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Antimicrobial Constituents Of Conyza Floribunda

Corresponding Author:
Prof. Lawrence Manguro,
Associate Professor, Chemistry, 40105 - Kenya

Submitting Author:
Prof. Lawrence O Manguro,
Associate Professor, Chemistry, 40105 - Kenya

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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* -b-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

Introduction

The genus *Conyza* (Asteraceae) comprises of about fifty species, which are mainly found in tropical and subtropical regions [1, 2]. In Kenya, the genus is represented by twenty four 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 400-2000m above sea level [3].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 [4]. 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 antiinflammatory [5-7], antitumor [8, 9] and antioxidants [10, 11]. 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.

Material 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 CDCl3 and CDCl3-DMSO-d6 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 Botanic 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 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 CH2Cl2 (3 L) and MeOH (3 L) by percolation for one week each time, with occasional shaking, thereafter filtered and then concentrated in vacuo to afford 65 g and 105 g of extracts, respectively.

Isolation and identification of compounds from CH2Cl2 extract

Approximately 60 g of the extract was dissolved in small amount of CH2Cl2 and adsorbed onto silica gel for column chromatography. Fractionation of the extract using gradient of n-hexane-ethyl acetate and MeOH afforded 300 fractions (20ml each) whose composition were monitored by TLC using solvent systems n-hexane-EtOAc (9:1; 4:1; 2:1) and CH2Cl2-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) [12]. 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-hydroxfriedooleanane (8, 165 mg) [13]. Pool IV (6.5 g) gave stigmasta-5, 22-dien-3-ol (5, 100 mg) and betulinic acid (10, 85 mg) [12].

Isolation and identification of MeOH extract constituents

The extract (75 g) was pre-adsorbed onto silica gel and chromatographed with CH2Cl2-MeOH gradient to pure MeOH affording 120 fractions of 50 ml each. The composition of the fractions were monitored by TLC using CH2Cl2-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 CH2Cl2-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, 55mg), 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (2, 45 mg) and quercetin 3-O-β-glucoside (11, 105 mg) [12, 14]. 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-H2O (35:65): mobile flow rate 10mLmin-1; injecting 10µl each time to afford pure 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (1'' → 4') rhamnoside (1) (tr=39 min, 93 mg) [13].

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 [17, 18]. The bacterial isolates were first grown on a nutrient broth (Oxoid) for 24 h before use. The inoculum suspensions were standardized to 107-108CFU/ml. Two hundred 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) and pooled fractions at concentrations of 200 µg/ml were separately introduced into the wells in the culture plates previously seeded with the test organisms, allowed to stand at room temperature for about one hour and then incubated at 37 0C 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 10µg/ml each.

Antifungal screening

The antifungal tests were done according to the known methods [19]. The fungal isolates were allowed to grow on a Sabouraud dextrose agar (SDA) (Oxoid) at 250C until they sporulated. The fungal spores were standardized before use and one hundred 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 6mm cork borer and filled with solutions of crude extracts and pooled fractions at concentrations of 500 and 200 µg/ml, 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 0C 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 each.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of pure isolates was determined using standard procedures [20, 21], whereby the isolates were dissolved in DMSO and different concentrations ranging between 1000-1 µg/ml 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

Phytochemical studies

Chromatographic fractionation of CH2Cl2 and MeOH extracts from C. floribunda whole plant afforded 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (1'' → 4') rhamnoside (1) , 24-ethylcholesta-5, 22E, 25-triene 3-O-β-glucoside (2), cyasterone (3), stigmasta-5, 22-dien-3-acetate (4), stigmasta-5, 22-dien-3-ol (5), spinasta-7, 22-dien-3-ol (6), 3-oxofriedooleanane (7), 3-hydroxfriedooleanane (8), 3-acetoxyfriedooleanane (9), betulinic acid (10),

quercetin 3-O-β-glucoside (11), quercetin (12), myricetin (13), kaempferol (14) [12-14].

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 CH2Cl2. 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 also be noted that the bacteria and fungi tested were relatively more susceptible to MeOH extract than CH2Cl2.

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 CH2Cl2-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. Pools III and IV both from CH2Cl2 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 CH2Cl2 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 50µg/ml against both S. pneumoniae and S. aureus while for E.coli it gave a value > 100 µg/ml. Similarly the compound exhibited MIC value of 25 µg/ml for C. albicans but values of 50 and 100 µg/ml were observed for T. mentagrophytes and M. gypseum, respectively. On the other hand, compound 3 inhibited the growth of both S. pneumoniae and S. aureus by showing a MIC value 50 µg/ml while the value for E. coli was 100 µg/ml. The same compound gave MIC value of 25 µg/ml against C. albicans and 50 µg/ml for both T. mentagrophytes and M. gypseum. The other compound that showed slight activity was 7 which gave MIC value of 100 µg/ml against both S. aureus and S. pneumoniae and almost no activity against E. coli (>200 µg/ml). Similarly, the same compound was observed to be active against C. albicans and T. mentagrophytes with MIC value of 100 µg/ml but not active against M. gypseum. Compound 10 isolated from CH2Cl2 showed MIC value of 100 µg/ml for both S. pneumoniae and S. aureus and greater than 200 µg/ml for E. coli. In the antifungal tests, it was found to be active against C. albicans with MIC value of 50µg/ml and moderately active against T. mentagrophytes (100 µg/ml) but inactive against M. gypseum.

Discussion and Conclusion.

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 [15,16]. 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-5,22E, 25-trienene 3-O-glucopyranoside (2), cyasterone (3), 3-oxofriedooleanane (7) and betulinic acid (10). 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.

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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Authors Contribution(s)

All others contributed equally

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Illustrations

Illustration 1

Table 1. Antibacterial activity of C. floribunda CH2Cl2 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

Illustration 2

Table 2. Antifungal activity of C. floribunda CH2Cl2 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

Illustration 3

Table 3. Antibacterial activity of C. floribunda CH2Cl2 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

Illustration 4

Table 4. Antifungal activities of C. floribunda CH2Cl2 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

Illustration 5

Table 5. Minimum inhibitory concentration (MIC, Âµg/ml) of pure compounds and standard antibiotics.

	Bacteria			**Fungi**		
compound/ 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

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