Journal of Ethnopharmacology 228 (2019) 92–98

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Antimicrobial activity of *Elytropappus rhinocerotis* (Asteraceae) against micro-organisms associated with foot odour and skin ailments

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ARTICLE INFO

Keywords:
Brevibacteria
Essential oil
Foot odour
Labdane diterpenoids
MIC
Staphylococci
Trichophyton mentagrophytes

ABSTRACT

Ethnopharmacological relevance: The twigs of *Elytropappus rhinocerotis* are widely used in the Cape region of South Africa to treat foot odour, perspiration and also itchy, chilblained and burning feet. However, no antimicrobial studies have hitherto been published on this popular Cape herbal medicine, which is also used for a wide range of ailments.

Aims of the study: To determine the antimicrobial activity of the extracts, essential oil and two major labdane diterpenes isolated from *E. rhinocerotis* against micro-organisms associated with foot odour and other conditions associated with skin infections.

Materials and methods: Leafy stems were harvested from three individual plants at three separate geographical localities, giving a total of nine plant samples. The samples were air-dried, powdered and extracted with a 1:1 mixture of methanol and dichloromethane, and also with sterile distilled water. A portion of each sample was also hydrodistilled to obtain nine samples of essential oil. Isolation of the major labdane diterpenes was performed using silica and ethyl acetate in hexane (3:7 v/v) as the mobile phase. Minimum inhibitory concentrations (MIC) were determined for nine crude extracts, as well as three essential oil samples and two labdane diterpenes obtained from a bulk sample. The test organisms used in this study were from Deutsche Sammlung von Mikrooganismen (DSM) and American type culture collection (ATCC) strains and included five bacterial species (*Brevibacterium agri* ATCC 51663, *B. epidermidis* DSM 20660, *B. linens* DSM 20425, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228) and one fungal species (*Trichophyton mentagrophytes* ATCC 9533) associated with the skin.

Results: The presence of essential oil is reported for the first time. Organic extracts and essential oil samples showed moderate to noteworthy activity against selected test organisms. For the Brevibacteria, the lowest MIC values for phenolic extracts were several times lower than the corresponding values for the positive control zinc (shown in brackets): 0.0031 mg/mL against *Brevibacterium agri* (0.33 mg/mL), 0.17 mg/mL against *B. epidermidis* (3.91 mg/mL) – both for sample 2 of Vanwyksdorp; 0.13 mg/mL against *B. linens* (2.28 mg/mL) – for sample 3 of Vanwyksdorp. Two isolated labdane diterpenoids (one of which is here first reported) were also antimicrobially tested and showed moderate activity but had high abundance in the extracts. Two major monoterpenes and four sesquiterpenes in the essential oil were identified as 1,8-cineole (4.6–12.3%), terpinen-4-ol (9.2–24.3%), germacrene A (3.9–15.6%), (−)-spathulenol (1.7–37.8%), viridiflorol (0.3–100%), and silphiperfol-6-en-5-one (4.5–26.8%).

Conclusion: The antimicrobial results particularly for the essential oils and against the Brevibacteria support the traditional topical use of *Elytropappus rhinocerotis* twigs to treat foot perspiration, foot odour and other related skin conditions.

1. Introduction

Elytropappus rhinocerotis (L.f.) Less. [=*Dicerothamnus rhinocerotis* (L.f.) Koek.] is commonly referred to as *renosterbos* and is one of the most frequently encountered plant species of the Fynbos Biome of South Africa. This small shrub is endemic to the Cape region and dominates in some areas (often on heavy soils), forming a distinct vegetation type known as renosterveld (Mucina and Rutherford, 2006).

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https://doi.org/10.1016/j.jep.2018.09.014
Received 9 July 2018; Received in revised form 6 September 2018; Accepted 10 September 2018
Available online 11 September 2018
0378-8741/

Renosterbos is an important component of Cape Herbal Medicine (Van Wyk, 2008), with a wide range of traditional medicinal uses (see Arnold et al., 2002, and references cited therein). Important references include Pappe (1868), Dykman (1908), Marloth (1917), Kling (1923), Watt and Breyer-Brandwijk (1962), Smith (1966), Palmer (1985), Dekker et al. (1988), Roberts (1992), Rood (1994), Shearing and Van Heerden (1994), Palmer (1995), Van Wyk et al. (1997), the Anonymous (1998), Van Wyk and Gericke (2000, 2018), Le Roux and Wahl (2005), Van Wyk (2008), Van Wyk et al. (2008) and Van Wyk et al. (2009). Medicinal uses that have been recorded include the treatment of indigestion, dyspepsia, lack of appetite, ulcers, kidney and bladder stones, stomach cancer, asthma, cough, diarrhoea, dysentery, fever, convulsions, influenza, back pain and leg cramps. Large quantities of plant material were burnt in the house as a fumigation treatment of both the house and its inhabitants, especially during the influenza epidemic of 1918 (Van Wyk et al., 2008; Britz, 2011) – old people from that era described how they barely survived the smoke – but they were sometimes the only survivors of the flu (Piet Cupido, cited by Van Wyk et al., 2008). Dry stumps of the shrubs are a favourite firewood because of its fragrance and the distinctive smoky flavour that it adds to food items which are prepared in an outside oven or on an open fire (De Beer and Van Wyk, 2011; Van Wyk, 2015).

Surprisingly, none of the older literature sources cited refers to a common folk use of the plant, namely to treat foot perspiration and foot odour. The only published information relating to this use is an ethnobotanical study in the Kamiesberg (Nortje, 2011; Nortje and Van Wyk, 2015), where it is used as a treatment for painful legs, as wash for burning feet and the fresh leaves are placed in shoes for chilblained feet, swollen feet and sweaty feet. The treatment of chilblained feet by smoke treatment also appears to have a single literature reference (Vlok and Schutte-Vlok, 2016). During recent ethnobotanical field work in the Little Karoo and in the Cederberg region, several anecdotes were recorded which show that it is a common practice to insert fresh leafy twigs into the shoes to treat foot perspiration and foot odour, as well as chilblained and burning feet. Feet are often treated by placing them in the smoke of burning twigs. Localities where these specific uses were recorded include Barrydale, De Rust, Haarlem, Uniondale, Vanwyksdorp, Volmoed and Zoar in the Little Karoo and Kleinvlei, Suurrug and Brugkraal in the Cederberg, South Africa.

No less than 44 anecdotes relating specifically to foot perspiration and foot odour were recorded in a book on South African folk remedies, first published by the South African Academy of Science and Art in 1965 (SAASA, 2010). *Elytropappus rhinocerotis* was not mentioned in this rather elaborate list of traditional remedies. The only plants were *Artemisia afra* Jacq. (wildeals) with three anecdotes, and *Eucalyptus globulus* Labill. (bluegum) leaves, *Elephantorrhiza elephantina* (Burch.) Skeels (*elandswortel*) and *Punica granatum* L. (pomegranate) fruit peels, each with one anecdote. The remaining 38 recommended treatments included boracic powder (six anecdotes), alum water (five anecdotes), carbolic soap, salt water and salt (three anecdotes each), bicarbonate of soda, bran water, copper sulphate and vinegar water (two anecdotes each) and one anecdote each for coal tar, “Little's Dip” – sheep dip, potassium nitrate, tobacco water, wood ash, and mixtures of powdered pomegranate fruit peels with alum, saltwater and *wildeals* (*Artemisia afra*), and talcum powder, cornflour and salicylic acid.

Foot odour can be caused by etiological factors such as wearing closed shoes, hyperhidrosis (Connolly and de Berker, 2003), the maceration of skin, fungal infections and the proliferation of bacteria (Sharquie et al., 2013). The *Brevibacterium* species are specifically related to foot odour (Sharquie et al., 2013; Van Vuuren et al., 2014). *Brevibacterium* species ingest dead skin on the feet which leads to the conversion of methionine (an amino acid) into methanethiol, which is responsible for the sulfuric aroma and cheesy odour (Sharquie et al., 2013). *Staphylococcus aureus* and *S. epidermidis* are Gram-positive micro-organisms; they are common skin pathogens and commensals (Van Vuuren et al., 2014). *Staphylococcus epidermidis* degrades leucine that is present in sweat into isovaleric acid (Kanda et al., 1990) which is responsible for the strong cheesy odour (Sharquie et al., 2013). *Trichophyton mentagrophytes* is a fungal dermatophyte (Van Vuuren et al., 2014) responsible for infections affecting the feet, face and body, such as *tinea pedis* (athletes foot) and ringworm.

There are apparently no published antimicrobial data for *Elytropappus rhinocerotis* (Zonyane et al., 2013). In a thesis, Knowles (2005) used disc diffusion to show that extracts of *E. rhinocerotis* were active against *Botrytis cinerea*, a fungal pathogen causing grey mould rot on a large number of economically and horticulturally important crops.

In this study, we evaluated the efficacy and scientific rationale for the topical use of *Elytropappus rhinocerotis* leafy twigs to combat foot perspiration and foot odour as well as other topical applications related to the skin.

2. Materials and methods

2.1. Materials

Three samples of aerial material were collected at each of three different localities, giving a total of nine plant samples. Voucher specimens were all lodged at the Herbarium of the University of Johannesburg (acronym JRAU):

Nieuwoudtville (three small samples from one locality: S 31°26′17.6″, E 19°08′36.8″, 705 m), date of collection 05/08/2016, voucher specimens: *Hulley & Van Wyk 38–16* (a, b and c). Vanwyksdorp (three bulk samples, one from each of three localities in close proximity: S 33°46′11.04″, E 21°40′1.82″, 322 m; S 33°46′47.6″, E 21°40′4.63″, 271 m and S 33°47′48.3″, E 21°37′56.7″, 148 m); date of collection 19/05/2016; voucher specimens: *Hulley 30–16* (a, b and c). Waboomskraal (three small samples from one locality: S 33°50′20.1″, E 22°20′57.5″, 566 m), date of collection 14/08/2016, voucher specimens: *Hulley 43–16* (a, b and c).

2.2. Hydrodistillation and extraction for antimicrobial testing

The leafy twigs were air-dried and the essential oils obtained by hydrodistillation using a Clevenger-type apparatus (European Pharmacopoeia, 2005). The oil yields were low (Table 2) and to ensure maximum recovery, hexane was used to recover the oil from the Clevenger apparatus. Oil samples were dried over anhydrous sodium sulphate and their yields calculated on a dry weight basis. Samples were stored in sealed amber vials at 4 °C awaiting analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

Both water and organic extracts were prepared by mixing 1 g of powdered leafy twigs with 10 mL of solvent and leaving it to soak overnight. The solvents were sterile distilled water and a 1:1 mixture of methanol and dichloromethane (MeOH:DCM). The extracts were filtered the next day – the organic solvents were evaporated off in a fume hood and the water solvents were lyopholized. The yields of all samples (in mg/g dry wt) are given in Table 2.

2.3. Antimicrobial studies

According to anecdotes collected during quantitative ethnobotanical interviews in the Cederberg, Kamiesberg and Little Karoo, one of the most common uses for *Elytropappus rhinocerotis* was against foot odour (caused by sweaty feet) and chilblained feet. Therefore, aqueous and solvent extracts for all nine plant samples and essential oils obtained from three bulk plant samples from one locality (Vanwyksdorp) were tested against bacteria that are responsible for foot odour. Zinc oxide (ZnO) with an active concentration of 250 mg/mL (ChemicalSafetyFacts.org) and zinc sulphate ($ZnSO_4$) with an active concentration in foot powders of 150 mg/mL (15% according to Sharquie et al., 2013) were used as positive controls for the Brevibacteria. Ciprofloxacin and amphotericin B were also used as controls

Table 1
Minimum inhibitory concentrations (MICs) for extracts and essential oils of *Elytropappus rhinocerotis*, tested on a selection of pathogens associated with foot perspiration, foot odours and other skin conditions. Samples 1 – 3 are from Vanwyksdorp, 4 – 6 from Nieuwoudtville and 7 – 9 from Waboomskraal. MIC values above 0.1 are given to two decimals; those below 0.1 are given to four decimals. No end point was obtained for the organic extract of sample 8.

Sample no	Sample type	Yield (mg/g dry wt)	MIC (mg/mL)					
			Brevibacterium agri ATCC 51663	*Brevibacterium epidermidis* DSM 20660	*Brevibacterium linens* DSM 20425	*Staphylococcus aureus* ATCC 25923	*Staphylococcus epidermidis* ATCC 12228	*Trichophyton mentagrophytes* ATCC 9533
1	H_2O extract	90.78	> 8	**2.00**	> 8	8.00	> 8	8.00
	MeOH:CH_2Cl_2	163.36	**0.19**	**0.25**	**0.42**	0.44	0.88	1.00
	Essential oil	1.03	> 8	**0.25**	**0.31**	**0.42**	**0.25**	**0.0160**
2	H_2O extract	72.03	> 8	> 8	> 8	2.00	> 8	> 8
	MeOH:CH_2Cl_2	1011.75	**0.0031**	**0.17**	**0.81**	0.22	0.31	**0.0098**
	Essential oil	3.06	> 8	**0.29**	**0.63**	**0.42**	**0.17**	**0.75**
3	H_2O extract	75.43	> 8	> 8	> 8	5.33	> 8	> 8
	MeOH:CH_2Cl_2	190.86	**0.0040**	**0.33**	**0.80**	**0.13**	0.41	2.13
	Essential oil	1.80	**0.0130**	**0.33**	3.00	**0.42**	**0.33**	**0.25**
4	H_2O extract	543.50	> 8	> 8	> 8	2.00	8.00	8.00
	MeOH:CH_2Cl_2	111.00	**0.13**	**0.84**	**0.13**	0.31	0.63	1.00
5	H_2O extract	508.92	> 8	> 8	> 8	8.00	8.00	> 8
	MeOH:CH_2Cl_2	88.60	**0.19**	**1.00**	**0.50**	0.63	1.00	0.25
6	H_2O extract	572.31	8.00	> 8	> 8	4.00	4.00	> 8
	MeOH:CH_2Cl_2	83.00	**0.25**	**0.88**	**0.63**	0.63	1.50	0.75
7	H_2O extract	90.77	5.33	> 8	> 8	> 8	> 8	4.00
	MeOH:CH_2Cl_2	167.70	**0.0890**	**0.81**	**0.13**	0.25	0.50	0.19
8	H_2O extract	75.53	> 8	8.00	> 8	> 8	> 8	6.00
	MeOH:CH_2Cl_2	134.10	**0.25**	0.69	**0.31**	< **0.0630**	1.00	**0.16**
9	H_2O extract	132.52	4.00	8.00	> 8	> 8	> 8	1.50
	MeOH:CH_2Cl_2	159.30	**0.16**	**0.69**	**0.25**	0.50	1.00	**0.0350**
Com-pound 2	(+)-13-epilabdanolic acid		0.058	0.23	0.46	0.46	0.12	ne
Com-pound 4	*ent*-labd-13-en-8β-hydroxy-15-oic acid		0.12	0.46	0.60	0.46	0.46	ne
ciprofloxacin (μg/mL)			n/a	n/a	n/a	0.86	0.47	n/a
zinc oxide			0.33	3.91	2.28	n/a	n/a	n/a
zinc sulphate			0.88	2.34	3.91	n/a	n/a	n/a
amphotericin B (μg/mL)			n/a	n/a	n/a	n/a	n/a	0.0244

n/a: not suitable control; ne: not evaluated (the isolated compounds were tested at a later date, when cultures of *T. mentagrophytes* were no longer available); **bold**: noteworthy activities.

for bacteria and fungi respectively.

2.4. Determination of minimum inhibitory concentrations (MIC)

Water extracts and methanol-dichloromethane extracts of all nine samples, as well three essential oil samples from Vanwyksdorp and two diterpenes (compound **2** and compound **4**) were investigated for antimicrobial activity using the minimum inhibitory concentration (MIC) microtitre plate method described by Eloff (1998) and according to the Clinical Laboratory Standard guidelines for bacteria and filamentous fungi (CLSI Clinical and Laboratory Standards Institute, 2010, 2012). The bacterial cultures (*Brevibacterium agri* ATCC 51663, *Brevibacterium epidermidis* DSM 20660, *Brevibacterium linens* DSM 20425, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228) were subcultured from stock agar plates and grown in Tryptone Soya broth (TSB) for 18–24 h at 37 °C, while *B. linens* was incubated at 30 °C for four days (Orchard et al., 2018). Sabouraud Dextrose broth (SDB) was used for growing the fungal culture *Trichophyton mentagrophytes* ATCC 9533. The aqueous samples were diluted in sterilized water, the diterpenes in DMSO (10% v/v) and the solvent extracts and essential oils in acetone. Hundred microliter (100 μl) samples were dispensed in the first row of the microtitre plate, at a starting concentration of 32 mg/mL for all the samples except the diterpenes at 1 mg/mL. Serial doubling dilutions were undertaken, resulting in concentration ranging from 8 mg/mL to 0.25 mg/mL. The diluted cultures (inoculum size of 1×10^8 colony forming units/mL) were loaded into all the wells of the 96-well microtitre plate. For positive controls, Ciprofloxacin at a starting stock concentration of 0.01 mg/mL was used against the Staphylococci, zinc oxide (starting concentration of 250 mg/mL against the Brevibacteria and amphotericin B, at a starting stock concentration of 0.1 mg/mL, against the fungal dermatophyte. All microtitre plates were sealed with a sterile adhesive and incubated for 24 h at 37 °C for all the bacterial test organisms except *B. linens* and *T. mentagrophytes,* which was incubated for four and seven days respectively at 30 °C. The indicator colour reagent, *p*-iodonitrotetrazolium violet (INT), was prepared at a concentration of 0.4 mg/mL and 40 μl was transferred to each of the inoculated wells after incubation except for *Trichophyton,* where INT was not needed because the growth was evident without INT. Each microtitre plate was examined for colour changes indicating microbial growth after different minimum INT development times, which varied from one hour to six hours, depending on the pathogen. The MIC value was recorded as the lowest dilution having no evidence of bacterial growth.

2.5. Isolation and structural elucidation of compounds

The presence of essential oil, albeit in low yields, is here reported for the first time. Viridiflorol (**1**) could easily be identified because it occurred as a pure compound in sample 8 from Waboomskraal. The identity was confirmed by spectroscopic assignment (1H and 13C NMR). Spectra were matched to those reported by Bombarda et al. (2001), using a Bruker Avance 500 MHz spectrometer. Other major essential oil compounds were not isolated but identified by GC-MS (see Section 2.6).

The three major diterpenes were isolated from a solvent extract (sample 2 from Vanwyksdorp). Approximately 300 g of aerial parts was extracted using dichloromethane and methanol at 1:1 vol ratio, which yielded 12.5 g of a brown resinous solid. All of the extract was applied to dry flash chromatography over 200 g of silica to produce three major fractions by washing with differential mobile phases. Fraction one

Table 2
Major essential oil compounds in nine leaf samples from three localities of *Elytropappus rhinocerotis* (**bold** = major compounds representing 10% or more in at least one sample; nd = not identified).

		Localities:		Vanwyksdorp			Nieuwoudtville			Waboomskraal		
		Plant samples:		1	2	3	4	5	6	7	8	9
		Oil yield (mg/g dry wt):		1.03	3.06	1.80	0.50	1.62	0.97	1.25	1.58	1.41
No	Compounds	AI	Published AI	%	%	%	%	%	%	%	%	%
1	**1,8-Cineole**	**1025**	**1026**	0.0	0.6	0.0	**12.3**	7.4	4.6	0.0	0.0	0.0
2	Santolina alcohol	1026	1034	0.0	0.0	0.0	0.0	1.4	4.4	0.0	0.0	0.0
3	Linalool oxide	1067	1067	0.0	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0
4	Pinocarveol	1139	1135	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
5	**Terpinen-4-ol**	**1182**	**1174**	0.0	0.0	0.0	**16.0**	9.2	**24.3**	0.0	0.0	0.0
6	**nd.**	**1277**	–	0.0	0.0	0.0	0.0	6.1	**22.6**	0.0	0.0	0.0
7	nd.	1322	–	0.0	2.9	2.7	6.6	5.3	0.0	0.0	0.0	0.0
8	nd.	1341	–	0.0	2.6	2.8	1.4	0.0	0.0	0.0	0.0	0.0
9	α-Ylangene	1377	1373	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	β-Bourbonene	1382	1387	0.0	0.0	0.0	0.0	0.9	9.1	0.0	0.0	0.0
11	Damascone	1392	1392	3.8	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	**Germacrene A**	**1515**	**1508**	**15.6**	5.0	3.9	0.0	0.0	0.0	0.0	0.0	0.0
13	**nd.**	**1524**	–	0.0	**12.1**	8.2	**10.5**	**12.6**	0.0	0.0	0.0	0.0
14	nd.	1553	–	3.7	2.1	3.2	0.0	0.0	0.0	0.0	0.0	0.0
15	Longipinonol	1568	1567	3.4	0.0	0.0	0.0	1.1	3.5	0.0	0.0	0.0
16	**(–)-Spathulenol**	**1576**	**1577**	**31.8**	**15.4**	**17.6**	4.5	1.7	9.4	**37.8**	0.0	0.0
17	**Viridiflorol**	**1597**	**1592**	0.0	0.5	0.3	**16.0**	**16.2**	4.4	**62.2**	**100.0**	**91.9**
18	Ledol	1601	1602	0.0	3.5	2.4	4.1	3.3	5.0	0.0	0.0	0.0
19	Humulane-1,6-dien-3-ol	1608	1622	2.6	1.1	1.2	0.0	0.0	0.0	0.0	0.0	0.0
20	**Silphiperfol − 6-en-5-one**	**1616**	**1624**	0.0	**18.2**	**13.2**	**20.1**	**26.8**	4.5	0.0	0.0	0.0
21	Dodedalactone	1676	1676	2.7	2.7	4.4	0.0	0.0	0.0	0.0	0.0	0.0
22	nd.	1741	–	3.4	2.0	5.2	0.0	0.0	0.0	0.0	0.0	0.0
23	Sclareoloxide	1881	1876	1.2	1.6	6.0	0.0	0.0	0.0	0.0	0.0	0.0
24	**nd.**	**1916**	–	4.4	4.3	**13.0**	2.9	0.0	0.0	0.0	0.0	0.0
25	**nd.**	**1957**	–	3.5	2.3	**10.0**	3.3	0.0	0.0	0.0	0.0	0.0
	Total %			**76.1**	**84.5**	**94.1**	**97.7**	**92.0**	**96.2**	**100.0**	**100.0**	**91.9**

(flash-1, 3.5 g) used only cyclohexane (200 mL), flash-2 (5.2 g) used 50% ethyl acetate in cyclohexane (200 mL) and flash-3 (2.3 g) used 100% ethyl acetate (200 mL).

Further fractionation of flash-2 (1 g) using flash column chromatography (200 g silica; 30% ethyl acetate in hexane) yielded three major diterpenes; (+)-13-epilabdanolic acid (**2**) (94 mg), (+)-*ent*-labdanolic acid (**3**) (157 mg) and *ent*-labd-13-en-8β-hydroxy-15-oic acid (**4**) (125 mg). Spectra (1H and 13C) were generated using the same spectrometer. For structural assignment, published spectra for **2** and **3** came from Martha et al. (1998) and Mshengu (2015). Assignment of **4** was made using spectra from Pacheco et al. (2009).

2.6. Major essential oil compounds

Preliminary GC-MS analyses of the nine essential oil samples were undertaken on a Shimadzu 2010 GC-MS system. A non-polar OV-1 (WCOT) column was used, with a detector interface at 250 °C, ion source 200, injector temperature 200 °C, with helium as carrier gas. Using an auto-sampler, 1 µl of sample was injected at a split ratio of 1:20. Column flow was set at 1 mL/min, with a column temperature starting at 60 °C and ramping without hold at 5 °C/min to 280 °C, and held for 5 min at this temperature. The main compounds (all those comprising 10% or more of the total oil) were identified by comparing their mass spectra and retention indices (calculated relative to *n*-alkanes) with Adams (2007), and the NIST and Wiley GC/MS Libraries (Wiley, 2012).

3. Results and discussion

3.1. Antimicrobial activity

3.1.1. Crude extracts

Noteworthy activity was observed where activities were ≤ 0.160 mg/mL (Van Vuuren and Holl, 2017), or having efficacy equivalent or better than the standard controls against test pathogens for the solvent extracts and essential oils (Table 1). Water extracts showed very low or no activity against any of the micro-organisms studied.

Against *B. agri,* noteworthy activity was recorded for almost all of the MeOH:DCM extracts. The most active Vanwyksdorp sample had an MIC value as low as 0.0040 mg/mL. One Nieuwoudtville sample had an average MIC value of 0.13 mg/mL and two Waboomskraal samples had MIC values of 0.0890 and 0.16 mg/mL. These MIC values are substantially lower than the values of the active ingredients commonly used in foot powder. Zinc oxide (ZnO) had an average MIC value of 0.33 mg/mL and zinc sulphate ($ZnSO_4$) an average MIC value of 0.88 mg/mL. Ciprofloxacin had an average MIC value of 3.98 µg/mL (0.00398 mg/mL) against *B. agri*. In the case of *B. epidermidis,* all MeOH:DCM samples, except one had noteworthy activity. Yet again these values illustrated far better activity than that of the commercial active ingredients (ZnO:3.91 mg/mL and $ZnSO_4$:2.34 mg/mL). The MeOH:DCM extracts from Nieuwoudtville (sample 4) and from Waboomskraal (sample 7) had noteworthy activity – both at 0.13 mg/mL. A similar trend was observed against *B. linens* and *S. aureus*. For *S. epidermidis,* not all of the MeOH:DCM extracts demonstrated noteworthy activity, however, the majority still showed good activity with the lowest MIC recorded at 0.31 mg/mL for a sample from Vanwyksdorp (population 2). In the case of *T. mentagrophytes,* two MeOH:DCM samples, one from the Vanwyksdorp (population 2) and one from the Waboomskraal population (sample 9) had excellent activity with MIC values as low as 0.0098 and 0.0350 mg/mL respectively.

3.1.2. Essential oil

Sufficient essential oil for antimicrobial testing was only obtained from the Vanwyksdorp population. In general the activities were broad-

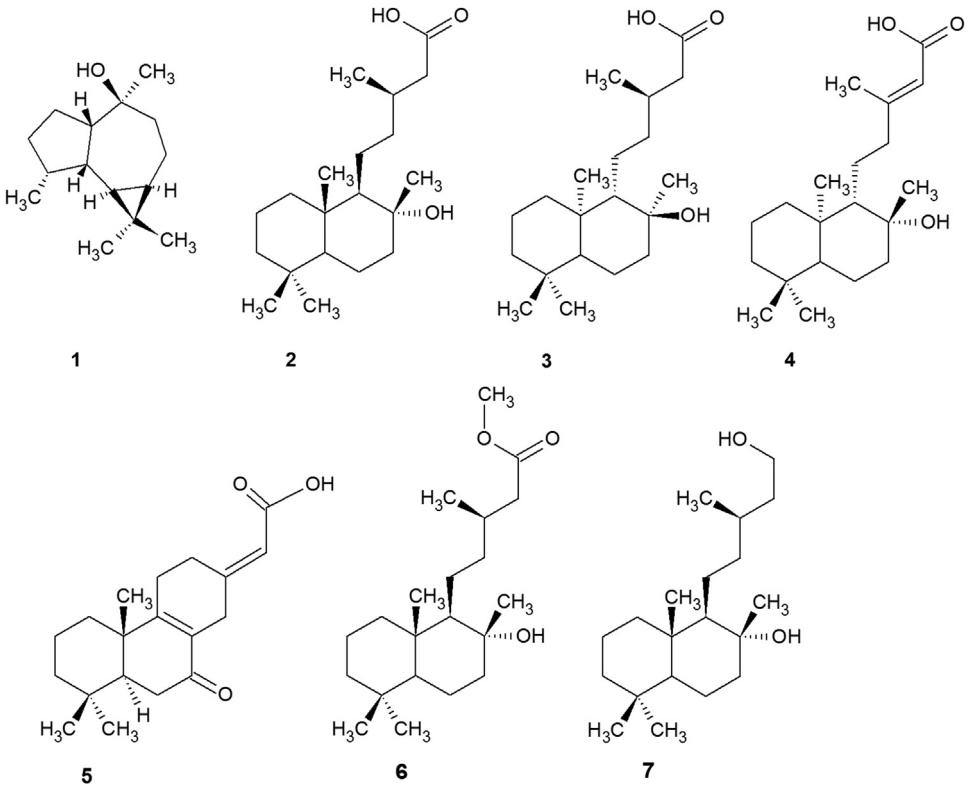

Fig. 1. Chemical structures of isolated compounds from the leafy twigs of *Elytropappus rhinocerotis*. Viridiflorol (**1**), (+)-13-epilabdanolic acid (**2**), (+)-*ent*-labdanolic acid (**3**), *ent*-labd-13-en-8β-hydroxy-15-oic acid (**4**), rhinocerotinoic acid (**5**), (+)-methyl 13-epilabdanolate (**6**) and (+)-(8*R*,13*R*)-labdan-8,15-diol (**7**).

spectrum with highest activities noted against *T. mentagrophytes* (0.0160 mg/mL sample 1) and against *B. agri* (0.0130 mg/mL sample 3). All three essential oil samples showed noteworthy activity against *B. epidermidis* with MIC values of 0.25–0.33 mg/mL. Samples 1 and 2 were the most active against *B. linens* (MIC values of 0.31 and 0.63 mg/mL, respectively). All the samples showed noteworthy activity against both *S. aureus* (MIC of 0.42 mg/mL for all three samples) and *S. epidermidis* (MIC ranging from 0.17 to 0.33 mg/mL). All the essential oil samples also showed noteworthy activity against *T. mentagrophytes*.

These results show that the essential oil probably contributes not only to the traditional use of the plant against skin micro-organisms but also to a wide range of other traditional uses that has been reported.

In a detailed study of the efficacy of essential oils (and various combinations of essential oils) against foot odour organisms, Orchard et al. (2018) recently reported noteworthy activity for several well-known commercial essential oils. The results reported here for *Elytropappus* oil are comparable to or even lower than the MIC values reported in that paper. The topical use of various aromatic plants, commonly referred to as buchu (or *boegoe*) is of considerable historical interest. The San and Khoi people of southern Africa used powdered plant material (often mixed with fat) as deodorants and as traditional cosmetics on the skin (Van Wyk and Gericke, 2018).

3.1.3. Isolated compounds

The antimicrobial activity of the diterpenes **2** and **4** may be considered only moderate (Table 1), with the best activity observed for compound **2** against *B. agri* with an MIC value at 0.0580 mg/mL. Although the activity was only moderate, the contribution of the diterpenes to antimicrobial outcomes in custom use should not be underestimated, since bioactivity may not be due to single compounds but rather to additive or synergistic effects with other compounds within the essential oil.

3.2. Isolated compounds

3.2.1. Structural assignment of the major diterpenes

The structures of the four assigned terpenes are presented in Fig. 1. The current study is the first report of the presence of the two known compounds **1** and **4** in *E. rhinocerotis*. Rhinocerotinoic acid (**5**) was previously reported by Dekker et al. (1988) in this species. The labdane diterpenes **2, 3, 6** and **7** were previously reported by Mshengu et al. (2017) in this species.

Although the quantity of diterpenes recovered in chromatography is substantially less than the actual yield, an estimate from such quantities demonstrates that diterpenes are major components in high yield. Compound **2** was recovered at 3.9% g/g of the extract yield; compound **3** at 6.5% and **4** at 5.2%, giving a total of 15.6% diterpene content in DCM:MeOH extracts. This means that the total diterpene yield relative to the total mass of plant material (twigs and leaves) exceeded 1.6%.

Dekker et al. (1988) reported the isolation of a diterpene called rhinocerotinoic acid (**5**) from *Elytropappus rhinocerotis* (Fig. 1), but subsequent workers (Grey et al., 2003; Mshengu et al., 2017) were not able to re-isolate this compound. Instead, they reported four other labdane diterpenes, namely (+)-13-epilabdanolic acid (**2**), (+)-*ent*-labdanolic acid (**3**), (+)-methyl 13-epilabdanolate (**6**) and (+)-(8 *R*,13 *R*)-labdan-8,15-diol (**7**). Several flavones, most of them methoxylated, have also been isolated from the plant (Ticha et al., 2015; Mshengu et al., 2017).

3.3. Essential oil composition

The preliminary results of the GC-MS analysis is summarised in Table 2. Further work is in progress to obtain more samples for detailed analyses of all compounds. The six major constituents (those representing 10% or more of the total oil) were 1,8-cineole (present in all three samples of Nieuwoudtville, 4.6–12.3%); terpinen-4-ol (only in the Nieuwoudtville samples, 9.2–24.3%); germacrene A (only in the

Vanwyksdorp samples, 3.9–15.6%); (−)-spathulenol (present in all except two samples, often in very high percentage yield); viridiflorol, another sesquiterpene alcohol, present in all except one sample, and in high percentage only in the Waboomskraal samples (62.2–100%); silphiperfol-6-en-5-one, present in all three samples of Nieuwoudtville (4.5–26.8%) and in two samples of Vanwyksdorp (13.2% and 18.2%). A geographically representative collection of samples will be required to gain meaningful insights into the chemical diversity of the species throughout its geographical range. The results presented here are the first for this species and give at least a glimpse of the chemical diversity and main compounds that can be expected. Bergh et al. (2007) reported considerable molecular genetic variation within and between populations of the plant and it seems that essential oil compositions may show similar uneven geographical distributions.

Elytropappus rhinocerotis has been described as eglandular (Levyns, 1935), probably because the glands of this species are partly covered by a dense layer of tomentose hairs. Koekemoer (2002) reported that two types of trichomes are present. Multicellular glands with two or four basal cells and two terminal rows of two to four cells each occur on the abaxial leaf surfaces. These glands are densely spaced but inconspicuous due to the co-occurring hairs. The second type of gland has a unicellular stalk and a multicellular head and occurs sporadically on the stems. It is likely that one or both of these multicellular glands are the site of essential oil production and/or accumulation but this aspect requires further detailed study.

4. Conclusion

The presence of essential oil in *Elytropappus rhinocerotis* has not been previously reported despite the importance of the plant in a wide range of medicinal applications. This study also revealed that the chemical composition of the plant has remained poorly known and that considerable variation can be expected. A comprehensive chemical variation study covering the entire distribution area of the species may yield interesting results.

The noteworthy antimicrobial activity supports the efficacy and scientific rationale of the traditional use of *Elytropappus rhinocerotis* to treat foot perspiration and foot odour. Some of the MIC values reported here are indeed amongst the lowest ever recorded for a natural product against Brevibacteria and *Trichophyton mentagrophytes*.

Acknowledgements

We would like to acknowledge the University of Johannesburg and the National Research Foundation (NRF) of South Africa for financial support (Grant UID: 88432 to MH; and Grant Number 8442 to BEvW, the SARChi National Research Chair in Indigenous Plant Use). CapeNature kindly provided a plant collecting permit (permit no. 0028-AAA008–00194). Mr A Britz (Hoogekraal Farm, Vanwyksdorp) is thanked for collecting some of the plant material used in this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.09.014.

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