentration (MIC): Minimum Inhibitory Concentration (MIC) of pure isolates was determined using standard procedures (Russeland and Furr, 1977; Igbinosa *et al*., 2009) whereby the isolates were dissolved in DMSO and different concentrations ranging between 1000-1 μg mL^{-1} were prepared. The MIC was taken as the lowest concentration that prevented the growth of the test microorganism. The antifungal and antibacterial activities were done in four replicates (n = 4).

RESULTS AND DISCUSSION

Phytochemical studies: Chromatographic fractionation of CH_2Cl_2 and MeOH extracts from *C. floribunda* whole plant afforded 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (1'→4') rhamnoside, 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside, cyasterone, stigmasta-5, 22-dien-3-acetate, stigmasta-5, 22-dien-3-ol, spinasta-7, 22-dien-3-ol, 3-oxofriedooleanane, 3-hydroxfriedooleanane, 3-acetoxy-friedooleanane, betulinic acid, quercetin 3-O-β-glucoside, quercetin, myricetin, kaempferol (Opiyo *et al*., 2009; Anjaneyulu and Narayana-Rao, 1980; Manguro *et al*., 2004).

Antibacterial and antifungal activities of extracts, fractions and pure compounds: Dichloromethane and MeOH extracts showed activities against all the three bacteria tested in the study (Table 1). The MeOH extract strongly inhibited the growth of *S. pneumoniae* (16±0.3) and *S. aureus* (16±0.4) while *E. coli* (12±0.5) was moderately inhibited. The MeOH extract was found to be more active in this respect than the CH_2Cl_2. Similarly, in the antifungal tests, methanol extract exhibited stronger activities against *C. albicans* (19±0.1) and *T. mentagrophytes* (16±1.1) than the dichloromethane extract (Table 2).

The MeOH extract also showed fairly moderate activity against *M. gypseum* (14±0.3). It can be noted that the bacteria and fungi tested were relatively more susceptible to MeOH extract than CH_2Cl_2.

Fractionation of dichloromethane extract as previously discussed in the experimental section gave four pools (I-IV) which were bioassayed. With the exception of pool I, the remaining three pools displayed moderate and weak activities against bacteria tested (Table 3). Similarly, the methanolic extract upon chromatography with CH_2Cl_2-MeOH gradient to pure MeOH constituted pools V and VI which exhibited similar antibacterial activities against all the bacteria tested (Table 3). In the antifungal tests (Table 4), pool II weakly inhibited the growth of *T. mentagrophtes* and *C. albicans* with values of 6±10 and 4±0.2, respectively. No activity was observed with this pool for *M. gypseum*. Pool III and IV both from CH_2Cl_2 extract moderately inhibited the growth of *C. albicans* and *T. mentagrophytes* but failed to show any activity against *M. gypseum*. Similarly, fairly strong inhibitions were experienced with pools V and VI against *C. albicans* and *T. mentagrophytes,* however against *M. gypseum* the pools showed moderate activities. The two pools which were from MeOH extracts were better fungal growth inhibitors than those from CH_2Cl_2 extract.

In the determination of MIC (Table 5), out of the fourteen compounds isolated from *C. floribunda* only four showed activities against the tested pathogens. Although, pool II showed weak activities against *S. aureus*, *C. albicans* and *T. mentagrophytes*, these activities were not observed with the pure compounds 4 and 9 isolated from this pool. Compounds 2 from MeOH extract showed MIC value of 50 μg mL^{-1} against both *S. pneumoniae* and *S. aureus* while for *E.coli* it gave a value >100 μg mL^{-1}. Similarly, the compound exhibited MIC value of 25 μg mL^{-1} for *C. albicans* but values of 50 and 100 μg mL^{-1} were observed for *T. mentagrophytes* and *M. gypseum,* respectively. On the other hand,

Table 1: Antibacterial activity of *C. floribunda* CH_2Cl_2 and MeOH extracts

	Zone of growth inhibition in mm (mean±SD)				
Bacteria	CH_2Cl_2	MeOH	Oflaxacin	Chlorophenicol	Streptomycin
S. pneumoniae	14±0.2	16±0.3	25±0.1	27±0.1	22±1.0
S. aureus	12±0.6	16±0.4	28±0.5	28±0.4	20±0.3
E. coli	8±0.8	12±0.5	25±0.16	19±0.5	0±0

Table 2: Antifungal activity of *C. floribunda* CH_2Cl_2 and MeOH extracts

	Zone of growth inhibition in mm (mean±SD)				
Fungi	CH_2Cl_2	MeOH	Micanazole	Amphotericin B	Fluconazole
C. albicans	16±1.0	19±0.1	29±1.0	32±1.1	29±1.2
T. mentagrophytes	14±0.5	16±1.1	22±0.2	29±0.4	20±0
M. gypseum	10±0	14±0.3	24±0.6	26±0.1	21±1.0

Table 3: Antibacterial activity of *C. floribunda* CH_2Cl_2 and MeOH extract fractions

	Zone of growth inhibition in mm (mean±SD)					
	CH_2Cl_2 fractions			MeOH fractions		
Bacteria	Pool I	Pool II	Pool III	Pool IV	Pool V	Pool VI
S. pneumoniae	0±0	0±0	10±0.3	12±0.5	14±0.1	14±0.2
S. aureus	0±0	4±0.5	12±0.4	12±0	14±0.2	14±0.3
E. coli	0±0	0±0	4±0.6	6±03	10±0.3	10±1.2

Table 4: Antifungal activities of *C. floribunda* CH_2Cl_2 and MeOH fractions

	Zone of growth inhibition in mm (mean±SD)					
	CH_2Cl_2 fractions			MeOH fractions		
Fungi	Pool I	Pool II	Pool III	Pool IV	Pool V	Pool VI
C. albicans	0±0	4±0.2	12±0.3	14±0.1	16±0.2	16±0.5
T. mentagrophytes	0±0	6±1.0	12±0.6	12±0.3	14±0.1	14±1.3
M. gypseum	0±0	0±0	0±0	0±0	14±1.0	12±1.2

Table 5: Mnimum inhibitory concentration (MIC, μg mL^{-1}) of pure compounds and standard antibiotics

	Bacteria			Fungi		
Cmpound/antibiotic	*S. pneumoniae*	*S. aureus*	*E. coil*	*C.albicans*	*T. mentagrophytes*	*M. gypseum*
2	50	50	>100	25	50	100
3	50	50	100	25	50	50
7	100	100	>200	100	100	>200
10	100	100	>200	50	100	100
Chorophenicol	6.25	3	1.5	ND	ND	ND
Ofloxacin	6.25	6.25	3	ND	ND	ND
Streptomycin	12.5	6.25	ND	ND	ND	ND
Micanazole	ND	ND	ND	6.25	6.25	3
Amphoterecin B	ND	ND	ND	6.25	3	6.25
Fluconazole	ND	ND	ND	12.5	12.5	6.25

ND = Not Detected

compound 3 inhibited the growth of both *S. pneumoniae* and *S. aureus* by showing a MIC value 50 μg mL^{-1} while the value for *E. coli* was 100 μg mL^{-1}. The same compound gave MIC value of 25 μg mL^{-1} against *C. albicans* and 50 μg mL^{-1} for both *T. mentagrophytes* and *M. gypseum*. The other compound that showed slight activity was 7 which gave MIC value of 100 μg mL^{-1} against both *S. aureus* and *S. pneumoniae* and almost no activity against *E. coli* (>200 μg mL^{-1}). Similarly, the same compound was observed to be active against *C. albicans* and *T. mentagrophytes* with MIC value of 100 μg mL^{-1} but not active against *M. gypseum*. Compound 10 isolated from CH_2Cl_2 showed MIC value of 100 μg mL^{-1} for both *S. pneumoniae* and *S. aureus* and <200 μg mL^{-1} for *E. coli*. In the antifungal tests, it was found to be active against *C. albicans* with MIC value of 50 μg mL^{-1} and moderately active against *T. mentagrophytes* (100 μg mL^{-1}) but inactive against *M. gypseum*.

The study showed that extracts from *C. floribunda* have antifungal and antibacterial activities and this is probably why the plant is widely used in traditional medicine. The extracts from the plant have broad spectrum activity since they are effective against both gram positive and gram negative bacteria. The extracts were also active against dermatophytic fungi, *T. mentagrophytes* and *M. gypseum*. This observation is of particular interest since many Kenyan traditional healers use the plant for treating ringworm, a type of infection caused by the two fungi above. The extracts were also found to be active against *C. albicans*, a ubiquitous fungi associated with the pathogenesis of urinary tract infections and oral thrush (Akinpelu and Kolawole, 2004; Widodo *et al*., 2008). Methanol extract exhibited higher activity compared to dichloromethane and this could be attributed to the fact that antibacterial and antifungal compounds in *C. floribunda* are polar compounds which could be extracted with polar solvents such as methanol and water.

The antibacterial and antifungal principles from *C. floribunda* were identified as (24S)-ethylcholesta-

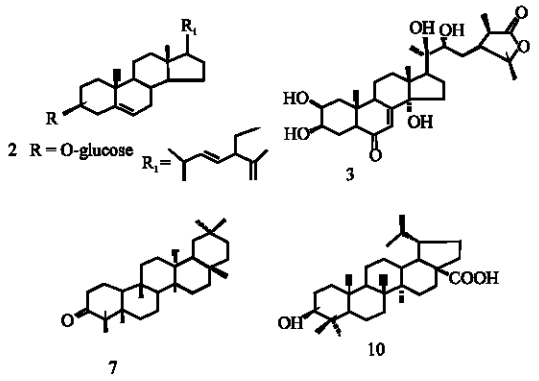

Fig. 1: The antibacterial and antifungal principals from *C. floribunda*

5,22E, 25-trienene 3-O-glucopyranoside (2), cyasterone (3), 3-oxofriedooleanane (7) and betulinic acid (10) (Fig. 1). Compounds 2 and 3 both from MeOH extract showed both antibacterial and antifungal activities and were more effective than the latter two. This observation suggests that the antifungal principles in the plant have broad spectrum antifungal activities.

CONCLUSION

The extracts and pure compounds from the plant were however, less active compared to ofloxacin, chlorophenicol and streptomycin which are known antibacterial compounds. Similarly for the antifungal tests, the extracts and pure compounds were less effective compared with known antifungal drugs such as amphotericin B, micanazole and fluconazole.

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