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Journal of Medicinal Plants Research

Table of Contents: Volume 10 Number 39 17 October, 2016

ARTICLES

Stem bark extracts of *Erythrina excelsa* (BAK) and their biological activities 686
N. M. Ombuna, A. K. Machocho, M. M. Ng'ang'a, G. K. Keru, A. W. Wanyonyi And O. Amuka

Phytochemical investigation, antinociceptive activity and cytotoxicity of crude extracts of *Calea uniflora* Less. 695
Vanessa Nicolau Rodrigues-Torres, Jéssica DeLuca Machado, Luan de Souza Ramos, Renato Paghan, Jacqueline Kautz, Isabelle Rouaud, Aurélie Sauvager, Sophie Tomasi, Françoise Lohézic-Le Dévéhat, Silvia DalBó and Patrícia de Aguiar Amaral

Documentation of indigenous knowledge on medicinal plants used to manage common influenza and related symptoms in Luwero district, central Uganda 705
Katuura E., Omujal F., Tumusiime R. H., Nabukalu D. and Byamukama R.

academicJournals

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Journal of Medicinal Plants Research

Full Length Research Paper

Stem bark extracts of *Erythrina excelsa* (BAK) and their biological activities

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The species is known from Southern Nyanza, Migori in Kenya. The extraction of the stem bark was done sequentially using organic solvents starting with the least polar; n-hexane then dichloromethane, ethyl acetate and finally the most polar methanol. Antibacterial and antifungal activities of the crude extract and that of isolated compounds from the stem bark of *E. excelsa* were investigated. The crude extracts had substantial activity against the tested micro-organisms. Methanol extract was highly active with inhibition zones of 15 mm against *S. aureus* and 14 mm against both *B. subtillis* and *E. coli*. Ethyl acetate and dichloromethane had mild activity. In antifungal test, methanol extract had highest activity of 15 mm against *A. niger* and 13 mm against *C. albicans.* Dichloromethane extract was also active with inhibition zones of 14 mm against *A. niger* and 12 mm against *C. albicans* while ethyl acetate had mild activity. A total of five compounds were isolated; glutinosalactone A (1), glutinosalactone B (2), lupinifolin (3), sitosterol (4) and 3β-stigimasterol (5). The compounds were active against the bacterial and fungal test strains used. Glutinosalactone A (1) had an activity of 15 mm against A. *niger*, 11 mm against both *B. subtillis* and S. *aureas*. Glutinosalactone B (2) had a high activity of 11 mm in both B. *subtillis* and C. *albicans*. and lupinifolin (3) had mild activity of 9 mm against both A. *niger* and C. *albicans.* Stigimasterol and sitosterol had mild activity of 8 mm against A. *niger*.

Key words: *Erythrina excelsa*, biological activities, ethnobotany, metabolites.

INTRODUCTION

Medicinal plants use in the world as phytotherapy is old as humankind. Methods of use are, however, regionally variable. The usages of plants are further influenced by cultural characteristics of the population, flora, and environmental factors (Murray and Pizzarno, 1995). *Erythrina excelsa* is one of the numerous species in the genus *Erythrina.* There is over 130 species of "coral tree" that belong to the genus *Erythrina* which has been widely studied and are distributed in tropical and subtropical regions of the world. (Hickey and King, 1981). *E. excelsa* is usually found in riverine and swampy forest up to 1500 m altitude. The species is known from Kisii, Migori

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and parts of Kisumu in Kenya. The leaves and the root decoction of the species *E. secluxii* are used in the treatment of malaria (Gessler et al., 1995). The stem bark of *E. abysinicca* is used to treat trachoma and syphilis while its root bark is used to treat malaria and colic (Ichimaru et al., 1996). Extracts of plants from this genus have proven highly bioactive (Normark and Normark, 2002); *E. glauca* and *E. lysistemon* have been reported to poses antiviral, antibacterial and estrogenic activity (Ito, 1999). Aqueous extract of the stem bark of *E. senegalensis* have proved to have analgesic and anti-inflammatory effects (Saidu et al., 2000). In addition, the leaf or bark decoction or tincture from *E. mulungu* is considered to calm agitation and other disorders of the nervous system, including insomnia (Vasconcelos et al., 2007). Several uses have been reported. Table 1 shows medicinal uses of the various parts of *E. excelsa* in East Africa.

Analysis of various parts of the plants from this genus has demonstrated the presence of flavonoids, especially, isoflavones, pterocarpanes, flavanones and isoflavanones (Chacha et al., 2005). Terpenes have also been isolated from the same genus (Nkengfack et al., 1997). Biologically active alkaloids are also found in a number of species of the genus. Many of the alkaloids have been reported to have anti-inflammatory, cardioactive, narcotic and sedative activities (Palframan and Parsons, 2010).

Erythrina burana is an endemic species in Ethiopia, where, it is widely distributed (Hanelt et al*.,* 2001). It is also cultivated across its natural distribution range; and in Kenya and Ethiopia as a shade tree for coffee plantations. The species is also used as fodder for livestock (Hanelt et al*.,* 2001). The bark sap of *E. excelsa* is administered as an anti-dote for snake bites (Owuor et al., 2005). Its stem and root decoction is used in treatment of arthritis. This study was aimed at investigating the bioactivity of the crude extract and that of pure compounds obtained from the stem bark of *E. excelsa* to substantiate its usage as anti-dote for snake bites as well as in treatment of arthritis.

MATERIALS AND METHODS

Plant materials

The stem bark of *E. excelsa* was collected from Migori County in February 2011 and taken to Plant Science Laboratory of Kenyatta University for identification. The plant was authenticated as *E. excelsa*, Voucher specimen NMO/KU/EE/SB/001 by Mr. Lucas Karimi of the Department of Pharmacy and Complementary Medicine of Kenyatta University. Voucher specimens of the plant were also taken to Kenyatta University Herbarium where they were held for future reference. The sample was air dried under a shade for one month to a moisture index of 13% and ground into fine powder to pass through 0.5 mm sieve using a manual grinder.

General extraction

The extraction of the stem bark was done sequentially using organic solvents starting with the least polar; n-hexane then dichloro-methane, ethyl acetate and finally the most polar methanol. 5 Kg of the air dried fine powdered sample was soaked in 4 L of n-hexane for 48 h then filtered and the filtrate concentrated under pressure using a rotary evaporator to obtain the hexane extract. The extraction was repeated once with n-hexane. The same procedure was followed replacing n-hexane with dichloromethane, ethyl acetate and then methanol in turn to obtain dichloromethane, ethyl acetate and methanol extracts. The extracts were the stored in a deep freezer.

Isolation of compounds

Methanol extract

Column chromatography was used to fractionate the extracts. The methanol extract (30 g) was fractionated using solvents of increasing polarity starting with n-hexane to methanol. 500 ml of the solvent system was used in each case. This gave a total of 21 fractions. Fraction 72 to 79 (dichloromethane: methanol = 9:1) was subjected to preparative TLC using 100% DCM yielding two compounds. 19.7 mg of white crystals of compound **1** and 17.5 mg of white crystals of compound 2.

DCM-hexane extract

The DCM-hexane extract weighed a total of 53.0 g. 50.0 g was adsorbed in 5.0 g of silica gel and subjected to column chromatography (diameter 4.0 cm and length 60 cm) over a column of silica gel 240 G using solvents of increasing polarities from hexane to methanol. 500 ml of each solvent system was used in each case. This yielded a total of 18 fractions. Fractions 36 to 54 (hexane: DCM = 2:8) was subjected to a Sephadex column (DCM: MeOH = 50:50) yielding seven sub-fractions. Sub-fraction 20 to 24 was subjected to preparative TLC using 100% DCM and this resulted in 18.0 mg of yellow crystalline solid of **3** preparative TLC was carried out on fraction sub-fraction 10 to 19 using 100% DCM yielding two compounds; 14 mg of white crystals **4** and 12 mg of white crystals **5**.

Spetral determination

Both one and two dimensional NMR spectra of compound 1 and 2 was done from the University of Kwazulu-Natal in South Africa using deuterated methanol as a solvent. The spectra for compound 3, 4 and 5 were determined from the University of Nairobi using chloroform. 15 mg of each the compounds; 1, 2, 3, 4 and 5 was packed in sample vial and taken for analysis.

Bioassays: Antibacterial and antifungal activities

The bioassays were done at the Microbiology Laboratory of Kenyatta University to determine the bioactivities of the crude extracts and that of the isolated pure compounds. Nutrient agar (NA) was used for antibacterial tests and potato dextrose agar (PDA) for antifungal tests. Petri-dish plates containing lids were used. All the microbial organisms were obtained from the Department. These were; *Staphylococcus aureus* (ATCC 35844) and *Bacillus subtillis* (ATCC 6051) that were Gram-positive and *Escherichia coli* (ATCC 11775) that was Gram-negative. Filter paper disc assay method was employed (Chhabra and Uiso, 1991) in which filter paper discs (6 mm) impregnated with hexane, dichloromethane, ethyl acetate and methanol extracts was applied on plates containing a cultured micro-organism. The plates were then incubated at 37°C for 24 h, and then zones of inhibition

Table 1. Medicinal uses of various species of *Erythrina.*

Use	Part utilized	Kind of extract/ way of administration	Species	Country	References
Trachoma	Bark	Unspecified, oral	*E. abyssinica*		
Malaria	Roots	Unspecified, oral	*E. abyssinica*	Kenya	Ichimaru et al. (1996)
Syphilis	Bark	Unspecified, oral	*E. abyssinica*		
Syphilis	Flowers	Infusion, oral	*E. abyssinica*	Uganda	Kamusiime et al. 1996)
Antimalarial	Leaves/roots	Decoction/ infusion, oral	*E. secluxii*	Tanzania	Gassler et al. (1995)

Table 2. Antibacterial test results for the crude extracts.

Solvent	Conc. (mg/disc)	*S. aureus*		*B. subtilis*		*E. coli*	
		Zone (mm)	MIC (mg/ml)	Zone (mm)	MIC (mg/ml)	Zone (mm)	MIC (mg/ml)
Hexane	0.5	6	ND	6	ND	6	ND
DCM	0.5	10	ND	9	ND	7	ND
EtOAC	0.5	13	ND	12	0.125	11	ND
MeOH	0.5	15	ND	14	0.125	14	ND
Gentamycin	10 µg/disc	17		17		17	

MIC = Minimum inhibitory concentration, ND = not done.

measured. This method was also used for pure compounds isolated which include glutinosalactone A, glutinosalactone B, lupinifolin, sitosterol and 3 β-stigimasterol. Gentamycin was used as a positive control. Two standard fungal strains namely; *Candida albicans* and *Aspergilus niger,* obtained from the Department of Plant and Microbiology of Kenyatta University were used. A standard antifungal drug, Nystatin, was used as positive control and DMSO as the negative control. 1 ml of each of the following extracts; hexane, dichloromethane, ethyl acetate and methanol were aseptically mixed with 15 ml of PDA. A five-day old fungal culture was then inoculated in an inverted position at the centre of the plate and then incubated at room temperature. This was done by streaking the fungal spores from the base plate to the new plate. The diameter of the zone of inhibition was measured in the third, fourth and fifth day. This method was also used for pure compounds.

RESULTS AND DISCUSSION

The DCM, EtOAc and MeOH extracts were active against all the three micro-organisms. MeOH extract showed highest activity of 15 mm against *S. aureus*. EtOAc was also highly active with its highest activity being 13 mm against *S. aureus*. DCM extract had mild activities of 10, 9, and 7 mm against *S. aureus, B. subtillis* and *E. coli* respectively (Table 2). The extracts were also active against fungi used in the tests (Table 3). Methanol extract showed highest activity against the tested micro-organisms. Methanol is the most polar solvent hence many compounds extracted in methanol have promotional and synergist effect.

Phytochemical analysis of the crude extracts yielded five compounds. The structures of the compounds were elucidated by standard spectroscopic techniques that included UV, IR, 1H NMR, 13C NMR, COSY, NOESY, HSQC and HMBC and comparison with published data.

Compound 1

White crystals; IR $\lambda_{max.}$(KBr) 3136 cm^{-1} hydroxyl (-OH), 2937 cm^{-1} methylene (-CH_2-), 1735 cm^{-1} δ-lactonic carbonyl and 1637 cm^{-1}olefinic group (>C=CH-); ^{1}H NMR (CD_3OD, 400 MHz) δ 6.00 (1H, *s*, H-12), δ 3.84 (1H, *d*, *J* = 9.56 Hz, H-30a), δ 3.76 (2H, *s*, H-30), δ3.68 (1H, *d*, *J* = 9.12 Hz, H-24a), δ3.41 (1H, *d*, *J* = 9.04 Hz, H-24b), δ 2.41 (2H, *d*, *J* = 13.2 Hz, H-16), 2.36 (1H, *d*, *J* = 13.2 Hz, H-15), δ 2.24 (1H, *d*, *J* = 8.56 Hz, H-9), δ 2.20 (2H, *s*, H-2a), δ 2.18 (2H, *s*, H-11a), δ 1.96 (3H, *m*, H-11a), δ 1.89 (3H, *s*, H-18), δ 1.83 (3H, *m*, H-22a), δ 1.78 (1H, *m*, H-2a), δ 1.70 (1H, *m*, H-11b), δ 1.58 (2H, *m*, H-21a), δ 1.57 (2H, *m*, H-7a), δ 1.45 (5H, *m*, H-16b), δ 1.45 (5H, *m*, 15b), δ 1.43 (5H, *m*, H-7b), δ 1.42 (5H, *m*, H-6a), δ 1.42 (5H, *m*, H-6b) δ 1.39 (1H, *m*, H-5), δ 1.37 (1H, *s*, H-29), δ 1.30 (2H, *m*, H-1), δ 1.18 (1H, *s*, H-27), δ 1.03 (1H, *s*, H-23), δ 0.91 (1H, *s*, H-25) and δ 0.81 (1H, *s*, H-26); ^{13}C NMR (CD_3OD, 150 MHz) δ 181.1 (C-8), δ 88.1 (C-20), δ 73.9 (C-19), δ 71.4 (C-3), δ 66.2 (C-24), δ 63.6 (C-30),δ 51.1 (C-18), δ 50.8 (C-5), δ 49.6 (C-9), δ 43.9 (C-4), δ 43.0 (C-14), δ 41.8 (C-8), δ 41.1 (C-17), δ 38.4 (C-10), δ 34.6 (C-7), δ 33.8 (C-1), δ 26.7 (C-15), δ 26.5 (C-16), δ26.0 (C-2), δ 25.9 (C-11), δ 25.9 (C-9), δ 25.6 (C-21), δ 25.2 (C-22), δ 23.4 (C-27), δ 22.8 (C-24), δ 19.8 (C-6), δ

Table 3. Antifungal test results for extracts from *E. excels.*

Solvent	Concentration (mg / disc)	Zones of Inhibition in mm	
		A. niger	*C. albicans*
Hexane	0.5	7	6
DCM	0.5	14	12
EtOAC	0.5	10	11
MeOH	0.5	15	13
Nystatin	10 µg / disc	16	16
DMSO	6	6	6

Standard Nystatin (10 µg / disc) was used as a control for the experiment and diameter of the disc was 6 mm; MeOH extract had strongest activity of 15 mm against *A. niger* and 13 mm against *C. albicans.* EtOAC extract exhibited an activity of 10 mm against *A. niger* and 11 mm against *C. albicans.* DCM extract exhibited a strong activity of 14 mm, respectively on *A. niger* 12 mm on *C. albicans* while hexane extract exhibited no activity on both micro-organism. Positive control Nystatin had an inhibition zone of 16 mm.

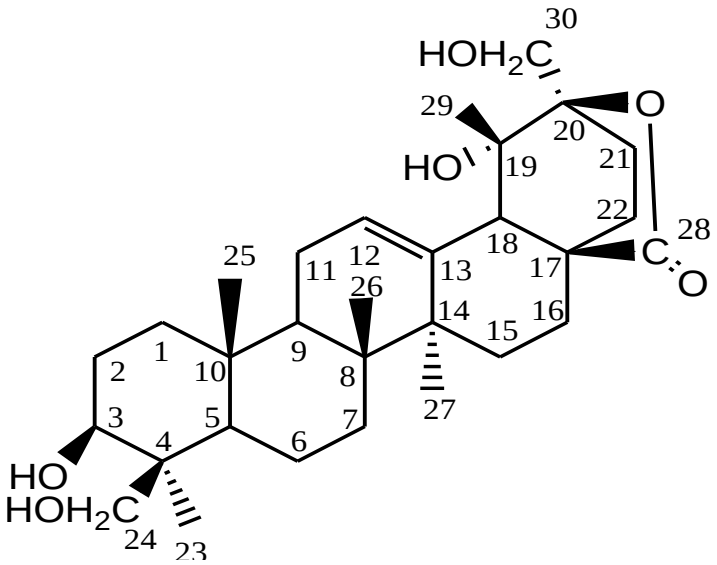

Figure 1. Compound 1: Gutinosalactone.

16.5 (C-26), δ 16.3 (C-25).

The isolated compound was white crystalline solid. It was visualized on TLC plate by spraying with anisaldehyde warming at 110°C and showed a characteristic purple colour confirming the presence of a terpenoid. The IR spectrum showed characteristic peaks at 3136 cm^{-1} (broad) for hydroxyl (-OH), 2937 cm^{-1} for methylene (-CH_2-), 1735 cm^{-1} for δ-lactonic carbonyl and 1637 cm^{-1} for olefinic group. The ^{1}H NMR spectrum revealed a total of 20 proton signals resolved by analysis of COSY, NOESY, DEPT and HSQC spectra. The proton singlet resonating at δ 6.00 (1H, *s*, H-12) showed significant correlation from COSY spectrum with (H-11a) and NOE correlation with (H-29) from the NOESY spectrum. The ^{13}C NMR spectrum showed 30 carbon signals, characteristic of a pentacyclic triterpenoid (Ahmad et al., 1996) as resolved by DEPT spectrum of which there were 8 quaternary carbons, three of which were highly deshielded oxygenated centers at δ 181.1 (C-28), δ 88.1 (C-20), δ 73.9 (C-19), four being points of attachment of methyl groups in the pentacyclic ring at δ 43.9 (C-4), δ 43.0 (C-14), δ 41.8 (C-8) and δ 38.4 (C-10), and one forming the lactonic carbonyl at δ 41.1 (C-17). There were 4 methine carbons identified at 71.4 (C-3), δ 51.1 (C-18), δ 49.6 (C-9), and δ 50.8 (C-5). The 11 methylene carbons were assigned to; δ 66.2 (C-24), δ 63.6 (C-30), δ 34.6 (C-7), δ 33.8 (C-1), δ 26.7 (C-15), δ 26.5 (C-16), δ 26.0 (C-2), δ 25.9 (C-11), δ 25.6 (C-21), δ 25.2 (C-22) and δ 19.8 (C-6).

The direct bonding of proton to carbon atoms were derived from the HSQC and HMBC spectra and the structure of compound 1 (Figure 1) was elucidated to be 3β, 19α, 20β, 24, 30-pentahydroxyurs 12-en-28-oic acid δ-lactone, $C_{30}H_{46}O_6$ commonly known as glutinosalactone

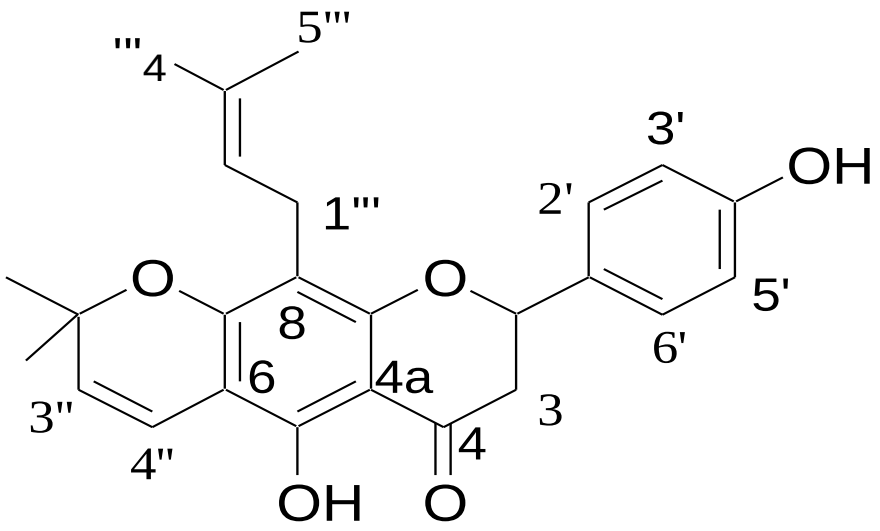

Figure 2. Compound 3: Lupinifolin.

A (Zhang et al., 2013). This is the first time extraction from this plant *E. excelsa.*

Compound 2

White crystals; IR $\lambda_{max.}$(KBr) 3137 cm^{-1} hydroxyl (-OH), 2937 cm^{-1} methylene (-CH_2-), 1735 cm^{-1} δ-lactonic carbonyl and 1637 cm^{-1} olefinic group (>C=CH-); ^{1}H NMR (CD_3OD, 400 MHz) δ 6.00 (1H, *s*, H-12), δ 3.82 (1H, *d*, *J* = 9.56 Hz, H-30a), δ 3.76 (2H, *s*, H-3), δ 3.68 (1H, *d*, *J* = 9.12 Hz, H-24a), δ 3.41 (1H, *d*, *J* = 9.04 Hz, H-24b), δ 2.41 (2H, *d*, *J* = 13.2 Hz, H-16), 2.36 (1H, *d*, *J* = 13.2 Hz, H-15), δ 2.24 (1H, *d*, *J* = 8.56 Hz, H-9), δ 2.20 (2H, *s*, H-2a), δ 2.18 (2H, *s*, H-11a), δ 1.96 (3H, *m*, H-11a), δ 1.89 (3H, *s*, H-18), δ 1.83 (3H, *m*, H-22a), δ 1.78 (1H, *m*, H-2a), δ 1.70 (1H, *m*, H-11b), δ 1.58 (2H, *m*, H-21a), δ 1.57 (2H, *m*, H-7a), δ 1.45 (5H, *m*, H-16b), δ 1.45(5H, *m*, 15b), δ 1.43(5H, *m*, H-7b), δ 1.42 (5H, *m*, H-6a), δ 1.42 (5H, *m*, H-6b) δ 1.39 (1H, *m*, H-5), δ 1.37 (1H, *s*, H-29), δ 1.30 (2H, *m*, H-1), δ 1.18 (1H, *s*, H-27), δ 1.03 (1H, *s*, H-23), δ 0.91 (1H, *s*, H-25) and δ 0.81 (1H, *s*, H-26); ^{13}C NMR (CD_3OD, 150 MHz) δ 181.1 (C-8), δ 88.1 (C-20), δ 73.9 (C-19), 71.4 (C-3), δ 66.2 (C-24), δ 63.6 (C-30), δ 51.1 (C-18), δ 50.8 (C-5), δ 49.6 (C-9), δ 43.9 (C-4), δ 43.0 (C-14), δ 41.8 (C-8), δ 41.1 (C-17), δ 38.4 (C-10), δ 34.6 (C-7), δ 33.8 (C-1), δ 26.7 (C-15), δ 26.5 (C-16), δ 26.0 (C-2), δ 25.9 (C-11), δ 25.9 (C-9), δ 25.6 (C-21), δ 25.2 (C-22), δ 23.4 (C-27), δ 22.8 (C-24), δ 19.8 (C-6), δ 16.5 (C-26), δ 16.3 (C-25).

The structure of compound **2** was found to be similar to that of **1** with the OH in C-24 replaced by H and was elucidated as 3β,19α,20β,30-tetrahydoxyurs-12-en-28-oic acid lactone $C_{30}H_{46}O_5$ commonly known as glutinosalactone B.

Compound 3

Obtained as yellow crystals; mp. 116-119°C. ^{1}H-NMR ($CDCl_3$, 200 MHz) 7.35 (*d*, J=8.8, H-2'/6'), 6.89 (*d*, J=8.6, H-3'/5'), 6.66 (*d*, J=10.0, H-4''), 5.52 (*d*, J=10.8, H-3''), 5.37 (*dd*, J=3.6, 12.6, H-2), 5.14 (*t*, J = 7.6, H-2'''), 3.22 (*d*, J=7.8, H-1'''), 3.05 (*dd*, J=12.6, 17.2, H-3ax), 2.82 (*dd*, J=3.2, 17.0, H-3eq), 1.65 (*s*, H-4'''), 1.65 (*s*, H-5'''); ^{13}C-NMR ($CDCl_3$ 50 MHz) δ 196.4 (C-4), 160.0 (C-7), 159.4 (C-8a), 156.5 (C-5), 156.0 (C-4'), 131.1 (C-1'), 127.7 (C-2'/6'), 126.0 (C-3'''), 122.5 (C-2'''), 115.6 (C-4'''), 115.5 (C-3'/5'), 108.5 (C-8), 102.6 (C-4a), 102.6 (C-6), 80.1 (C-2''), 78.5 (C-2), 43.3 (C-3), 25.8 (C-4'''), 21.5 (C-1'''), 17.8 (C-5''') (Figure 2).

The compound was isolated from the DCM crude extract as yellow crystals; mp. 116-119°C. It was analyzed by ^{1}H NMR spectrum as follows; the first signal was identified as a hydroxyl proton singlet stabilized by chelation with the carbonyl group and resonating at δ 12.24 (1H, *s*, H-1). The second signal integrating into two protons was identified as an aromatic proton doublet resonating at δ 7.35 (2H, *d*, *J* = 8.8Hz, H-2´, H-6´) which showed significant correlation from COSY spectrum with (H-3´, H-5´). The third signal integrating into two protons was aromatic proton doublet resonating at δ 6.89 (2H, *d*, *J* = 8.6 Hz, H-3´, H-5´). The fourth signal was a vinylic proton doublet resonating at δ 6.66 (1H, *d*, *J* = 10Hz, H-4´´). The fifth signal was a proton doublet δ 5.52 (1H, *dJ* = 10.8 Hz, H-3´´). The sixth signal was a proton double doublet δ5.37 (1H, *dd*, *J* = 3.6 Hz, *J* = 12.6 Hz, H-2). The seventh signal was a proton assigned as δ 5.14 (1H, *t*, *J* = 7.8 Hz, H-2´´´) which showed COSY correlation with (H-1´´´). The eighth signal was assigned as δ 3.22 (2H, *d,* *J* = 7.8 Hz, H-1´´´). The ninth signal was a double doublet assigned 3.05 (1H, *dd*, *J* = 12.6 Hz, *J* = 17.2 Hz, H-3ax).

The tenth signal was assigned as δ 2.82 (1H, *dd*, *J* = 3.2Hz, *J* = 17.0 Hz, H-3eq). The eleventh signal was identified as a proton singlet δ 1.65 (6H, *s*, H-4´´´/ H-5´´´). The twelfth signal was assigned as δ 1.45 (3H, *s*, H-Me_2) (Yenesew et al., 2009).

The ^{13}C NMR spectrum showed 21 carbon signals. The hydroxylated methine carbon was identified as δ 78.5 (C-2) and a methylene carbon resonating at δ 43.3 (C-3). The highly deshielded quarternary carbon was identified as a carbonyl carbon at δ 196.4 (C-4). The quartenary aromatic carbons were identified as, δ 102.6 (C-4a), δ 156.5(C-5), δ 102.8 (C-6), δ 160.0 (C-7), δ 108.5 (C-8) and δ 159.5 (C-8a). The other aromatic carbon atoms were identified as δ 131.1 (C-1´), δ 127.7 (C-2´/6´) and δ 115.6 (C-3´/5´). The prenyl ring showed shifts at δ 80.0 (C-2´´), δ 126.0 (C-3´´), δ 115.6 (C-4´´). The hydroxylated olefinic group attached to benzene showed chemical shifts at δ 21.5 (C-1´´´), δ 122.5 (C-2´´´), δ 25.8 (C-4´´´) and δ 17.8 (C-5´´´). Most chemical shifts were in close agreement with those of lupinifolin (Yenesew et al., 2009).

Compound 4

White crystals in chloroform; mp. 131-133°C. The IR λ_{max} (KBr) 3423 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.20 (1H, t), 3.22 (1H, m), 1.06 (3H, s), 0.90 (3H, s), 0.85 (3H, s), 0.80 (9H, s); ^{13}C-NMR (Chloroform 75 MHz) δ 139.6 (C-5), 124.4 (C-6), 79.1 (C-3), 59.1(C-17), 55.2 (C-14), 47.7 (C-9), 46.9 (C-24), 42.1 (C-4), 41.5 (C-13), 39.7 (C-12), 37.1 (C-1), 37.0 (C-10), 34.8 (C-20), 33.8 (C-22), 31.9 (C-8), 31.3 (C-2), 31.1 (C-7), 29.4 (C-25), 28.1 (C-16), 26.2 (C-23), 23.4 (C-28), 23.3 (C-19), 23.3 (C-21), 23.3 (C-15), 21.4 (C-11), 18.0 (C-26), 17.5 (C-27), 14.1 (C-18), 14.1 (C-29).

The compound was obtained from hexane/ DCM extract, with a melting point of 128 to 131°C. On the TLC plate the compound had an R_f of 0.6 in hexane-DCM (1:1). When the plates were sprayed with p-anisaldehyde the spot turned purple suggesting that the compound was a triterpenoid (Dey and Harborne, 1991). The ^{1}H-NMR spectrum displayed three regions namely; aliphatic, hydroxylated and allylic regions. The signals appearing as triplet at δ 5.20 suggested the presence of a double bond at a quaternary carbon atom. A multiplet centred at δ 3.22 was a characteristic of a proton germinal to a hydroxyl group at C-3 of triterpenoids. Six signals representing methyl groups were observed at δ 0.90, 0.96, 0.98, 1.00, 1.06 and 1.25 which are characteristics of a triterpenoid (Ahmad et al., 1996).

^{13}C-NMR spectra displayed signals of olefinic carbon atoms at δ 139.6 and 124.4 supporting the presence of a double bond at the end of a fused ring between C-5 and C-6 (Ahmad et al., 1996). The shifts at 14.1, 15.5, 16.8, 18.0, 23.4 and 18.4 were assigned to the methyl groups. The spectral data of compound 59 were in close agreement with those of 3β-sitosterol, whose structure is shown below (Alam et al., 1996).

Compound 5

White crystals soluble in chloroform, mp. 174-176°C; The IR λ_{max} (KBr) 3423 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.35 (1H, m), 5.15 (1H, dd), 5.05 (1H, dd), 3.51 (1H, m), 1.10 (6H, s), 1.05 (3H,s) 1.00 (3H, s), 0.90 (6H, s); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 140.8 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), 56.9 (C-17), 56.0 (C-14), 51.3 (C-24), 50.2 (C-9), 42.3 (C-4, 13), 39.8 (C-12), 37.3 (C-1), 36.5 (C-10), 36.1 (C-20), 31.9 (C-26), 31.9 (C-25), 31.9 (C-2), 28.3 (C-16), 25.4 (C-28), 24.3 (C-15), 21.1(C-11, 21), 19.4 (C-19), 19.1 (C-27), 12.2 (C-29), 11.9 (C-18).

Compound 60 also obtained from hexane/ DCM extract, had a melting point of 173 to 175°C. On the TLC plate the compound had an R_f of 0.6 in hexane-DCM (1:1). When the plates were sprayed with p-anisaldehyde the spot turned purple suggesting that the compound was a triterpenoid (Dey and Harborne, 1991). The ^{1}H-NMR spectrum displayed several signals between 1.25 and 0.64 characteristic of a triterpenoid. The diagnostic chemical Shift values of the methyl protons for C-18 and C-19 appeared as singlets at δ 1.07 and 1.25 respectively. Four methyl groups appeared at δ 0.92, 0.93, 1.00 and 1.02 (Dey and Harborne, 1991). The multiplet at δ 3.51 suggested the presence of a proton attached to a hydroxylated carbon atom. A doublet at δ 5.35 suggested the presence of a double bond at a quaternary carbon atom. Two doublets of a doublet signal at δ 5.15 and 5.02 suggested the presence of a double bond on the side chain of a triterpenoid.

^{13}C-NMR spectra displayed 29 carbon atoms confirming compound **5** (Figure 4) was a modified triterpenoid. It showed signals at δ 140.8 and 121.7 confirming the presence of olefinic carbon atoms with more deshielded signals assignable to quaternary carbon at the bridge. The signal at δ 138.3 and 129.3 that lacked in the spectra of compound **4** (Figure 3) represented the olefinic carbons at the side chain. The peak at δ 71.8 was assigned to C-3 due to the presence of the hydroxyl group common to this class of compounds. Three signals at δ 36.1, 36.5 and 42.3 were associated with the three quaternary carbon atoms. The spectral data compared closely to that of 3β-stigmasterol, whose proposed structure is shown below (Alam et al., 1996). This compound is being reported from *E. excelsa* for the first time. Glutinosalactone A (**1)** and glutinosalactone B (**2)** exhibited high activity against all the three micro-organisms in which glutinosalactone A (**1**) had activities of 11, 11 and 10 mm against *S. aureus*, *B. subtillis*, and *E. coli*, respectively while glutinosalactone B 2 had activities of 10, 11 and 10 mm against *S. aureus*, *B. subtillis* and *E. coli,* respectively. Lupinifolin (**3**) had mild

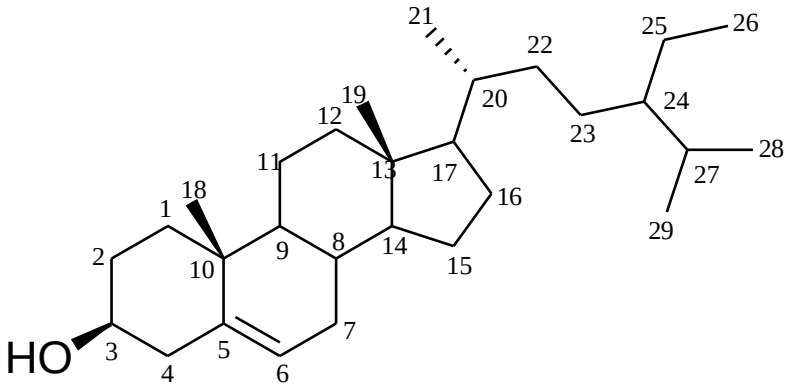

Figure 3. Compound 4: Sitosterol.

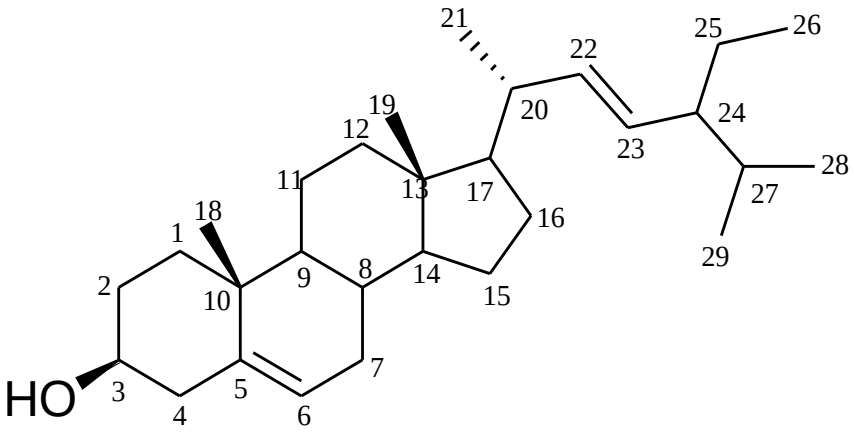

Figure 4. Compound 5: Stigimasterol.

Table 4. Antibacterial and antifungal activities of the isolated compounds from the stem bark.

Compound	*S. aureus*	*B. subtillis*	*E. coli*	*A. niger*	*C. albicans*
Glutinosalactone A **1**	**11**	**11**	**10**	15	11
Glutinosalactone B **2**	**10**	**11**	**10**	10	11
Lupinifolin **3**	**8**	**8**	**8**	9	9
3β- sitosterol **4**	**7**	**6**	**6**	8	6
3β- stigimasterol **5**	**7**	**6**	**6**	8	6
Gentamycin (+ve control)	**17**	**17**	**17**	16	16
Nystatin (+ve control)	**-**	**-**	**-**	16	16

activity of 8 mm against all the three micro-organisms. 3β-sitosterol (**4**), 3β- stigimasterol (**5**) had mild activity on *S. aureus* only. Glutinosalactone A (**1**) had the highest activity with inhibition zones of 15 mm against *A. niger* and 11 mm against *C. albicans* while glutinosalactone B (**2**) had activity of 10 mm against *A. niger* and 11 mm against *C. albicans*. Lupinifolin **(3)** was moderately active with inhibition zone of 9 mm against the two micro-organisms. 3β sitosterol and 3β stigimasterol were both inactive in C. albicans but had mild activities of 8 mm respectively against *A. niger* (Table 4).

E. excelsa has a wide range of phytochemical

components responsible for its biological activity. Amongst the phytochemicals identified are flavonoids and terpenoids. From these results it is evident that the crude extracts had substantial activity against tested micro-organism but the activities of the isolated compounds were much lower. This is attributed to the synergistic, antagonistic and promotional effects resulting from the various compounds that were present in the crude extracts (Gathirwa et al., 2008). At the same time the methanol extract showed highest activity because it is the most polar solvent hence during extraction several compounds in this extract brings about synergism.

Conclusions

The reported activities of crude extracts and isolated compounds justify the usage of *E. excelsa* as an anti-dote for snake bites and in treatment of arthritis by the communities in Western Kenya. The plant extracts have diverse compounds, flavonoids and terpenoids included. Methanol, ethyl acetate and dichloromethane extracts were active against some bacteria and fungi. Methanol extract had strongest activity of 15 mm against *S. aureus*, 15 mm on *A. niger* and 13 mm on *C. albicans.* 14 and 12 mm were recorded against *A. niger* and *C. albicans* respectively in the dichloromethane.

Phytochemical characterization of compounds isolated from the stem bark of the plant resulted in the isolation of four triterpenoids and one flavonoid. Methanol extracts contained, triterpenoids; glutinosalactone A (**1**) and glutinosalactone B (**2)** were isolated. From hexane-DCM extract, Lupinifolin (**3**), sitosterol (**4**) and 3β-stigimasterol (**5**) were obtained. Glutinosalactone A (**1**) had highest activity of 11 mm against *S. aureus* and *B. subtillis* while glutinosalactone B (**2)** had high activity of 11 mm against *B. subtillis*. This justifies the greatest activity of the MeOH crude extract. Lupinifolin **(3)** had mild activity of 8 mm against all the three micro-organisms. Glutinosalactone A (**1**) exhibited an inhibition zone of 15 mm against *A. niger* and 11 mm against *C. albicans* while glutinosalactone B (**2**) had an inhibition zone of 10 mm against *A. niger* and 11 mm against *C. albicans.* Flavonoid **(3)** had an activity of 11 and 9 mm against *A. niger* and *C. albicans*, respectively. Sitosterol (**4**) and 3β-stigimasterol (**5**) had mild activity against *A. niger* and were inactive against *C. albicans*.

It is evident that crude extracts contains many compounds and this accounts for the high activity of the crude compared to the isolated pure compounds. This many compounds bring about promotional and synergist effects (Gathirwa et al., 2008).

RECOMMENDATIONS

Further studies should be done on other parts of the plant such as the leaves, roots and the seeds to isolate more bioactive compounds

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical investigation, antinociceptive activity and cytotoxicity of crude extracts of *Calea uniflora* Less.

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***Calea uniflora* Less. is a medicinal plant used in the treatment of inflammation and haematomas in southern Brazil. The aim of this study was to investigate the antinociceptive effects and cytotoxicity of *C. uniflora*. Regarding phytochemical evaluation, the crude extracts of plant were analysed by high-performance liquid chromatography (HPLC). Antinociceptive activities utilised models on chemical and thermal stimuli *in vivo*. To evaluate cytotoxic activity, the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide method (MTT test) was utilised *in vitro*. Phytochemical analyses verified the presence of flavonoids and sesquiterpenes. Regarding antinociceptive activity, the models produced significant results that correspond to chemical at doses of 100 and 300 mg/kg of the crude extract as compared to the control groups, respectively. The rota rod model showed satisfactory results, since the extract did not cause motor incoordination and sedation in this experiment. In the *in vitro* cytotoxic tests, the crude extracts and ethyl acetate and butanolic fractions produced IC_{50} values greater than 58 µg/mL with the HaCaT lineage and 48 µg/mL with the B16-F1 lineage; thus, these values did not produce cytotoxic effects. According to these results, flavonoids and alkaloids were found in *C. uniflora* extracts. Pharmacological activities were also detected as reported by the local population that uses this plant in traditional medicine, especially antinociceptive and cytotoxic activities.**

Key words: Antinociception, cytotoxicity, phytochemistry, *Calea uniflora*, medicinal plant.

INTRODUCTION

Medicinal plants are a means of obtaining molecules to be exploited therapeutically, and many compounds isolated from plants remain promising as new drugs (Atanasov et al., 2015). Many species are used

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empirically, without scientific support for efficacy and safety, which shows that in a country like Brazil, with enormous biodiversity, there is a huge gap between the supply of plants and little research (Oliveira et al., 2014; Santos et al., 2014). Among the great diversity of plants, there is the Astereaceae family, which includes different *Calea* genera (Youssef et al., 2013). The genus *Calea* consists of 110 species that are distributed from northern to southern Brazil (Moura and Roque, 2014).

Moreover, some *Calea* species have been studied for their biological activity, which suggests that great scientific interest in this genus exists (Mondin et al., 2015; Nascimento and Oliveira, 2004; Nascimento et al., 2002). Some species of this genus were investigated for their medicinal properties including the following: *Calea pinnatifida* (Lima et al., 2015) with leishmanicidal activity; *Calea urticifolia* (Yamada et al., 2004) used to treat oliguria and gastroenteritis; *Calea zacatechichi* (Leonti et al., 2003) used for dermatological/respiratory ailments and gastrointestinal purposes; *Calea serrata* Less. (Ribeiro et al., 2008) used to treat ulcers and liver problems and *Calea glomerata* (Guerrero et al., 2002) used as an antihypertensive.

Among these species, *Calea uniflora* has gained popularity due to its use for the treatment of diseases (Ferraz et al., 2009). *C. uniflora* Less. is native to the southern region but is also found in the southeast and midwest regions of Brazil (Mato Grosso do Sul, Minas Gerais and São Paulo), Argentina, Uruguay and Paraguay (Nakajima and Semir, 2001). This plant, popularly known as ‘arnica da praia’ or arnica, is used as a medicine in the southern region of Santa Catarina (Mondin et al., 2015). The local population of the region uses the plant for anti-inflammatory activity, pain, bruising and rheumatism (Rossato et al., 2012).

A study has shown that the most commonly used pharmaceutical form in this region is the flowers in an ethanol tincture (Rossato et al., 2012). *C. uniflora* has been little studied. Previous studies showed that this plant is rich in constituents such as lactones, sesquiterpenes, flavonoids, saponins and derivatives of p-hydroxyacetophenone (Lima et al., 2015). Other phytochemical studies by Nascimento and Oliveira (2004) showed that *C. uniflora* contains the compounds, glucoside-5-deoxyflavone, 3’,4’,7-trihydroxyflavone-7-O-β-glucopyranoside, 2’,4-dihydroxy-3-methoxychalcone-40-O-β-glucopyranoside and quercetin-3-O-β-galactopyranoside. Regarding biological activities of *C. uniflora*, leishmanicidal activities (Nascimento et al., 2007), antifungal properties (Nascimento and Oliveira, 2004), trypanocidal action (Nascimento et al., 2002) and genotoxicity (Ferraz et al., 2009) have been studied.

So, in this study, the preliminarily phytochemical profile of crude extracts and fractions, antinociceptive and muscle relaxant activity and cytotoxic potential of crude extracts and fractions obtained from flowers of *C. uniflora* were analysed.

MATERIALS AND METHODS

Plant material

The aerial parts (inflorescence) of *C. uniflora* were identified by Dr. Vanilde Citadini Zanette and Mara Rejane Ritter and were collected in January 2013 in Balneário Rincão (GPS position 28°48'20.0" S 49°14'45.3" W). The voucher specimen was deposited in the CRI Herbarium of Dr. Raulino Reitz of the Universidade do Extremo Sul Catarinense (UNESC-SC), Brazil, CRI 10304.

Preparation of extracts

The plant material was dried at approximately 50 to 60°C and cut into small pieces. The flowers and leaves were crushed on a type mill, and the resulting matter was extracted with ethanol (70%) for 15 days with occasional stirring, followed by filtration and concentration of the filtrate by rotary evaporation. The flower extract was subsequently subjected to a process of liquid-liquid partition with solvents of increasing polarity: ethyl acetate, dichloromethane and *n*-butanol. So, three fractions were obtained for use in the experiments earlier. The partition methods were based on that adapted from Cechinel-Filho and Yunes (2009).

Phytochemistry

Crude extracts of leaves and flowers of *C. uniflora* fractions were subjected to HPLC to investigate the compounds present. For the high-performance liquid chromatography (HPLC) analysis, measurements were performed on a Waters Spherisorb ODS-2 C18 column (150 × 4.6 mm, 5 µm particle size) (Waters Technology Ireland, Ltd., Wexford, Ireland) protected by an in-line filter and set at room temperature. Peak detection was performed online using a diode array detector (HPLC 540 DAD, Kontron instruments, Montigny-le-Bretonneux, France) at 280 and 310 nm, and absorption spectra (210 to 400 nm) were recorded every second directly on the HPLC-separated peaks. The solvents used for separation were HPLC-grade acetonitrile (solvent A) and 1% phosphoric acid (concentrated)/10% acetic acid (glacial)/5% acetonitrile (v/v/v) in water (solvent B). The linear gradient elution program was ran as follows: 0 min, 100% (B); 30 min, 70% (B); 40 min, 100% (A) (Giusti et al., 1999).

An amount of 1 mg of crude extract of *C. uniflora* was mixed with 1 mL of ethanol at room temperature, producing a freshly prepared solution of 1 g/L. The extract was stirred in a vortex for 10 min until diluted, filtered with a single-use filter unit, and directly injected (20 µL in volume) into the HPLC system.

Pharmacological assays

Animals

Swiss mice weighing between 20 and 30 g were purchased from the vivarium at UNESC. The animals were housed under controlled light (12:12 h light-dark cycle) and temperature conditions (23 ± 1°C) with access to water and food *ad libitum*. The groups received treatments of appropriate doses of *C. uniflora* crude flower extracts or vehicle, administered orally (p.o.). The experimental protocol was approved by the local ethics committee (Ethics Committee on Animal Use, CEUA of UNESC 019/2013).

Antinociceptive activity

Acetic acid-induced writhing in mice

Acetic acid (0.6%) was injected into the peritoneal cavities of mice,

which were placed in a large glass cylinder. The intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. *C. uniflora* was administrated at doses of 30, 100 and 300 mg/kg (p.o.) given 60 min prior to acetic acid injection. The antinociceptive activity was expressed as writhing scores during a 20 min period (Choi et al., 2003; Koster et al., 1959).

Formalin test

Mice were pre-treated with a vehicle (saline) or *C. uniflora* (30, 100 or 300 mg/kg p.o.) 60 min before the start of the experiment. The positive control, morphine (10 mg/kg s.c.), was administrated 30 min before the start of the experiment. For nociception induction, an intraplantar injection of a 2.5% formalin solution (20 µL) was injected into the hind paw plantar surface, and the animals were individually placed in transparent observation chambers. The time spent licking the injected paw was recorded and expressed as the total licking time in the early phase (phase 1; 0 to 5 min) and late phase (phase 2; 20 to 30 min) after formalin injection (Hunskaar and Hole, 1987).

Hot plate test

Mice were pre-treated with *C. uniflora* (30, 100 or 300 mg/kg, p.o.) 60 min earlier, or morphine (10 mg/kg, s.c.), the positive control, 30 min before being placed on a metal plate warmed to 52 ± 0.5°C. The time that elapsed between the start of the experiment and the appearance of reactions (latency, in seconds) to the thermal stimulus, such as lifting or licking the paws, was recorded as an index of nociception. To avoid damage to the animals, the maximal time standing on the plate was limited to 30 s (Le Bars et al., 2001).

Rotarod

The animals were treated with *C. uniflora* (30, 100, or 300 mg/kg, p.o.) 60 min after being subjected to the equipment, according to the methodology of Oliveira et al. (2008). The cut-off time used was 60 s. The animals had been selected 24 h previously by eliminating those mice that did not remain on the bar for 60 s.

Cytotoxicity assay

Cytotoxicity bioassay and cell culture

Murine cancer (B16-F1) and keratinocyte-type cell lines (HaCaT) were used. The cells were maintained as previously described except for the use of RMPI as medium culture with 5% FBS instead of 10% FBS. Diluents (300 mM) of test compounds were prepared in dimethyl sulfoxide (DMSO) and added to each well 1 day after seeding. The amount of DMSO was adjusted to give a final concentration lower than 0.1%. Cells were cultured according to the methodology described previously (Millot et al., 2007).

Cytotoxic assay

Cytotoxic activity was determined in B16 cells seeded at 20 000 cells/mL at day 0. Compounds were serially diluted in RPMI 1640 at day 1 in a 96-well plate; with concentrations ranging from 2.5 to 300 µM. Incubation was performed at 37°C in an atmosphere of 10% CO_2. After 48 h of incubation, corresponding to day 3, compounds were added a second time. After a new 48 h incubation period, cell growth and viability were measured at day 5, using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Millot et al., 2007). Each experiment was repeated at least three times, and three different wells were used for each concentration.

Statistical analysis

The results are expressed as means ± SEM. Statistically significant differences between groups were measured using one-way analysis of variance (ANOVA) followed by Dunnett's test. *$p < 0.05$ or **$p < 0.01$ was considered statistically significant. The geometric mean IC_{50} values were determined by nonlinear regression from individual experiments using GraphPad Prism software.

RESULTS

Phytochemistry

It was found that the crude extracts of flowers had more peaks (Figure 1A and B), which indicated the presence of more compounds in this extract.

In addition, the crude extract of *C. uniflora* flowers was subjected to preparative thin-layer chromatography (TLC). The results showed the presence of flavonoids and sesquiterpenes, compared with the references used in HPLC analysis.

Antinociceptive activity

Acetic acid-induced writhing in mice

The treatment of mice with *C. uniflora* crude extract given by the oral route, at doses of 100 and 300 mg/kg, significantly reduced the writhing response induced by injection of 0.6% acetic acid, as shown in Figure 2.

Formalin test

In the formalin test (Figure 3), the pre-treatment of animals with *C. uniflora* promoted a significant reduction in the nociceptive response in the second phase only at higher doses (B), while in the first phase, it was ineffective (A).

Hot plate

The treatment with *C. uniflora* at doses of 300 mg/kg by the oral route showed significant results of tolerance to pain on the hot plate (52°C) compared to the group treated with morphine (10 mg/kg, s.c.; positive control), since morphine and *C. uniflora* extract (300 mg/kg) increase the latency time, as shown in Figure 4.

Rotarod

In addition, the extract of *C. uniflora* had no significant

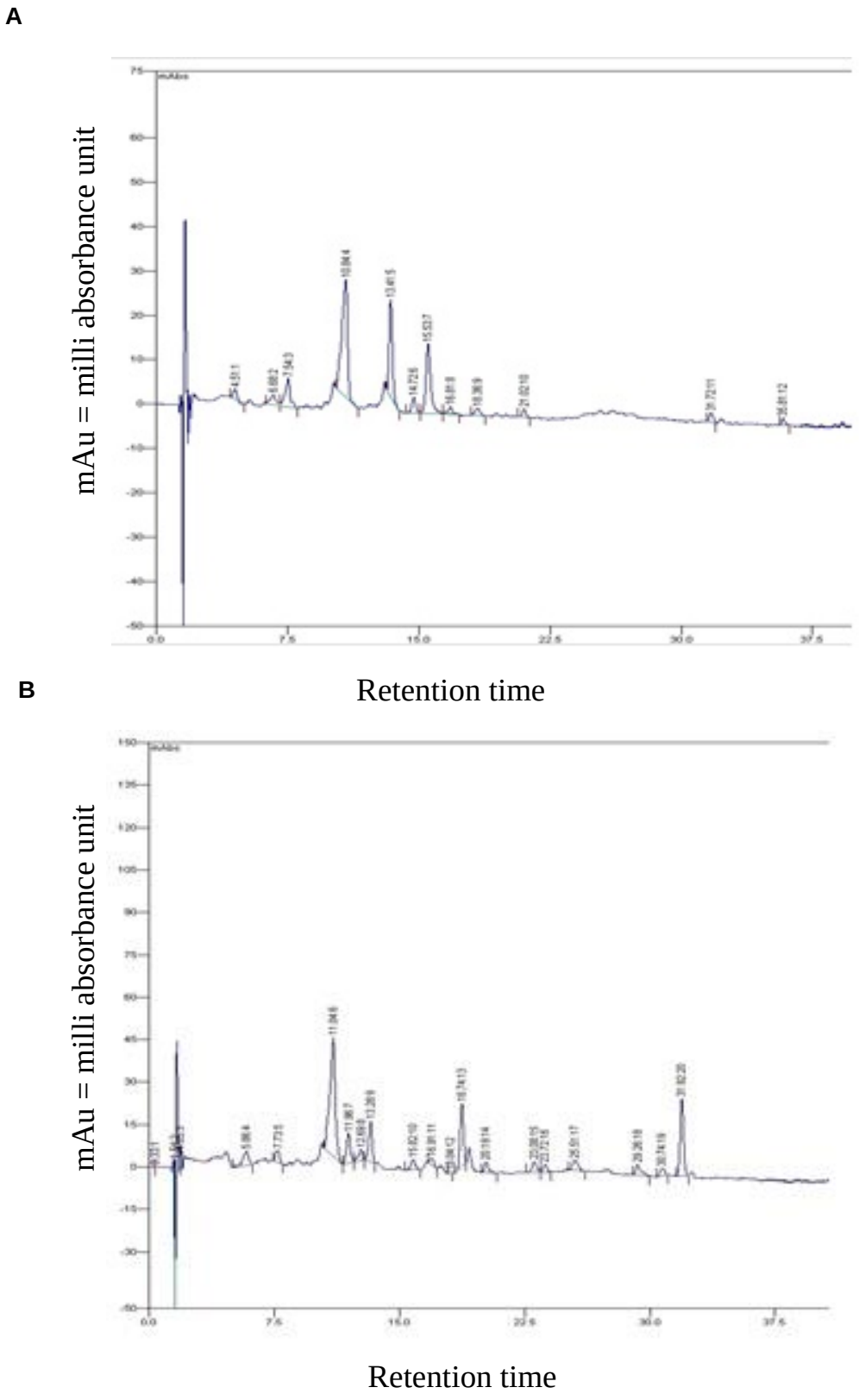

Figure 1. Chromatogram of the crude extracts of leaves (A) and flowers (B) of *C. uniflora* detected with a DAD 540 HPLC at 280 nm with a runtime of 60 min. Mobile phase: solvent A, 100% acetonitrile; solvent B, phosphoric acid (1%), acetic acid (10%), acetonitrile (5%) and water (84%).

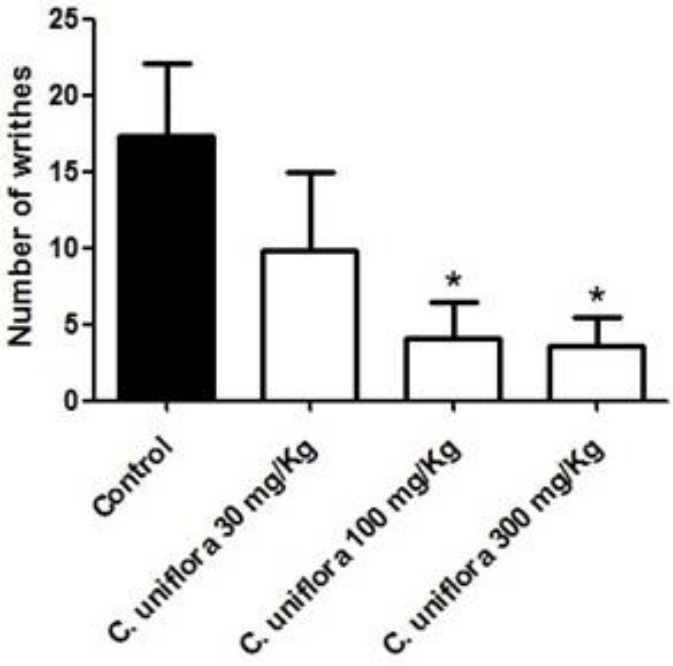

Figure 2. Effects of *C. uniflora* (30, 100 and 300 mg/kg) given orally 60 min before the administration of acetic acid to induce abdominal constriction. Each column represents the mean ± SEM of 8 to 10 animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (via analysis of variance [ANOVA] and Dunnett's test).

effect in the rotarod test, dismissing possible unspecific effects such as motor incoordination in mice (Figure 5).

Cytotoxicity assay

The crude extracts of leaves and flowers did not show a high degree of cytotoxicity as compared to the controls. The ethyl acetate and butanol fractions of the flowers did not show a high degree of cytotoxicity, but the dichloromethane fraction showed a significant inhibition in both B16-F1 and HaCaT cells as compared to the vincristine and doxorubicin controls (Figure 6). The controls used have different mechanisms of action: vincristine acts in the inhibition of the mitotic spindle by binding to the microtubule proteins and consequently disrupting cell division in metastasis, while the doxorubicin mechanism of action specifies on cell cycle arrest by intercalating DNA and inhibiting topoisomerase II.

DISCUSSION

C. uniflora is widely known and used in Brazilian population as a medicinal plant in wound healing, muscle pain, bumps and hematomas, flu and colds, insect bites and toothache. Despite its vast use in southern Brazil, this plant has been the subject of only a few studies and so we decided to analyse the crude extract in order to ensure safe use of the species and validate its popular use.

In the phytochemical analysis, it was observed that the chromatograms obtained by HPLC showed differences in the amounts of compounds of the crude extracts of flowers as compared to the crude extracts of the leaves (the extracts of flowers showed more peaks than the leaf ones). These differences may be related to the plant organs in which the compounds are stored (Gobbo-Neto and Lopes, 2007). Chromatographic analyses by TLC of the ethyl acetate fraction of *C. uniflora* after purification in preparative TLC indicated the presence of some chemical constituents. The ethyl acetate fraction included predominantly flavonoids and sesquiterpenes.

Flavonoids exhibit a great variety of therapeutic properties including antioxidant, anti-inflammatory, antifungal, antimicrobial and anticancer (Favarin et al., 2013; Kim et al., 2004; López-Posadas et al., 2008; Nijveldt et al., 2001). According to the Pastoral da Saúde Regional Sul IV, *C. uniflora* is popularly used for

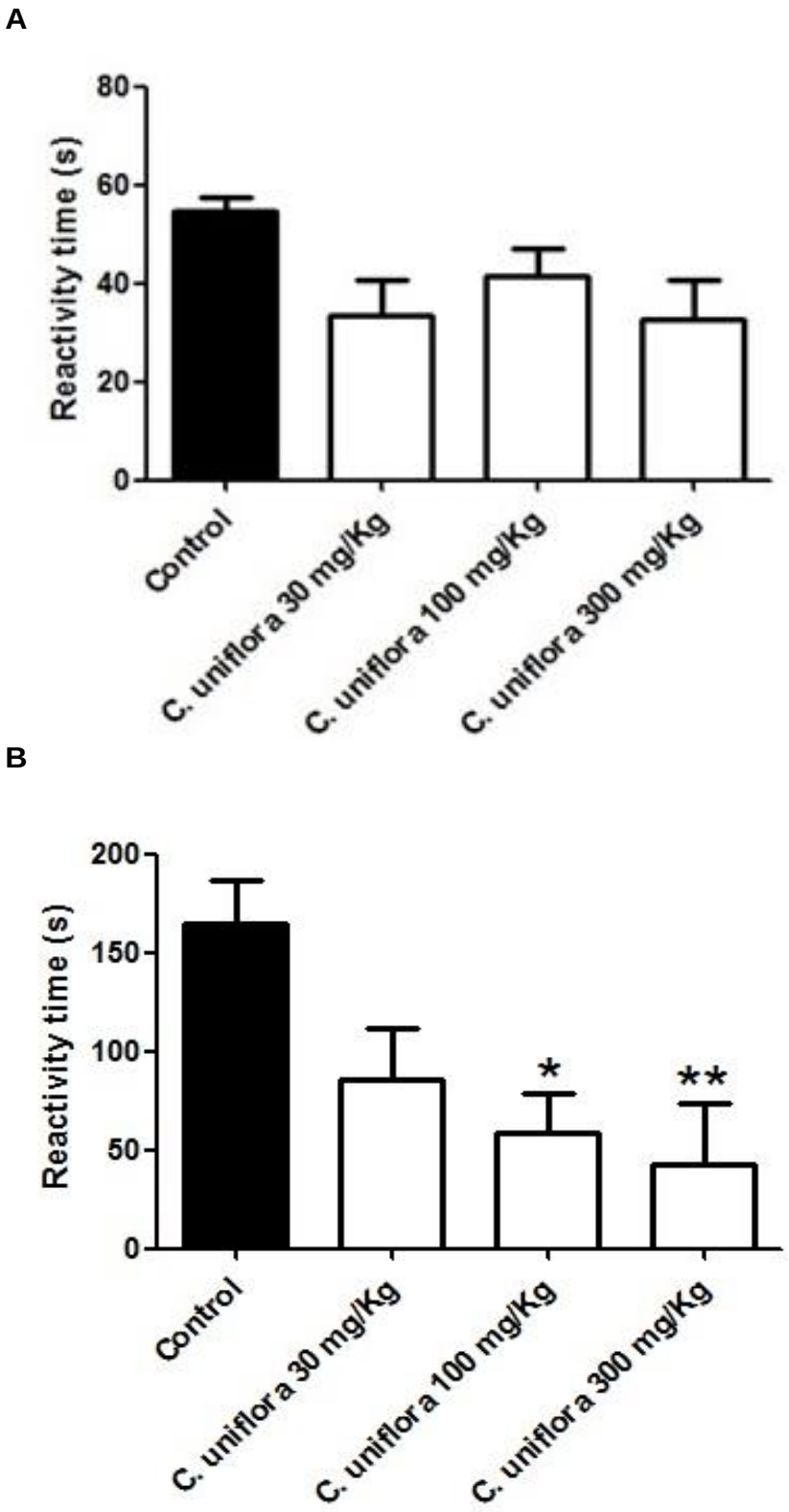

Figure 3. Effects of *C. uniflora* (30, 100, and 300 mg/kg) on the formalin test in the first (A) and second phases (B) administered orally 60 min before formalin-induced nociception. Each column represents the mean ± SEM of eight animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (using ANOVA and Dunnett's test).

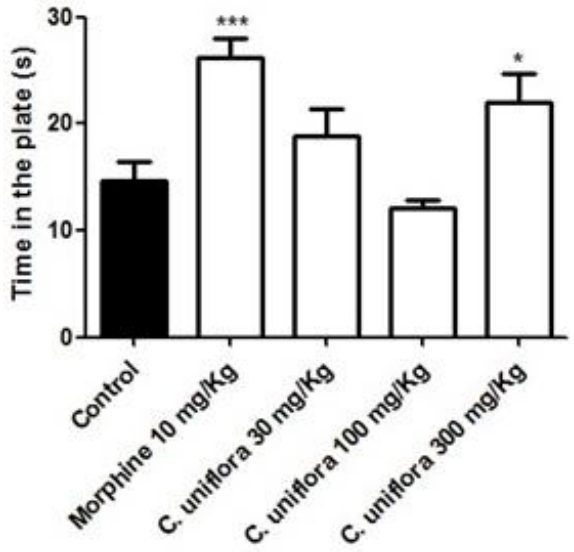

Figure 4. Effects of *C. uniflora* administered orally in the hot plate test. Animals were pre-treated orally with a vehicle, morphine (10 mg/kg, s.c.), or crude extracts of *C. uniflora* (30, 100, or 300 mg/kg) on a hot plate at 52°C. Each column represents the mean ± SEM of latency time of eight animals per group. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: **p* < 0.05 and ***p* < 0.01 as compared to respective control values (using ANOVA and Dunnett's test).

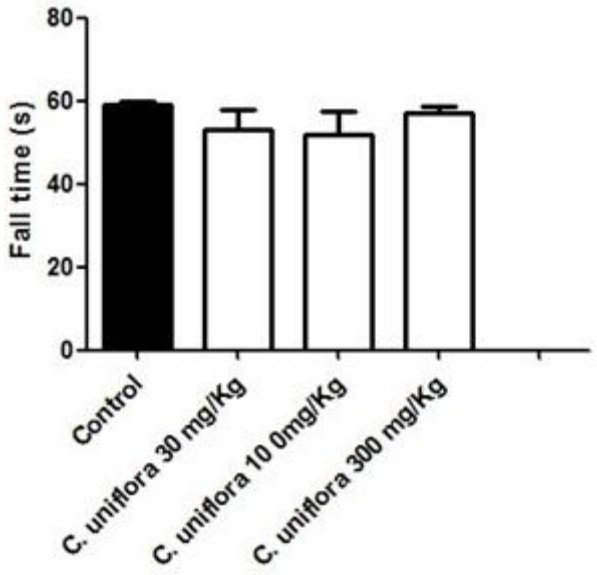

Figure 5. Effects of *C. uniflora* given 60 min prior to rotarod motor incoordination after administration of crude extract (30 to 300 mg/kg, p.o.) or vehicle. Each column represents the mean ± SEM of eight animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: **p* < 0.05 and ***p* < 0.01 as compared to respective control values (using ANOVA and Dunnett's test).

inflammatory processes and has a healing action.

The phytochemical composition and popular use, it was proposed to study the antinociceptive effects of *C. uniflora* extracts. The pharmacological studies were performed with the crude extracts of the flowers of *C. uniflora* to verify antinociceptive activity. Some experimental models based on chemical stimuli (writhing and formalin) and thermal stimuli (hot plate) can detect this effect. The writhing test is based on the number of twists that occur in response to peritoneal irritation produced by acetic acid, a similar inflammatory pain to peritonitis (Le Bars et al., 2001). The data obtained in this model were satisfactory for antinociceptive potential because crude extracts of *C. uniflora*, administered orally at doses of 100 and 300 mg/kg, reduced the number of writhes as compared to the controls. According to Rodrigues et al. (2011), plants indicated by 26 indigenous groups in Brazil are used for analgesic properties and contain predominantly alkaloids but also triterpenoids, phenolic compounds and coumarins in their chemical composition. Alvarenga et al. (2013) reported that metabolites, such as saponins and flavonoids, can be directly related to peripheral analgesia. According to the phytochemical experiments in this study, the chemical composition of *C. uniflora* contains alkaloids and flavonoids, and these compounds may be related to the antinociceptive action observed in the writhing model.

The formalin model allows for evaluation of two distinct phases of pain. In the first phase (up to 5 min after injection), called the neurogenic phase, activation of C and Aδ fibres occurs. After the first phase, the silent phase is initiated (5 to 15 min after injection), where the animal has no nociceptive behaviour. In the second phase, an inflammatory reaction in the peripheral tissue, referred to as the neuropathic phase, occurs (15 to 30 min after injection) (Hunskaar and Hole, 1987). In this experiment, the plant extracts showed significant results only in the neuropathic phase at doses of 100 and 300 mg/kg. The second phase is characterised by inflammation, which is caused by local inflammation and release of hyperalgesic and inflammatory mediators that can cause inflammatory responses. Thus, it can be concluded that *C. uniflora* can exhibit anti-inflammatory activity which is related to the presence of secondary metabolites such as flavonoids. As reported by Mutoh et al. (2000), flavonoids, such as quercetin and apigenin, showed anti-inflammatory action, as these compounds cause inhibition of cyclooxygenase (COX-2) and nitric oxide synthase.

In order to verify the analgesic action at the central level, the hot plate test was performed. This model is characterised by producing a rapid response to noxious stimuli. The heat stimulates thermoreceptors, and these activate an unalterable activation sequence. In practice,

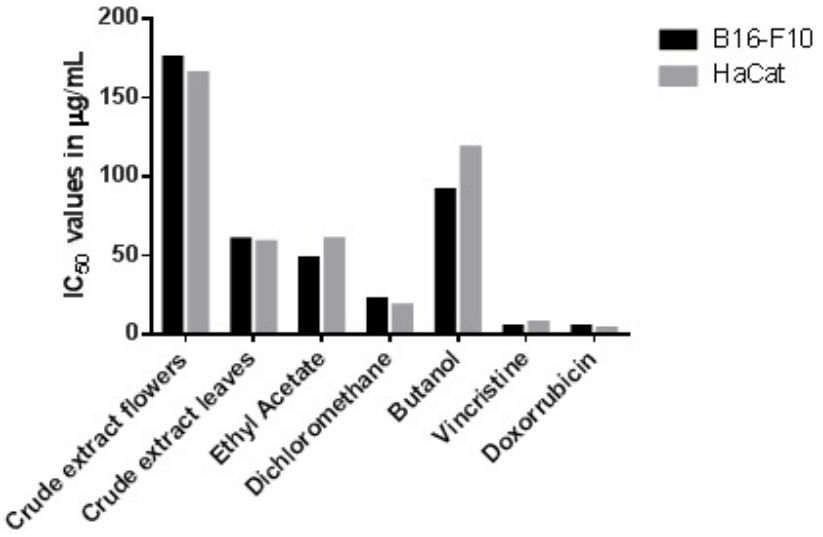

Figure 6. IC_{50} values of leaf and flower crude extracts of *C. uniflora* and of the ethyl acetate, dichloromethane and butanol fractions of flowers compared to the standards doxorubicin and vincristine of two cell lines, B16-F1 and HaCaT, made in triplicate. The IC_{50} was calculated using linear regression.

an animal quickly withdrawing its paw from the stimulus is a result of action at the central level. This model evaluates the antinociceptive activity of opioid drugs (Ankier, 1974; Hiruma-Lima et al., 2000; Le Bars et al., 2001). The results of this test indicated that *C. uniflora* has a central analgesic effect at doses of 300 mg/kg as compared to morphine. In studies of analgesic substances, possible alterations of motor performance, which can be produced by some potentially analgesic drugs, must be disclosed. The largest source of error in studies of drugs that interfere with the transmission of nociceptive response is the change in motor performance of the animal (Millan, 2002). Therefore, motor incoordination was evaluated through the rotarod test. The results showed that the crude extracts of *C. uniflora* did not alter the motor performance of animals at the doses tested (30, 100 and 300 mg/kg). Thus, it was shown that the reduction of nociceptive behaviour in animals was due to the analgesic effect and not changing motor performance.

In addition to the phytochemical and antinociceptive analysis, the cytotoxic potential of extracts and fractions of *C. uniflora* was evaluated, since this plant is widely used in the local region. The *in vitro* culture of cells is an important tool for studying the cytotoxic activity of substances with potential therapeutic activity (Freshney, 2001).

The evaluation of the cytotoxicity of *C. uniflora* showed that crude extracts of flowers showed a lower cytotoxic potential than the crude extract of leaves. The ethyl acetate and butanol fractions also showed no significant degree of cytotoxicity according to the National Cancer Institute of the United States criteria, which confirms the plant is safe for popular use, especially important since it is widely used in the region (Geran et al., 1972).

The screening program of plants requires that plant extracts with IC_{50} less than 20 mg/mL for crude extracts and 4 µg/mL for pure compounds exhibit cytotoxic potential. However, the dichloromethane fraction has more affinity with these types of compound, extracting more lipophilic substances. This may be responsible for exhibiting a greater cytotoxic potential when compared with other extracts and fractions (Geran et al., 1972). Therefore, the dichloromethane fraction becomes more interesting as it may present a promising effect.

Conclusion

Results from this study indicate that the crude extracts of *C. uniflora* flowers have more chemical compounds than the crude extracts of leaves. Flavonoids and alkaloids were identified in the ethyl acetate fractions; these plant compounds have been demonstrated to have pharmacological action.

The results also showed that the extracts and fractions of ethyl acetate and butanol of *C. uniflora* had IC_{50} values greater than 58 mg/mL for the HaCaT lineage and 48 mg/mL for strain B16-F1. Thus, these values exhibited no cytotoxicity, allowing people to use the plant safely. The dichloromethane fraction exhibited an IC_{50} of 18 mg/mL, showing significant inhibition as compared to the controls, vincristine and doxorubicin. Because dichloromethane showed a potential cytotoxicity higher than the other fractions, it requires further study.

The antinociceptive activities for both thermal and

chemical stimuli showed significant results for crude extracts of *C. uniflora* flowers at doses of 100 and 300 mg/kg. These effects can be related to the chemical compounds present, with sesquiterpenes at the central level and flavonoids at the peripheral level. The extracts of *C. uniflora* do not induce motor incoordination or muscle relaxant activities.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Full Length Research Paper

Documentation of indigenous knowledge on medicinal plants used to manage common influenza and related symptoms in Luwero district, central Uganda

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Herbs are used in treatment of various diseases in Uganda. Influenza is one of the diseases that is treated by herbs, the disease previously known to be non fatal is progressively becoming a threat due to immune complications in people living with HIV, poor diets and the fear of fatal introduced strains like Bird Flu. An ethno botanical survey was conducted to document medicinal plants and preparations used in the management of influenza and related symptoms in Luwero, Central Uganda. Information was obtained using questionnaires, focus group discussions, key informant interviews, direct observations in households and field excursions. Information on plants/parts used, method of preparation, mode of administration, commonly used formulations and their dosages was documented. Twenty nine medicinal plants distributed in 17 families were documented; *Mangifera indica* family Anacardiaceae, *Ocimum basilicum* L., (Lamiaceae), *Psidium guajava* L. (Mytaceae) and *Mormodica feotida* L. (Curcubitaceae) were the commonly used plants respectively. The leaves were the most used part of the plants while decoctions were the commonly used method of preparation and the oral route was the main mode of administration. Antimicrobial assay and nutritional profile of some formulae is underway to standardize formulae that can be used against respiratory infections at household level.

Key words: Medicinal plants, influenza, malaria, indigenous knowledge.

INTRODUCTION

Influenza, commonly known as flu in Uganda, is a contagious respiratory infectious disease which is air borne and is widely spread especially in the hot windy temperatures (Hudson, 2009; Centre for Disease Control, 2016). For a long time, influenza has been a non-fatal disease in the Tropical African countries. However

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in the recent past; the disease has caused serious health concerns throughout the world, Tropical Africa being at the highest risk because of its devastating consequences on human and animal health (Qiao-Feng et al., 2012). Consequently, many Sub-Saharan African countries are likely to be at risk of influenza epidemic due to migration of Asian and European nationals for investments in the industrial and other sectors. Most importantly, it is necessary to take precaution and prepare for the influenza epidemic outbreak. As most countries in sub Saharan Africa continue to face challenges of HIV epidemic, patients have compromised immunities which put them at risk of influenza infection. The problem is aggravated by the change of diets from the nutritious half cooked or raw fruits and vegetables, staple boiled or steamed foods to fried and junk diets that have less immune stimulating properties and fewer minerals and vitamins that fight off various diseases including influenza. Although chemotherapy is the mainstay in the management of various diseases including influenza in Uganda, plants remain an important source of medicines especially for the rural poor. The use of plants in the treatment of various diseases has been embedded in many indigenous societies, leading scientists to depend on such plants for drug development. Among the various indigenous medicines used in the treatment of common diseases such as malaria are plants used in treatment of influenza. The use of plants as medicine has been attributed to the presence of bioactive components such as flavanoids, alkaloids, saponins and tannins (Evans, 2002; Farnsworth, 1994). Several studies have confirmed antiviral activities of medicinal plants (Rajbhandari et al., 2001; Gebre-Mariam et al., 2006; Qiao-feng et al., 2012).

Like other sub Saharan African countries, majority of the rural communities in Uganda use medicinal plants in the treatment of diseases including influenza. Considering the rate at which natural vegetation which are source of medicinal plants are being depleted due to increased population pressure on the land and climate change effects, many important medicinal plants are disappearing before they are even documented, leave alone being scientifically validated. Moreover, dyeing off of the old people who are the custodians of indigenous knowledge even worsens the situation. Because of poor conservation of indigenous knowledge, scientists have used ethno pharmacological survey as the main approach to search for important plant species that can be evaluated for development of efficacious drugs (Farnsworth, 1994). Although many of these ethno botanical surveys have been carried out in Uganda to document plants used in treatment of various diseases, no systematic study has particularly been carried out for documenting in depth information on medicinal plants used by communities in treatment of influenza and related ailments. In this study, we obtained indigenous peoples perception of the disease and documented the plants, parts used, mode of preparation and administration of medicinal plants and practices used by the local communities to treat influenza and related symptoms in Luwero District in Central Uganda. We also documented common formulae as used by the respondents in the surveyed areas.

MATERIALS AND METHODS

Study area

An ethno botanical survey was conducted in Luwero district (Figure 1), central Uganda in the two sub counties of Luwero and Butuntumula. Luwero district was chosen because there are organized Village Health Team (VHTs) members and herbalists at the community centre for traditional medicine, supported by the Ministry of Health. The research team also has established good working relationship with the leaders and some respondents. Luwero District is also one of the districts of Uganda that had experienced the Ebola viral disease outbreak in the recent past (Figure 1). The survey was carried out between May and June, 2012 to document community perception of the influenza and related infections symptoms, forms, causes and plants used for its management by the indigenous communities and related infections. Information was collected using individual interviews with semi structured questionnaires, focus group discussions, key informants and direct observation in the field from consenting adults who included both males and females of 18-60 years of age. A total of 60 households that mainly comprised of a village health team representative who are members of the community selected by Ministry of Health, Uganda to Coordinate Health programme at village level (MoH, 2010) and a few Traditional Health Practitioners (THPs) were interviewed. Information on community perception of manifestation of influenza disease, plants and parts used in the treatment, their preparation and dosage was documented. Family heads/representatives that had at least two children below the age of five were selected using Snowball method (Hardon, 2001) and interviewed. This was because children below five are the most vulnerable to influenza infection (Mbonye, 2004). After the individual survey, focus group discussions (FGD), each consisting of 10 members (both females and males) was conducted at the community centre for traditional medicine located at the district.

The FGD was to document priority plant, methods and formulae used in the treatments of influenza related symptoms. In depth interviews with key informants that included two elderly and five renowned THPs in the area were carried out to obtain in depth knowledge on community perception of influenza manifestation and its management. In addition, Allopathic Health officials at national health facility (Kasana Government Health Center in Luwero District) were also interviewed in order to get in depth knowledge of influenza frequency of occurrence among patients and its management at the health centre. Field excursions were carried out to identify the plants reported by VHTs and THPs and voucher specimen were collected. Voucher specimens were identified by a taxonomist at Makerere University National Herbarium, Kampala and then deposited at the Natural Chemotherapeutic Research Institute, herbarium in, Kampala.

RESULTS

The results of socioeconomic demographic characteristics of the respondents in this study are presented in Table 1. Most of the respondents (88.9%) were aged between 18 years to 55 years and. Majority (70.0%) were female with 87% being married. Approximately 57.0% of respondents

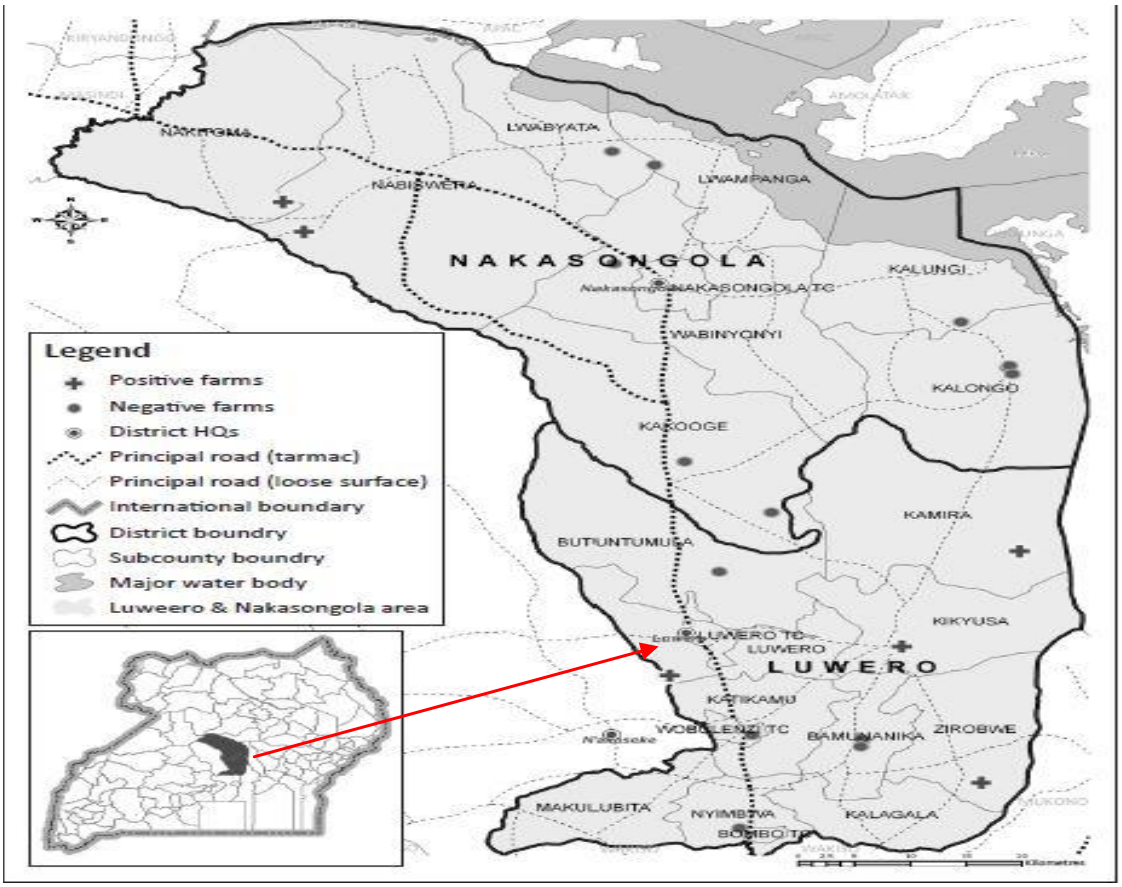

Figure 1. Map showing Luwero district in Uganda.

had family size of between 5-9 members. The education of most respondents indicated majority (56%) having completed primary level of education with most of the respondent (84.7%) being farmers as their main occupation.

Community knowledge of Influenza and its symptoms

The symptoms that were related with influenza by respondents are presented in Table 2. Most respondent (54.3%) identified running nose while sneezing was reported by 52.2% as the symptoms associated with influenza. Other symptoms reported by respondents included headache (39.1%), fever (34.1%), cough (28.3%), itchy throat (28.3%), red eyes (13.0%), change in breathing (10.9%). Other symptoms included blocked nostrils (8.7%), loss of appetite (8.7%), body weakness (6.5%), itchy nose (6.5%), body pain (4.3%), chest pain (4.3%), swollen eyes (4.3%), voice changes (4.3%), tears in eyes (4.3%), itchy ears (4.3%). Also mentioned by respondents were diarrhea, dry mouth, high body temperature, itchy eyes, salivating and the body filling cold (chills).

Nomenclature of influenza in Luwero district

Respondents further categorized influenza disease and gave it names according to the differences observed in its manifestation. The different categories and the proportion of there occurrence are summarized in Figure 2. The common occurrence of the disease (86%) was the mild infection termed "*Senyiga*" (Luganda dialect) and was mainly associated with symptoms such as running nose, mild headache, sneezing and tears in eye, however the respondents said that the patient has no other complications. Patients manifesting with "*Senyiga"* rarely visit the health centre because the condition allows them to do their daily activities. The other common type of manifestation (52.3%) was *"Lubyamira",* described by respondents to be severe illness that impairs the person from work. The patients manifesting with *Lubyamira* are likely to be bed ridden for a while, and to have productive cough in association. While the third type of manifestation was referred to as "*Yegu"* and this had symptoms like "Lubyamira" but mainly affects the elderly. Unlike "*Senyiga",* the respondents reported similarity in the symptoms of "*Yegu* and "*Lubyamira"* the difference was age of the individual. Lukusense and Seseba were rare

Table 1. Socio-economic demographic characteristics of respondents.

Variable	% respondents
Age	
18-35 years	46.7
36-55 years	42.2
>55years	11.1
Sex	
Female	69.6
Male	30.4
No. of people in house hold	
0-4 members	14.3
5-9members	57.1
10-14members	23.8
>14members	4.8
Marital status	
Single	2.2
Married	87.0
Divorced	4.3
Widowed	6.5
Education	
None	15.6
Primary	55.6
Secondary	28.9
Occupation	
Farming	84.8
Civil service	2.2
Student	2.2
Nursery teaching	2.2
Building	2.2
Business	2.2

Table 2. Knowledge of the respondents, on the symptoms of influenza in Luwero district.

Symptom	% respondents
Running nose	54.3
Sneezing	52.2
Headache	39.1
Fever	34.8
Cough	28.3
Itchy/sour throat	28.3
Red eyes	13.0
Change in breathing pattern	10.9
Blocked nostrils	8.7
Loss of appetite	8.7
Body weakness	6.5
Itchy nose	6.5
Body pain	4.3
Chest pain	4.3
Swollen eyes	4.3
Voice changes	4.3
Tears in the eyes	4.3
Itchy ears	4.3
Diarrhea	2.2
Dry mouth	2.2
High body temperature	2.2
Itch eyes	2.2
Salivating	2.2
Body feeling cold	2.2

N=60.

manifestations reported by respondents.

Mode of transmission of common influenza

The community knowledge on mode of transmission of common influenza are presented in Table 3. The respondents gave a diversity of the possible causes of influenza. Most respondent (40.0%) identified wind as major cause of influenza while 20% reported that sometimes a person is vulnerable to get infected with influenza due to diseases such as asthma. About 15% reported that a virus is the cause of influenza, but 13.3% said it was taking un-boiled water, smoke (11.1%), lack of body fluids (6.7%), dust, aeroplanes and staying near already sick people, transmission by birds, coldness, and dry seasons were cited by 4.4 and 2.2% of the respondents respectively.

Infection according to age

According the respondents, infection with influenza is not segregate as presented in Figure 3. Nearly half of the respondents reported that influenza affect all age groups. Although, 44.4% reported that it affects mostly children, 17.8% reported that affects new born babies. Meanwhile 15.6% reported those elderly people were most affected by influenza while 8.9% reported that pregnant women were also commonly infected by influenza.

Frequency of infection with influenza

The number of times and individual could be infected by annually was given by respondents as presented in Figure 4. Twenty percent of respondents reported that a person is infected by influenza any time and every 3 month respectively. About 14% and 12% of respondents said it be weekly and monthly, respectively. Eleven percent of the respondents reported that the disease affects an individual in almost every four months. Four percent could not estimate the number of times a person could be infected by influenza annually.

Method used by communities to treat influenza

The respondents reported various methods used to

Table 3. Community and traditional health practitioner's knowledge of possible causes of influenza.

Cause	% respondents
Wind	40.0
Vulnerability due to diseases e.g. asthma	20.0
Virus	15.6
Un boiled water	13.3
Smoke	11.1
Lack of fluids	6.7
Dust	4.4
Germ	4.4
Aero plane fumes	4.4
Being around sick people	2.2
Birds	2.2
Coldness	2.2
Dry seasons	2.2

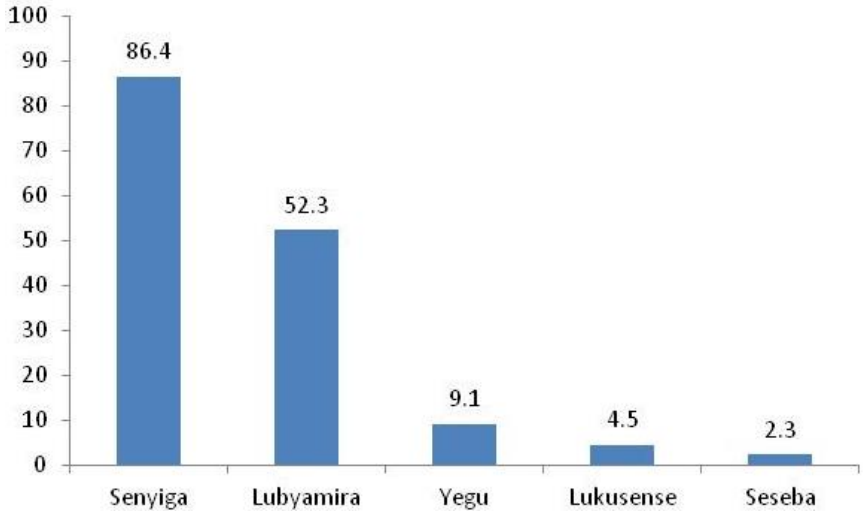

Figure 2. Different local names of influenza in Luwero district.

manage influenza at household in community are presented in Figure 5. Most respondent (45.2%) reported using allopathic while 19% used herbal medicine. About 35.7% reported that they combined both herbal medicine and allopathic medicine.

Medicinal plants used in treatment of influenza

The medicinal plants used for treatment of influenza and related symptom are presented in Table 4. The study revealed 29 medicinal plants distributed in 17 families. *Mangifera indica* (family Anacardiaceae), was the most commonly mentioned (67.4%) medicinal plant, followed by *Ocimum basilicum (39.5%), Psidium guajava (34.9%), Mormodica feotida (30.2%), Callisteon rigidus (27.9%) and Citrus limon (23.3%)* in that order. The family Myrtaceae had the highest number of plants, followed by Rutaceae, Lamiaceae and Asteraceae, respectively.

Information on percent availability, formulation and administration of herbal mecicines

The respondents gave information on the availability, formulation and administration of herbal medicine to treat influenza as presented in Table 5.

Habitats of the medicinal plants

The medicinal plants used for treatment of influenza and

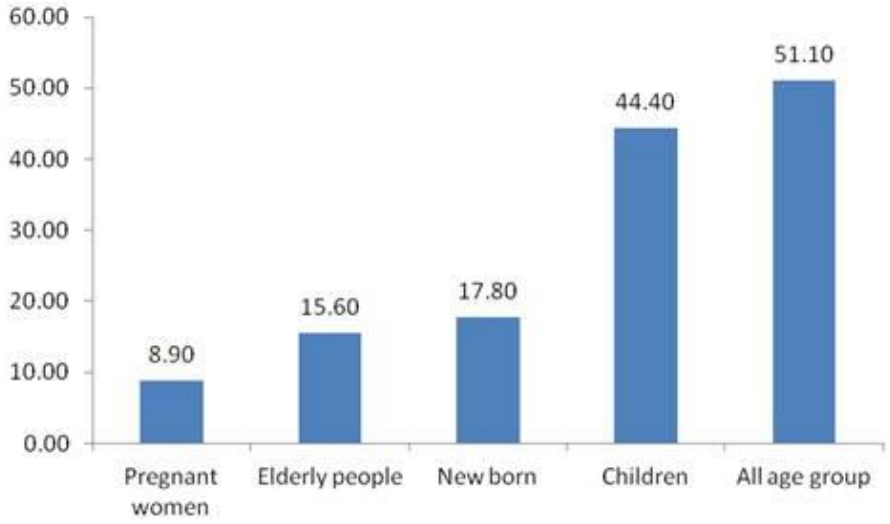

Figure 3. Age group affected by influenza in Luwero district.

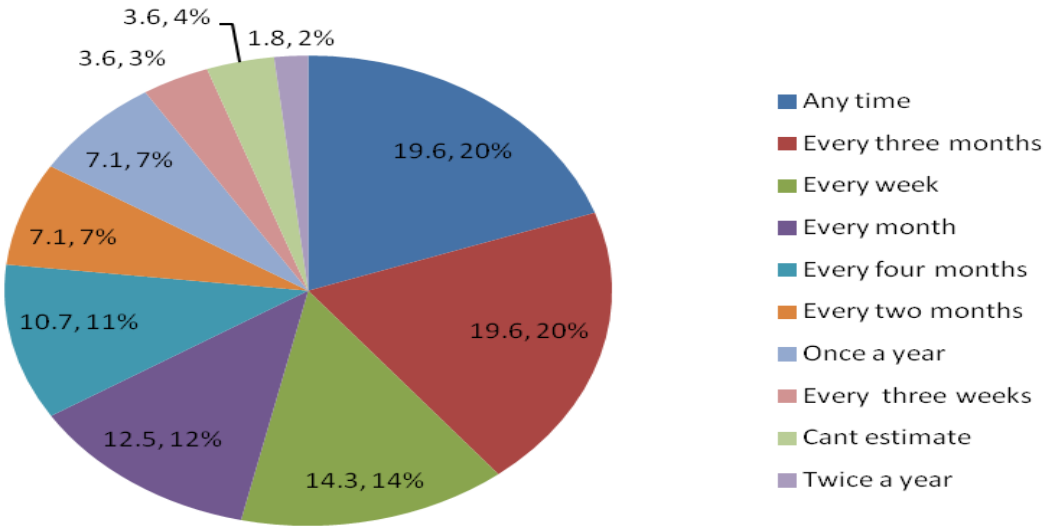

Figure 4. Frequency of infection with influenza.

related symptoms are still accessible to the households. Majority (54%) of the respondents reported to collect them from the wild while about 46% harvest them in backyard gardens. The respondents also said that majority (97%) of the plants are abundant and only about 3% were said to be rare.

Type of herbal formulation

Among the formulations, majority (55%) were reported to be formulated from single plants while 45% formulated from mixtures of plants that range from two to six in the respective formulae. In these formulations, the three most regularly used plant parts were; leaves (90.2%), followed by the stem bark (63.4%) and fruit (31.7%) in order of preferred use by respondents. Also used were the root bark (22.0%) bulb (7.3%) and whole plant (4.9%), as shown in Figure 6. A part from medicinal plants, other natural products reported to be added in some formulae include; egg york, ash, salt, cow ghee, milk, mushrooms, cold sponge and water.

Method of preparation and administration

The most common methods preparation of the herbal medicines reported by respondents included; decoction

Table 4. Frequently used medicinal plants, parts used, method of preparation and mode of administration for the treatment of influenza and related symptoms, in Luwero district.

Scientific name, (Authority) Voucher No., family, local name and route of administration	Method of preparation and part of the plant used	% Frequency of use (N=60)	Scientific studies on the plant against respiratory infections
Magnifera indica L. EKM/80,(Anacardiaceae), Muyembe [O, S]	Decoction[SB], Steaming [L,RB,SB]	67.4	Antibacterial activities (Bossa et al, 2007) Antimicrobial activity (Dabur et al. 2007)
Ocimum basilicum L. EKM/90 (Lamiaceae),Kakubansiri [O, M, S]	Decoction [L], Steaming [L], Juice [L]	39.5	Antibacterial activity (Siddiqui et al., 2012) Antibacterial activity (Chah et al., 2006)
Psidium guajava L. EKM/81 (Mytaceae, Mapeera [O]	Decoction [L], Steaming [L]	34.9	Review of phytochemistry, ethnobotany and pharmacology (Gutierrez et al., 2008)
Mormodica feotida L.EKM/82 (Curcubitaceae), Bombo [B,M,O]	Decoction[WP], Juice [L]	30.2	Ethnobotanical (Segawa and Kasenene 2007)
Callistemon rigidu EKM/75 (Mytaceae), Mwambalabutonya [O,S,]	Decoction	27.9	Ethnobotanical use against cough (Kakudidi et al, 2000)
Citrus limon L. EKM/69 (Rutaceae), Niimu/endimu [O]	Decoction [F, L], Chewing [F], Steaming [L,F], , Juice [F]	23.3	Ethnobotany, (Camejo-Rodrigues et al.,2003)
Syzygium cuminii (L) Skeels EKM/84 (Myrtaceae) Jambula [O]	Decoction [L,SB]	20.9	Antioxidative properties(Glover et al,.1961; Kokwaro 1976)
Vernonia amygdalina L. EKM/95 (Asteraceae), Mululuuza [O,S]	Decoction [L,RB,SB]Juice [L]	16.3	Antiplasmodial and cytotoxic activity (Lacroixa et al., 2011) Antibacterial (Erasto et al.,2006)
Digitaria scaralum (A. Rich.) Stapf EKM/96 (Poaceae) Olumbugu [O,S]	Decoction [RB,WP], Infusion [RB,WP]	16.3	
Combretum molle R. Br.ex G.Don f. EKM/97 (Combretaceae) Endagi [O]	Decoction [SB], Steaming [SB]	14.0	Biological activity (McGaw et al.,2001; Eloff et al., 2008)
Citrus aurantiifolia (Christm) EKM/91 (Rutaceae) Enimawa [O]	Decoction [F,L] Chewing [F], Juice[F]	14.0	Used to treat cough and influenza (Kokwaro 1976; Adjanohoun et al 1993; Tabuti at al; 2003b)
Azadirachta indica. A. Juss EKM/ 87 (Meliaceae), Neem tree [O]	Decoction, [L, SB] Steaming [L, SB], Juice [L]	14.0	Antiviral (Faccin-Galhardi et al.,2012) , Toxicity (Mbaya et al., 2010)
Persea americana Miller EKM/85 (Lauraceae), Avocado [O, S]	Decoction [L,SB], Steaming [L,SB]	14.0	
Eucalyptus sp. L'Herit. EKM/99 (Myrtaceae), Kalitunsi [O, M, S]	Decoction [L,SB]	14.0	Used to treat cough and fever (Adjanohoun, 1993)
Manihot esculenta Crantz EKM/76 (Euphorbia) Muwogo [B, M, O, S]	Decoction [L]	9.3	
Hoslundia opposita Vahl EKM/75 (Lamiaceae), Kamunye	Decoction [L,,A]	9.3	Used to treat colds (Bally 1937; Kokwaro 1976)
Erigeron floribundus Sch. Bip.EKM/28 (Asteraceae), Kafumbe [O, S]	Decoction [L, A]	7.0	
Acacia polyacantha EKM/27(Fabaceae), Kibeere [O]	Decoction [SB]	7.0	
Piliostigma thonningil EKM/ 100 (Caesalpiniaceae) Omugaali [o]	Decoction [L,SB], Steaming [L,SB]	7.0	
Leonotis nepetifolia (L) R. Br. EKM/30 (Lamiaceae), Ekifumufumu [O, S]	Decoction [L, A]	4.7	
Morus alba L. EKM/31 (Moraceae), Enkenene[O]	Decoction [L]	4.7	Antimicrobial ant-oxidative activity (Wang et al, 2012)
Artocarpus heterophyllus Lam. EKM/56 (Moraceae), Fene[O]	Chewing [F], Decoction [L,SB]	4.7	
Lantana camara L.EKM/54 (Verbanaceae), Kayukiyuki[O]	Decoction [L], Steaming [L]	4.7	Antimicrobial activity (Dabur et al., 2007)
Maytenus senegalensis (EKM/27) Celastraceae), Naligwalimu [O]	Decoction [RB, SB]	4.7	
Ageratum conyzoides L. (EKM/32) (Asteraceae),Namirembe [O, S]	Decoction [L,A]	4.7	Used to cure cough(Kokwaro 1976;Geissler et al.2002)
Albizia coriaria Welw. ex Oliver EKM/20 (Fabaceae), Omugavu [B, O]	Decoction [SB]	4.7	Anti-parasitic activity and cytotoxicity (Kigondu et al., 2009)
Lycopersicon esculentum Miller EKM/22 (Solanaceae) Nyanya [O]	Chewing [F]	4.7	
Citrus sinensis (L.) Osbeck EKM/30 (Rutaceae), Micwunga [O]	Decoction [F,L], Steaming [F,L]	4.7	Ethnobotany, (Camejo-Rodrigues et al.,2003

Key: Part of plant used: SB, Stem bark; L, Leaves; RB, Root bark; F, Fruit; WP, Whole plant; A, Arial part;
Method of preparation: Decoction, boiling of the dry plant for some minutes and leaving to cool; Steam Bathing, Boiling of the herbs in water and covering to inhale steam; Juice is squeezing a liquid from the plant part; Infusion, bringing to boil; Chewing, eating of the plant part raw. **Route of administration:** [O] Oral; [B] Bathing; [S] steaming; [M] massaging.

(62%), juice from leaves and fruits (17%) and steaming (13%). The routes of administration of the medicines reported by respondents were; oral, inhalation, steam baths and massage at 58, 19, 13 and 10% respectively.

Some formulae used in the treatment of influenza and related symptoms in Luwero district

The study documented sixty formulae, of these six formulae that were given through the oral route of administration, with detailed information that was compiled in the focus group discussions are given as follows;

(1) Formula
Ingredients (plants)

Scientific name	Local name	Part used	Quantity
Maytenus senegalensis	Naligwalimu	Leaves	Handful
Wabugia ugandensis	Abaasi	Bark	Handful
Eucalyptus sp.	Kalitunsi	Leaves	Handful
Callisteon rigidus	Mwambalabutonya	Leaves	Handful

Preparation
(i) Mix the four plants and pound them together.
(ii) Pick two handfuls of the mixture, using one hand.
(iii) Add six cups of water (3 L) in the saucepan
(iv) Boil until water remains to three cups (1.5 L).

Dosage: Two spoons (10 mls) three times a day (i.e. morning, afternoon, evening.)

(2) Formula
Ingredients/plants

Scientific name	Local name	Part used	Quantity
Chenopodium opunifolium	Omwetango	Bark	4 pieces
Magnifera indica	Omuyembe	Bark	4 pieces
Canarium schweinfurthii	Omuwafu	Bark	2 pieces
Acacia polyacantha	Kibeere	Bark	2 pieces
Eucalyptus sp.	Kalitunsi	Bark	1 pieces
Syzygium cuminii	Jjambula	Bark	3 pieces

Preparation: Boil the mixture in five liters of water until it remains with two liters.

Dosage: *Adults:* Take two spoons (10 ml) x three daily
Children: take one spoon (5 ml) x three daily

(3) Formula
Ingredients/plants

Scientific name	Local name	Part used	Quantity
Tagetes minuta	Kawunyira	Leaves	8 leaves
Mormodica feotida	Bombo	Leaves	4 leaves
Ocimum basilicum	Kakubansiri	Leaves	4 leaves
Carica papaya	Pawpaw	Leaves	2 leaves
Citrus limon	Lemon	Fruit	1 fruit
Callisteon rigidus	Mwambalabutonya	Leaves	3 handfuls

Preparation: Add four cups (500 ml) of water and boil until it remain two cups

Dosage: *Adults:* 2 spoons (10 ml) x three times
Children: One spoon (2.5 ml) x 3 times
Note : Take Namirembe plant for stomach pains

(4) Formula
Ingredients/plants

Scientific name	Local name	Part used	Quantity
Mormodica feotida	Ebombo	Leaves	8 leaves

Preparation: Crush 8 leaves in ½ L (500 ml) of water *Dosage:* Take all 500 ml in one day

(5) Formula
Ingredients/plants

Scientific name	Local name	Part used	Quantity
Vernonia amygdalina	Mululuuza	Root bark	Handful
Magnifera indica	Mango	Leaves/bark	Handful
Psidium guajava	Mapeera	Leaves	Handful
Piliostigma thonningi	Omugaari	Leaves	Handful
Combretum molle	Endagi	Leaves	Handful

***Preparation*:** Boil one handful of each in five liters of water until you remain with three liters.

Dosage: Adult ½ a tumpeco (250 ml) 3 times a day. Note: A difference in 3 days, 7 days for complete healing.
Dosage: Drink one cup 2 times (one litre) a day, morning and evening.

(6) Formula
Ingredients/plants

Scientific name	Local name	Part used	Quantity
Rhus vulgaris	Obukwansokwanso	Fruit	Four fruits

Preparation: Eat the fruit

Dosage: Four fruit x 3 daily

Table 5. Presents percent availability, formulation and administration of herbal mecicines used by the respondents of Luwero

Habitat of plants	% response
Wild	54
Gardens	46
Formulation of herbal medicine	
Single plants	55
Mixtures	45
Method of Preparation of medicinal plants	
Decoctions	62
Juice	17
Steaming	13
Chewing	5
Infusion	3
Mode of administration	
Oral	58
Inhilation	19
Bathing	13
Massaging	10

DISCUSSION

Clinically, influenza disease in human is associated with symptoms such as; cough, malaise and fever and this is normally accompanied with sore throat, nasal obstruction and sputum production (Hudson, 2009). Nevertheless, in severe cases it causes bronchitis, pneumonia, asthma and chronic obstructive pulmonary disease (Morens et al, 2007). Children under five years and elderly are more vulnerable, because of their immunity is low (Bearden et al., 2012; Yu et al., 2013). Until the recent past in most tropical African countries, influenza as a disease has been considered a mild infection that clears without any/strong medication. In view of this, community knowledge perception of the influenza epidemic is important since community knowledge of influenza symptoms and it management can be used to develop strategies for its control. The fact that common influenza virus causes low immunity among patients could also lead to the patients getting secondary infections, particularly in manifestation described as "*Lubyamira* and *Yegu"* by the respondents in this study. While knowledge of influenza manifestation is important, understanding its management particularly in the rural settings is important too. For centuries, knowledge of medicinal plants in management of diseases has been a key to almost every community. In sub Saharan African countries like Uganda, herbal medicine is used by majority due to the poor infrastructure of the national health care system which leads to inaccessibility of allopathic drugs and also high cost for the available conventional health care. Herbs have also been proven efficacious against influenza and other viruses (Xiuying, et al., 2012; Mousa, 2015) and communities have strong belief in herbal medicine with high level of acceptability due to the perception that they

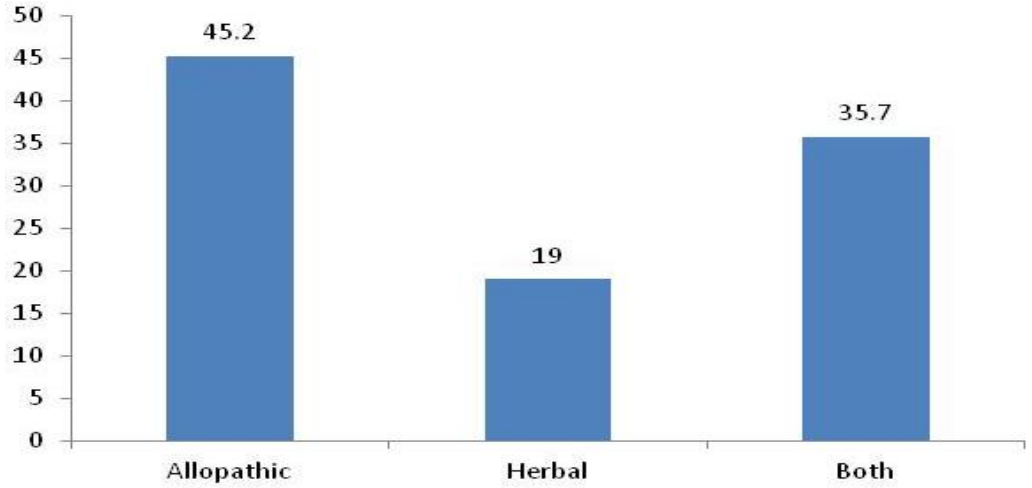

Figure 5. Method used by communities to treat influenza.

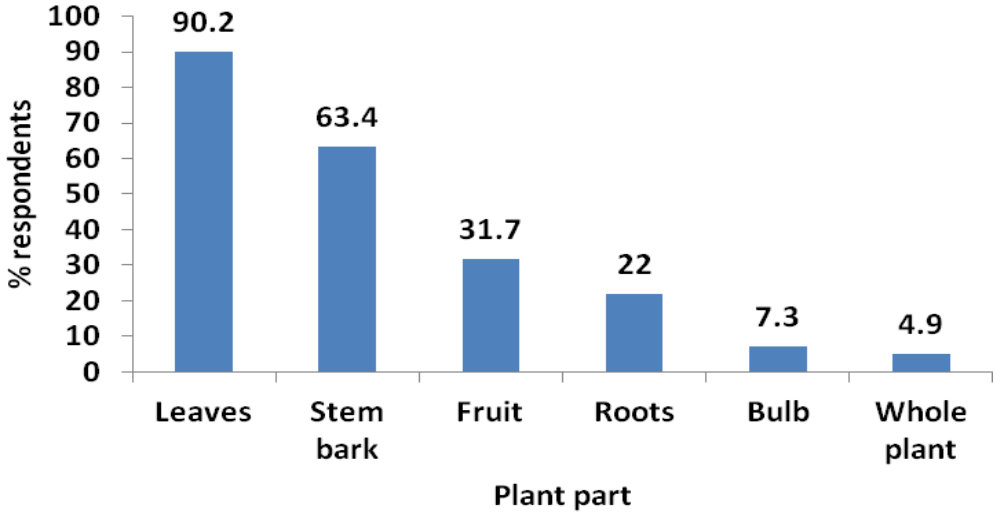

Figure 6. Parts of medicinal plant used for treatment of influenza and related symptoms.

are efficacious and safe makes its use widely acceptable (WHO, 2003). The plants reported in this study have been reported in previous ethno botanical surveys , and a few of them have also been proven scientifically to possess immune boosting properties, antibacterial, anti-flammatory, antiviral activities, vitamins and minerals. For example, *Citrus* fruits and *Psidium guajava* have bioactive compounds, vitamin C, and micro minerals that are good immune boosters (Gutierrez et al., 2008).

In addition other ethno botanical studies have indicated plants in families Mytaceae, Rutaceae, Lamiaceae and Anacardiaceae to have been used in traditional medicine and to possess therapeutic properties (Koudouro et al., 2011). According to Menkovic et al., (2011), *Nepeta cataria* (Lamiaceae) is ethno botanically reported to treat cold and fevers associated with influenza. On the other hand there has been observed strong co-movements between influenza and Malaria in some tropical countries which suggest that humidity which is an important factor in transmitting malaria also plays a role in influenza transition (Vaisenberg and Noymer, 2011). This is coupled with the fact that Influenza has symptoms related to those of malaria. In fact the symptoms are not that dramatic and can easily be mistaken for an attack of influenza (Netcare, 2014). In the rural areas of Uganda, most people in the local communities take medicines including herbal medicines without diagnosis. It is possible that some of the cases that were reported to be influenza could be malaria cases. It is therefore probable that these herbal formulae used for treatment of influenza

also have antimalarial activity. The team will in future validate antimalarial activities of these formulae to ascertain their efficaciousness. Unlike Allopathic medicine which has clear guidelines and is standardized the world over. Herbal medicine especially in most African countries including Uganda is not standardized at almost all stages of development and use. Majority of the measures documented in this study such as handful, pieces, leaves are not uniform and this brings about a major discrepancy in formulation and dosage. It is intended that these formulae will be standardized as well as be evaluated for their effectiveness if in addition to their safety and efficacy in treatment of influenza and related infections.

Conclusion

This study conducted in Luwero district in Central Uganda, identified and documented 29 medicinal plants and about six formulae used for treatment of influenza and related symptoms by the respondents. The Indigenous knowledge has been organized to support conventional measures in the management of influenza and related symptoms and the evaluation of the efficacy of priority medicinal plants/ herbal formula *in vivo* is ongoing. All together, the information obtained in the study will be used in the standardization of herbal medicine that can be recommended for used against respiratory infections at household level in Luwero. Phytohemical investigations are recommended to determine the active compounds in the plants. While clinical trials to determine the efficacy of the plant formula in human are suggested. There is high chance that an active molecule with antiviral or antibacterial inhibiting properties can be identified and used as a template in developing a cheaper and affective medicine that can be used in the management of influenza and related illnesses. Tested and efficacious formula in clinical trials can also be integrated in the national health care system for treatment of influenza and other bacterial infections at household level, particularly in the rural areas where access to allopathic medicine is still a challenge.

Conflict of Interests

The authors have not declared any conflict of interests.

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