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Chemical components of the volatile and non-volatile extractives of *Croton* species and their microbial activities

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Essential oil compounds of *Croton pseudopulchellus* and *Croton gratissimus* were analysed using Gas Chromatography/Mass Spectrophotometry and screened for antimicrobial activity against *Bacillus pimilus* (ATCC 29212), *Bacillus cereus* (ATCC 10702), *Staphylococcus aureus* (ATCC 3983), *Streptococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983) and *Pseudomonas aeruginosa* (ATCC 19582) using Agar gel disk diffusion test and minimum inhibitory concentrations. The susceptibilities of all isolates of different essential oil compounds were standardised by National Committee for Clinical Laboratory Standards (NCCLS 1998). The cytotoxicity test was also carried out to determine the toxicity levels of essential oil compounds. These plants were selected based on their use by traditional healers for treatment of upper respiratory tract, gastrointestinal tract and urinary tract infections. The essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* were found to be active against all the test microorganisms, while the preliminary assessment of essential oil compounds from these plants exhibited low cytotoxic activity.

Key words: Essential oil, sesquiterpenes, chemical composition, antimicrobial activity.

INTRODUCTION

The emergence of multidrug resistance to antimicrobials, has become an important public health issue in many developing countries as treatment of ailments require the use of more expensive drugs for a longer treatment period (Buwa and Afolayan, 2009; Oyedeji et al., 2010). Therefore, this study focused on the use of essential oil compounds derived from *Croton pseudopulchellus* and *Croton gratissimus* as alternative therapeutic agents or new inexpensive antimicrobial drugs which are more effective and with less side effects for treatment of upper

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respiratory and gastrointestinal tract infections. Antimicrobial agents inhibit the growth of microorganisms by interfering with the specific physiological characters or metabolic functions of microorganisms (Ndip et al., 2008). Based on the ethnomedical information on these plants they were screened against four Gram-positive bacteria, namely, *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Staphylococcus aureus* (ATCC 3983), and *Streptococcus faecalis* (ATCC 29212). Gram-negative bacteria were *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983) and *Pseudomonas aeruginosa* (ATCC 19582). The four Gram positive bacteria were included because of their opportunistic properties in the upper respiratory tract infections, while the other three Gram negative bacteria are custodians of the urinary and gastrointestinal tract infections. It is expected that essential oil compounds derived from *C. gratissimus* and *C. pseudopulchellus* showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens (Ndip et al., 2008). *C. gratissimus* and *C. pseudopulchellus*, though not widely distributed in Africa have shown in traditional medicine that their leaves can be used in treating several ailments; therefore its versatile use in traditional medicine necessitated this research.

Croton is a large family genus of Euphorbiaceae comprising about 700 species as trees, shrubs and herbs (Salatino et al., 2007). The leaves are glossy and aromatic in nature (Van Wyk, 2008; Compagnone et al., 2010). *C. pseudopulchellus* Pax is commonly known as small lavender fever – berry, a pale white-yellow-flowered, bark grey, smooth to roughish, reddish brown branchlets covered with hairy scales, shrubby, perennial plant up to 4 m tall with a sweet smell that attracts many insects, while *C. gratissimus* Burch is known as Lavender Croton. Both are found to grow widely in the North-Eastern regions of South Africa along the coastal belt, rocky hillside and along rivers and streams, from Kwazulu-Natal, extending further North through Swaziland to Mpumalanga and Limpopo province. Leaves and bark of *Croton* species are often used in folklore medicine for the treatment of syphilitic ulcers and chest-complaints. Root-decoction is also used for Asthma while powdered root is taken as snuff for head colds (Neves and da Camara, 2012).

According to the review of *Croton* spp. by Salatino et al. (2007), several *Croton* spp. are well known as medicinal plants in the traditional medicinal practices for the treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight-loss across Africa, Asia and South America. Other studies reported that oil derived from *Croton* spp. was commonly used as a therapeutic tool to treat acne and skin infections (Bikanga et al., 2010; Mulholland et al., 2010). Mulholland et al. (2010) further reported that *Croton gratissimus* Burch (Lavender Croton) also contains organic compounds such as pumarane, kaurane, labdane, clerodane, cembrane, diterpenoides, isoquinoline, alkaloids and triterpenoides that enhance treatment of headache, coughs, fever, cold, syphilic ulcers and chest pains, while the leaf sap of *C. pseudopulchellus* is drunk to treat abdominal pains, painful respiratory conditions, bleeding gums, asthma, headache, coughs, fever and colds (Ndip et al., 2008). Decocted leafy twigs are drunk for the treatment of gonorrhea and cytotoxicity tests showed an ID_{50} of 64 µg/mL against vervet monkey cells (Langat et al., 2012).

Essential oil compounds derived from plants have been widely used in the treatment of respiratory tract, urinary tract, gastrointestinal tract infections as well as skin infections (Bikanga et al., 2010; Mulholland et al., 2010; Leite et al., 2015). Since the essential oil compounds of *Croton* spp. are of economic and medicinal value (Viljoen et al., 2006). The aim of this study was to investigate the chemical composition and antimicrobial activity of *C. psedopulchellus* and *C. gratissimus* essential oil compounds against four Gram positive and three Gram negative test microorganisms.

MATERIALS AND METHODS

Plant Collection

Croton pseudopulchellus samples were collected from Twin Stream Indigenous Nursery and Landscaping, Mtunzini, while *C. gratissimus* samples were collected from a private garden in Mtunzini, Kwazulu Natal. Mrs A. Hutching of the Botany Department, University of Kwazulu Natal authenticated the samples. Voucher specimens were deposited at the University of Zululand Herbarium. The Eastern Cape *Croton* spp. were collected from Flagstaff by Mr Iwopa of the Botany Department and Dr Morobe of Medical Microbiology Department, Walter Sisulu University and the plants were authenticated by Dr Immelman, KL, a taxonomist of the Department of Botany, Walter Sisulu University (WSU), Mthatha, Eastern Cape, South Africa. Voucher specimens were deposited at the Walter Sisulu University Herbarium.

Extraction of essential oil

One kilogram of fresh leaves of each species was subjected to hydro distillation in a Clevenger apparatus for 4 h. This technique is based on the evaporation of volatile compounds induced by steam. The essential oil was collected in amber vials after 4 h, weighed, sealed and stored in the refrigerator (4°C) until use (Oliveira et al., 2020).

GC/MS analysis

GC/MS analyses of the oils were performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with Hewlett Packard 5973 mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The initial temperature of the column was 70°C

and was heated to 240°C at a rate of 5°C min^{-1}. Helium was usedas the carrier gas at a flow rate of 1 mL/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. 1 µL of the diluted oil was injection for analysis. *n*-Alkane of C_8-C_{30} was run under the same condition for Kovat indices determination (Ndukwe and Okhiku, 2018). The components of the oils were identified by matching their spectra and retention indices (Kovat Index) with those of the authentic samples and literature values (Oyedeji et al., 2010).

Cytotoxic screening of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus*

MAGGI CCR5+ cells were used for cytotoxic screening of the essential oil compounds. All cell lines were purchased from ATCC, Manassas, VA 20108, USA. Cell lines were cultured in Advanced Modified Eagle's Medium (DMEM) with 10% 5 Mm L-glutamine (Gibco BRL) and grown at 37°C in a 5% CO_2 humidified incubator (Thermo Fisher Scientific, Wakenyaku Co. Ltd, Japan). Cells were subcultured every 2 days after the confluent growth was observed, MAGI cells were then seeded into two 96 well µL plates with 10^4 cells/well in 100 µL of DMEM supplemented with 10% foetus bovine serum (FBS). 11 µL of oil was added into 2 wells of row B with final concentration of 1/20. Another 11 µL of mixture was removed from B to C and then to D, E, F and 10 µL was discarded from F. 100 µL of medium was added into each well from B to G. After 48 h, cells were observed and 150 µL of supernatant from each well was discarded and then 10 µL of MTT was added into each well. The plates were incubated at 37°C for 4 h. 100 µL of stop solution was added into each well and OD_{570} was checked and then CC_{50} were determined as previously reported (Morobe et al., 2012).

Biological activity

Antimicrobial assay

Agar gel disk diffusion: The essential oil compounds were tested for antibacterial activity using modified Kirby-Bauer agar gel disk diffusion test according to Kose et al. (2010) and the MIC breakpoints of all isolates were determined using the E-test strips (Morobe et al., 2013). The susceptibilities of all isolates of different essential oil compounds were standardised using National Committee for Clinical Laboratory Standards (NCCLS, 1998).

Microorganisms were grown overnight at 37°C in 20 mL of Müller-Hinton broth (Oxoid). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland No. 5 standard (1.0×10^8) CFU/mL. 90 mm Petri dishes (Merck, South Africa) containing 12 mL of sterilized Mueller-Hinton agar (Oxoid) were inoculated with these microbial suspensions. Sterile Whatmann No. 1 (6 mm) discs papers were individually placed on the surface of the seeded agar plates and 10 µL of essential oil compound in DMSO was applied to the filter paper disk. The plates were incubated at 37°C for 24 h and the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. Ampicillin (10 µg) and Chloramphenicol (10 µg) were used as positive controls, while hexane and DMSO served as negative controls.

The essential oil compounds were tested against seven reference bacterial strains obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice. Gram-positive bacteria: *B. cereus* (ATCC 10702), *B. pumilus* (ATCC 14884), *S. aureus* (ATCC 3983), and *S. faecalis* (ATCC 29212). Gram-negative strains were *Escherichia coli* (ATCC 4983), *K. pneumoniae* (ATCC 2983), and *P. aeruginosa* (ATCC 19582).

The stock cultures were maintained at 4°C in Mueller-Hinton agar (Oxoid) (Morobe et al., 2018).

Minimum inhibitory concentration of essential oil compounds

The minimum inhibitory concentrations (MICs) of the essential oil compounds were determined using 96-well µL dilution method as described by Oyedeji et al. (2010) and Eloff et al. (2011). Bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C and a 1:1 dilution of each culture in fresh MH broth was prepared prior to use in the micro dilution assay. Sterile water (100 µL) was pipetted into all wells of the µL plate, before transferring 100 µL of essential oil compound into DMSO. Serial dilutions were made to obtain concentrations ranging from 10 to 0.078 mg/mL. 100 µL of bacterial culture of approximate inoculums size of 1.0×10^8 CFU/mL was added to all well and incubated at 37°C for 24 h. After incubation, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) solution was added to each well and incubated at 37°C. Plates were examined after 60 min of incubation. Microbial growth was indicated by the presence of a reddish colour which was produced when *p*-iodonitrotetrazolium violet (INT), a dehydrogenase activity detecting reagent, was reduced by metabolically active microorganisms to the corresponding intensely coloured formazan (Oyedeji et al., 2010). Solvent controls (DMSO and Hexane) and the standard antibiotics ampicillin (10 µg) and chloramphenicol (10 µg) were included in the assay.

RESULTS

Chemical analysis of essential oil compounds

In this study, the chemical profile of *C. pseudopulchellus* and *C. gratissimus* oils showed a high amount of monoterpenes and sesquiterpenes similar to that reported for samples collected worldwide. Analysis of the oils was performed using GC/MS (Table 1). The leaf oils of *C. pseudopulchellus* had germacrene (24.2%), β-phellandrene (17.4%), myrcene (13.4%) and β-caryophyllene (11.4%) as the prominent compounds. The chemical composition of the leaf oil of *C. gratissimus* was characterized by sabinene (14.6%), β-phellandrene (12.3%), α-pinene (6.0%) and germacrene D (5.9%), respectively.

Cytotoxic screening of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus*

In this study, a systematic evaluation of cytotoxic activities of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus* were conducted and exhibited a minimal toxic activity on cell lines (Table 2).

The results from both *Croton* spp. (Table 2) revealed germacrene (0.2 Cc_{50}) and β-phellandrene (0.2 Cc_{50}) as the most toxic oils and induced over 50% cell death, followed by α-phellandrene (0.19 Cc_{50}) and β-caryophellene (0.18 Cc_{50}), respectively. The oils that induced the least cell death were α-pinene (0.15 Cc_{50}),

Table 1. Percentage composition of essential oil compounds of *C. pseudopulchellus* and *C. gratissimus*.

Compound	KI	Percentage composition of essential oil compound			
		CpECP	CgECP	CpKZP	CgKZP
α-thujene	936	-	-	-	1.2
α-pinene	943	4.5	-	3.7	6.0
sabinene	977	-	-	-	14.6
1-octen-3-ol	983	-	-	6.7	-
myrcene	993	11.3	4.6	13.4	2.4
α-phellandrene	1003	1.0	15.5	-	12.3
α-terpinene	1019	0.5	0.7	-	1.5
β-phellandrene	1037	9.2	5.0	17.4	T
trans-β-ocimene	1040	1.0	1.1	-	2.8
γ-terpinene	1069	1.4	1.6	-	2.1
cis-sabinene hydrate	1097	-	1.0	-	1.4
α-terpinolene	1098	-	3.0	-	1.9
linalool	1101	1.0	0.3	1.2	4.1
α-terpineol	-	1.8	0.2	-	-
α-cubebene	-	1.5	0.5	-	-
Eugenol	-	0.2	2.0	-	-
α-copaene	1376	3.2	5.3	2.2	2.5
β-bourbonene	1387	1.0	1.8	1.2	0.7
β-elemene	1391	4.0	1.0	3.6	0.5
β-caryophyllene	1442	10.2	12.9	11.7	4.2
β-cubebene	-	-	0.6	-	-
Viridiflorene	-	-	0.8	-	-
α-humulene	1460	3.0	2.7	3.6	1.1
Aromadendrene	1470	1.3	1.9	0.4	2.0
germacrene D	1481	28.1	16.0	24.2	5.9
Bicyclogermacrene	1497	4.0	2.6	3.1	1.6
γ–cadinene	1518	-	1.9	-	1.0
γ–muurolene	-	-	1.6	-	-
δ–cadinene	1526	1.0	1.3	2.3	0.9
α–cadinene	1538	0.9	-	1.0	-
germacrene-D-4-ol	1574	2.7	7.8	0.9	-
caryophyllene oxide	1586	1.6	1.4	1.3	2.4
Spathulenol	1589	1.0	0.8	0.8	T
Total % (no. of cpd)	-	95.3 (24)	96.0 (28)	78.8 (18)	82.1 (24)

KI = Kovat indices; t = trace amount; - = not detected, CpECP = *C. pseudopulchellus* Eastern Cape Province, CpKZP = *C. pseudopulchellus* KwaZulu-Natal Province, CgECP = *C. gratissimus* Eastern Cape Province, CgKZP = *C. gratissimus* KwaZulu-Natal Province.
Source: Authors

cytotoxic activity of 21%.

Antimicrobial activity of essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* against seven microrganisims

In this study, results obtained (Table 3) revealed the varying levels of the antimicrobial activity of *C. pseudopulchellus* and *C. gratissimus* essential oil compounds against bacterial isolates studied.

The essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* were tested for antibacterial activity against seven microorganisms using agar gel disc diffusion test. The essential oil compounds of the two *Croton* spp. showed activity against all test microorganisms (Table 3) and the zones of inhibition of essential oil compounds varied from 0 to 12 mm and the largest zone of inhibition was obtained for *E. coli* (12 mm) and the lowest for *B. pimilus* (2 mm).

Table 2. Cytotoxic screening of seven essential oil coumpounds from *C. pseudopulchellus* and *C. gratissimus*.

Compound	Essential oil compound concentration (Cc50)	
	C. pseudopulchellus	***C. gratissimus***
A-phellandrene	0.16	0.19
β-phellandrene	0.14	0.2
Germacrene	0.17	0.2
β-caryophellene	0.15	0.18
α-pinene	0.13	0.15
Myrcene	0.14	0.13
Sabinene	0.09	0.13

Source: Authors

Table 3. The zones of inhibition of essential oil compounds of two Croton species against seven microorganisms.

Compound	Inhibition zones of essential oil compound against seven microorganisms (mm)						
	S. aureus	***S. faecalis***	***B. cereus***	***B. pumilus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
Germacrene	9	7	3	2	12	11	8
A-phellandrene	6	5	3	2	10	9	11
β-phellandrene	5	6	4	3	5	7	9
β-caryophyllene	6	6	2	2	7	5	5
α-pinene	3	2	2	2	4	3	2
Myrcene	9	7	3	3	8	8	7
Sabinene	5	5	2	2	5	3	2
Ampicillin (10 µg)	30	25	20	19	18	28	19
Chloramphenicol (10 µg)	21	20	15	14	26	17	8

Source: Authors

Minimum inhibitory concentration of essential oil compounds

The Minimum Inhibition Concentration method showed that essential oil compounds of the two *Croton* spp. were active against all test microorganisms (Table 4).

The MIC values of the essential oil compounds ranged from 2 to 19 µg/mL (Table 4), with the most prominent being *E. coli* (19 µg/mL), *K. pneumoniae* (17 µg/mL), *S. aureus* (16 µg/mL) and *P. aeruginosa* (15 µg/mL), with the least active being *B. cereus* (2 µg/mL).

DISCUSSION

Leaves of *Croton* spp. are used in traditional medicine for the treatment of syphilitic ulcers and chest-complaints (Compagnone et al., 2010). In this study, the three major compounds among sesquiterpene were germacrene (24.2%) and phellandrene (17.4%). The chemical profile of the oils had germacrene (5.0-28.1%) and β-caryophyllene (4.2-12.9%) as the two most prominent compounds in all the oil extracts. α-phellandrene was found in trace amount in the oil extract of *C. gratissimus* from Kwazulu-Natal province, while other oil samples had significant amount of the compound (5.0- 17.4%).

In previous studies on the essential oil compounds from other samples of sesquiterpene hydrocarbons, Oliveira et al. (2007) showed germacrene as a major compound (66.0%), while the monoterpene hydrocarbons (phellandrenes) were present only as trace constituents (1.1%). The essential oil from the leaves of *C. gratissimus* gave fenchyl acetate (25.3%), β-caryophyllene (20.7%), α-selinene (12.8%) and β-bourbene (9.3%) as major constituents. In contrast to the aforementioned findings, in the present study germacrene was identified as a major compound in the essential oil of South African *Croton* spp. from Kwazulu-Natal and the Eastern Cape provinces. These findings strongly suggest that the germacrene content in the sample analysed in this study was due to environmental conditions, since the seasonal, climate and soil conditions are different in various geographical areas, supporting the existence of two different chemotypes for germacrene and phellandrene. Furthermore, this suggests that there are different chemotypes for these species. However, it is also known

Table 4. The Minimum Inhibition Concentration values of essential oil compounds against seven microorganisms.

Compound	MIC values of essential oil compounds against seven microorganisms (µg/ml)						
	S. aureus	***S. faecalis***	***B. cereus***	***B. pumilus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
Germacrene	16	7	2	3	18	17	10
A-phellandrene	13	5	2	3	19	10	15
β-phellandrene	12	8	3	5	17	13	9
β-caryophyllene	11	7	4	4	14	10	11
α-pinene	2	6	2	2	9	7	6
Myrcene	13	7	3	5	12	17	12
Sabinene	3	4	2	2	5	2	4
Ampicillin (10 µg)	6	6	6	6	9.4	9.4	9.4
Chloramphenicol (10 µg)	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Source: Authors

that cultivation conditions can affect secondary metabolite production (Edris, 2007). The preliminary bioassay assessment of *C. pseudopulchellus* and *C. gratissimus* essential oils exhibited low cytotoxic activity 21%.

The essential oil compounds were tested for antibacterial activity by the agar gel disc diffusion method (Kose et al., 2010). Disc diffusion is one of the most common assays used in the evaluation of antimicrobial activity of essential oil compounds. In this study antimicrobial activity by disc diffusion method showed that the essential oil compounds of the *C. gratissimus* and *C. pseudopulchellus* were active against *E. coli* followed by *K. pneumoniae* and *P. aeruginosa*. The zones of inhibition of essential oil compounds varied from 2 to 12 mm. The largest zone of inhibition was obtained for *E. coli* (12 mm) and the lowest for *B. pimilus* (2 mm).

The MIC values of the essential oil compounds ranged from 2 to 19 µg/mL (Table 4), with the most prominent being *E. coli* (19 µg/mL), *K. pneumoniae* (17 µg/mL), *S. aureus* (16 µg/ml) and *P. aeruginosa* (15 µg/mL), with the least active being *B. cereus* (2 µg/mL). According to Burt (2004), both chemotypes (germacrene and phellandrene) appear to make the cell membrane permeable and are able to disintegrate the outer membrane of Gram negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to adenotriphosphate (ATP). Furthermore, in this study Minimum Inhibition Concentration method showed that the essential oil compounds of the two *Croton* spp. were active against all test organisms.

Therefore essential oil compounds from *C. gratissimus* and *C. pseudopulchellus* may be suitable for treatment of infections caused by designated pathogens and this is consistent with a previous finding (Morobe et al., 2012). According to Nanyonga et al. (2013), the antimicrobial activity of essential oil compounds is linked to its chemical composition. The essential oil compounds of *C. gratissimus* had a broader inhibitory effect of the bacteria, compared to the essential oil compound of *C. pseudopulchellus*. However, the antimicrobial activity of *C. gratissimus* and *C. pseudopulcellus* are slightly related to the major compounds of the essential oil compounds of germacrene and phellandrene.

Conclusion

The essential oil compounds from *C. pseudopulchellus* and *C. gratissimus* leaves exhibited variable activities against seven different microorganisms tested in this study and in some cases showed equivalent or better activities than some antibiotics. The potency of these compounds against test microorganisms and on cell lines suggests their potential to be used as a source of alternative medicine, new pharmaceutical and health care product that can be used as a therapeutic agent in the face of antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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