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Antimicrobial activity of volatile and non-volatile isolated compounds and extracts from the bark and leaves of *Warburgia salutaris* (Canellaceae) against skin and respiratory pathogens

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ABSTRACT

Warburgia salutaris is one of the most popular trees used in traditional medicine throughout tropical southeast Africa, because of its ability to treat a wide variety of illnesses caused by fungi, bacteria, viruses and insects. The aim of the study was to test the antimicrobial activity of *W. salutaris* and its isolated compounds against selected skin and respiratory pathogens. Furthermore, to investigate the main chemical compounds of this species and compare their antimicrobial activities with previously published studies. Crude extracts, essential oils and isolated compounds from the bark of *W. salutaris*, showed antimicrobial activity against skin pathogens (*Pseudomonas aeruginosa* ATCC 743971 and *Staphylococcus aureus* ATCC 25923) and respiratory tract pathogens (*Klebsiella pneumoniae* ATCC 13883 and *Moraxella catarrhalis* ATCC 23246). Bioassay guided fractionation of the dichloromethane extract (muthi market bark sample) and essential oil (from the bark of the cultivated tree) yielded six compounds, which included the two major essential oil components drimenol (**1**) and *E*-nerolidol (**2**) and the less volatile drimane sesquiterpenes from the muthi market bark: 12α-acetal-polygodial (**3**), polygodial (**4**), ugandensidial (**5**) and warburganal (**6**). Noteworthy activity against *M. catarrhalis* was observed by **2** with a minimum inhibitory concentration of 31 μg/ml and by **4** with an MIC of 25 μg/ml against *K. pneumoniae*. This is the first report of **1**,**2** and **3** isolated from *W. salutaris* and the identification of these compounds could explain the traditional use of this species as an inhalation therapy to treat respiratory ailments. Despite numerous studies, the chemical composition and biological activities of the leaves and bark of *W. salutaris* are not yet fully explored.

1. Introduction

South Africa is faced with a serious need for improved health care services, particularly to lower socioeconomic groups. The country is one of the world leaders in the epidemic of contagious infections such as human immunodeficiency virus (HIV) or acquired immune deficiency syndrome (AIDS) (Kumarasamy, 2004) and multi-drug resistance in tuberculosis (WHO, 2013). However, even on a global scale, treatment of contagious disease is becoming more difficult due to the development of resistant pathogenic strains (Sekyere, 2016). Hence, further drug development is necessary in the search for suitable alternatives.

According to Mander (1997), about 72% of black South Africans depend largely on traditional medicine, which is predominantly of botanical origin. Southern Africa has one of the world's richest species diversity indexes and from that is a high representation of medicinal plants, with nearly 3000 species used in treatment of a wide variety of ailments (Van Wyk and Gericke, 2000; Arnold et al., 2002). *Warburgia salutaris* (Bertol. f.) Chiov. is one of the more popular plant species of the 16 that have been fully commercialised as medicinal products of southern Africa (Van Wyk, 2011).

Warburgia salutaris is a small evergreen tree growing up to 10 m tall, occurring in savanna woodland, coastal forest and Afromontane forests of KwaZulu-Natal, Limpopo, Mpumalanga, Swaziland, Mozambique and Zimbabwe (Van Wyk et al., 2009; Leonard and Viljoen, 2015). The genus *Warburgia* is comprised of four species, also including *W. elongata* Verdc., *W. ugandensis* Sprague, and *W. stuhlmannii* Engl. (Maroyi, 2014). The last three are located in East Africa, outside the more southern geographical distribution of *W. salutaris*.

The 'health giving' benefits of *W. salutaris* have led to its drastic decline in the wild because of over harvesting and exploitation of the bark by muthi market traders, herbalists and traditional healers (Williams, 1996). As a result, the plant is considered endangered in South Africa, vulnerable in Mozambique, critically endangered in Swaziland and facing extinction in the wild in Zimbabwe (Maroyi,

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2014). One of the most common uses of *W. salutaris* is the inhalation of smoke from the powdered bark, for treatment of upper respiratory tract infections, such as coughs, colds, tuberculosis and influenza (Van Wyk and Gericke, 2000; Madikane et al., 2007). An infusion or decoction of the bark is also used for abdominal pains, backache, blood disorders, malaria, toothache, venereal diseases, constipation, stomach ulcers or as an emetic or a purgative for febrile complaints (Hutchings et al., 1996; Rabe and Van Staden, 2000; Grace et al., 2003). Furthermore, powdered bark is extracted in fat and applied directly onto the skin to treat sores, inflammation, snakebites, rheumatism and skin eruptions (Mabogo, 1990; Van Wyk and Gericke, 2000; Van Wyk et al., 2009; Maroyi, 2014). According to traders on the Faraday and Kwa Mai-Mai Traditional Medicine (*Muthi*) Markets in Johannesburg, an infusion of the bark is taken orally (one cup per day) to treat diarrhoea and influenza. A common method of administration is also to lick the powdered bark from the back of the hand (a traditional Zulu practise called *khotha*), in this case to treat the symptoms of colds and influenza by loosening the phlegm in the chest and promoting healing (Khumalo, unpublished data).

Previous chemical investigations on *W. salutaris* revealed the presence of drimane sesquiterpenes such as isopolygodial, warburganal, polygodial, mukaadial, salutarisolide (Mashimbye et al., 1999), muzigadial (Rabe and Van Staden, 2000) and 11α-hydroxycinnamosmolide (Madikane et al., 2007). Another study identified furans and furanones (Mohanlall and Odhav, 2009). Drimane sesquiterpenes possess antimycobacterial, antibacterial, antifeedent, antifungal, anti-inflammatory and antimycotoxigenic activity (Maroyi, 2014; Leonard and Viljoen, 2015). Other prominent international botanical sources of drimane sesquiterpenoids are *Polygonum* L. (Fukuyama et al., 1982), *Drymis winteri* J.R.Forst & G.Forst (Montenegro et al., 2014), *Canella* P.Browne (Ying et al., 1995) and *Tasmannia* R.Br. (Mathie et al., 2017), just to name a few. The drimane-type sesquiterpenes give a peppery flavour to these species (Mathie et al., 2017), which influences their vernacular and botanical names, such as Tasmanian pepper [*Tasmannia lanceolata* (Poir.) A.C.Sm., water pepper (*Polygonum hydropiper* L.) and pepper bark tree (*Warburgia salutaris*)].

The aim of the current study was to identify the active components responsible for antimicrobial effects against respiratory and skin pathogens. Furthermore, we also examined the chemistry of both volatile and non-volatile fractions. The chemistry of essential oils is complex, and this study therefore also aimed to identify major components of the essential oils, which could be responsible for the antimicrobial activity observed when smoke inhalation is used as a form of administration. The full chemical characterisation of the essential oils was beyond the scope of this study.

2. Methods

2.1. Plant material and hydrodistillation

Bark samples of *W. salutaris* were obtained from cultivated specimens and purchased from both the Faraday and KwaMai-Mai muthi markets, in Johannesburg (South Africa). Leaves were collected from two cultivated trees (ex hort., B-E Van Wyk). Bark and leaves were hydrodistilled using a Clevenger-type apparatus. Vouchers specimens of the cultivated trees (*Khumalo 1a, 1b*) were lodged at the University of Johannesburg Herbarium (JRAU).

2.2. Extraction, isolation and chemical assignment

Methanol (MeOH) and dichloromethane (DCM) extracts were prepared from dried plant material. Based on antimicrobial activity, a ratio of 1:1 (MeOH:DCM) was selected as the extractant for further isolation. The powdered bark from the muthi market (717.7 g) was extracted using 1500 ml of solvent. Essential oils were extracted by hydrodistillation for 24 h, using a Clevenger-type apparatus.

Isolation of components from essential oils (bark of cultivated trees) and bark extract from the muthi market sample (MeOH:DCM) was performed using flash column chromatography exclusively over silica gel with cyclohexane (Cyclohex) and ethyl acetate (EthOAc) as the mobile phase. Essential oils were studied by gas chromatography (GC-FID) and the less complex oil from the cultivated trees were subjected to column chromatography with 1:9 (EthOAc:Cyclohex). The 1:1 (DCM:MeOH) bark extract was chromatographed with 1:9 (EthOAc:Cyclohex) and was gradually increased to 2:8 (EthOAc:Cyclohex). Fractions of *ca.* 5 ml were collected in test tubes and those containing pure compound were combined.

The identities of all isolated compounds were confirmed by comparison of 1H and 13C NMR spectra with published values using a Bruker Avance 500Mhz NMR. Pure compounds that were identified included drimenol **1** (24 mg) (Urban and Capon, 1996), *E*-nerolidol **2** (114 mg) (Machado et al., 1998), 12α-acetal-polygodial **3** (103 mg) (Ying et al., 1995), polygodial **4** (72 mg) (Mashimbye et al., 1999), ugandensidial **5** (188 mg) (Cortes et al., 1990) and warburganal **6** (58 mg) (Mashimbye et al., 1999).

2.3. Minimum inhibitory concentration determination

The antimicrobial activity was determined using methods described by Eloff (1998). The minimum inhibitory concentration (MIC) was obtained for plant extracts, essential oils and isolated compounds against *Pseudomonas aeruginosa* ATCC 743971, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883 and *Moraxella catarrhalis* ATCC 23246. Bacterial cultures were obtained from Davies Diagnostics (South Africa) and inoculated in Müller Hinton (MH) broth. Crude extracts were tested at a concentration range from 0.06–8.0 mg/ml, while isolated compounds ranged from 0.01 to 1.25 mg/ml. All test samples were dissolved in acetone. Crude extracts isolated compounds (100 μl) were transferred to the first row of a 96-well micro-titre plate together with 100 μl MH broth which was inserted in all wells. Dimethyl sulfoxide (DMSO) was used where solubility was problematic, and was added at no higher than 6.25%. Acetone and DMSO were used as negative controls and ciprofloxacin (Sigma–Aldrich) at a starting concentration — 0.01 mg/ml) was used as the positive control. Two fold serial dilutions were performed, followed by the addition of 100 μl of culture (1×10^6 colony forming units per ml (CFU/ml) into all the wells. Each plate was then sealed with a sterile adhesive sealing film. The plates were then incubated for 18 h at 37 °C. Following incubation, 40 μl of *p*-iodonitrotetrazolium chloride (INT) (Sigma–Aldrich) was added as a growth indicator to each well of the micro-titre plates. The MICs were recorded as the lowest concentration of test compounds and crude extracts required to inhibit microbial growth. Microbial growth was observed by the formation of a red colour.

3. Results

The two major components isolated from the bark essential oils (Fig. 1) were drimenol **1** and *E*-nerolidol **2**. Further components isolated from the bark by solvent extraction included the known non-volatile sesquiterpenes 12α-acetal-polygodial **3**, polygodial **4**, ugandensidial **5** and warburganal **6** (Fig. 1).

The essential oil component *E*-nerolidol is the only non-drimane isolated in the current study and was reported in the genus *Warburgia* for the first time. This is not surprising since the chemistry of essential oils from *W. salutaris* is poorly documented. This compound also demonstrated noteworthy antimicrobial activity with an MIC of 0.031 mg/ml against *M. catarrhalis* (Table 1). 12α-Acetal-polygodial was also newly identified in the genus *Warburgia*, with an average MIC value of 0.15 mg/ml against *K. pneumoniae*.

The DCM crude extract from the stem bark showed better overall antimicrobial activity when compared to the MeOH extract. The DCM

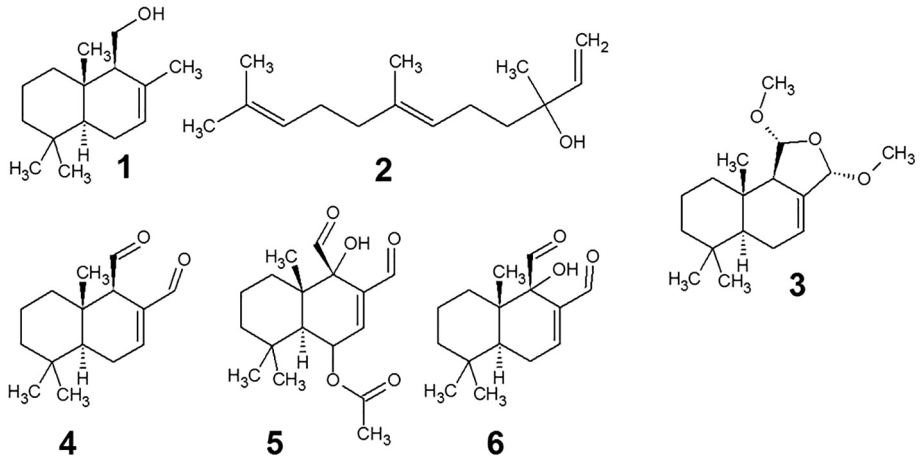

Fig. 1. Six antimicrobial compounds isolated and identified from *Warburgia salutaris* bark: essential oil hydro-distilled from the bark yielded drimenol (**1**) and *E*-nerolidol (**2**), while solvent extraction of the bark yielded the non-volatile sesquiterpenes 12α-acetal-polygodial (**3**), polygodial (**4**), ugandensidial (**5**) and warburganal (**6**).

extract was most active against *P. aeruginosa* with an MIC of 0.25 mg/ml (Table 1). This provides evidence that the activity is most likely related to the less polar components, such as the drimane sesquiterpenes isolated in the current study. Acetone and DMSO were used as negative controls and at the highest percentage, no inhibition was observed (these results are therefore not shown in Table 1).

Essential oils from the bark and leaves of the cultivated trees showed similar MIC values against all tested bacteria, while the muthi market bark was slightly more active, with the lowest concentration of 0.25 mg/ml against the Gram negative *P. aeruginosa*. The DCM extract from the bark of the muthi market sample was more active compared to the MeOH extract, with the lowest MIC value of 0.25 mg/ml against *P. aeruginosa*. However, antimicrobial activities of individual components isolated from the stem bark (muthi market sample) were more active compared to the crude extracts or whole essential oil (see Table 1), with the lowest average value of 0.025 mg/ml by polygodial. Drimenol demonstrated some activity against both *M. catarrahalis* and *S. aureus* at a concentration of 0.062 mg/ml. Activity of warburganal ranged from 0.104 mg/ml to 0.208 mg/ml against the four bacterial species.

4. Discussion

Three previous studies of *W. salutaris* demonstrated chemical variation. A study of specimens from the Zoutpansberg mountain of the Venda region (Limpopo Province) identified warburganal, polygodial, mukaadial, isopolygodial and a new lactone, named salutarisolide (Mashimbye et al., 1999). Another study of cultivated specimens from Silverglen Nature Reserve near Durban reported muzikadial (Rabe and van Staden, 2000), which was not isolated in the earlier study. A study by Madikane et al. (2007) from cultivated trees at Silverglen identified the presence of the component 11α-hydroxycinnamosmolide. This compound showed potent anti-mycobacterial activity. The crude extract itself also demonstrated anti-mycobacterial activity (Leonard et al., 2010).

In the current study, chemical variation was observed, since the two cultivated trees differed from the muthi market samples. Furthermore, both 12α-acetal-polygodial and ugandensidial were reported in *W. salutaris* for the first time. These constituents were also the dominant components identified in the DCM:MeOH extract. Ugandensidial was previously isolated from *W. ugandensis* and *W. stuhlmannii* (Leonard and Viljoen, 2015). However, the current study constitutes the first reported occurrence of 12α-acetal-polygodial in the genus *Warburgia*. It was previously isolated from another member of the family Canellaceae, *Canella winterana* (L.) Gaertn. (Ying et al., 1995).

Warburganal is one of the well-known compounds in terms of its biological activities. It has potent antifungal, molluscicidal and antifeedant activity (Kubo et al., 1977). However, polygodial and muzikadial have also been examined and these compounds showed antifungal activity (Kubo and Taniguchi, 1988). Muzikadial has shown excellent activity, with values as low as 12.5 μg/ml (Rabe and Van Staden, 2000), which is more active than the isolates tested in the current study. In the study by Rabe and Van Staden (2000), muzikadial was also tested against the respiratory pathogen *K. pneumoniae* but had low activity. In the current study, *K. pneumoniae* was also the most susceptible microorganism. Warburganal, polygodial and muzikadial have also been reported to possess strong cytotoxicity against cancer cell lines (Fukuyama et al., 1982; Leonard and Viljoen, 2015; Montenegro et al., 2014). The compound polygodial does not induce any mutagenic effects, giving it the advantage (Anke and Sterner, 1991). Toxicity studies

Table 1
Minimum inhibitory concentrations (mg/ml) of solvent extracts, essential oils and isolated compounds from *Warburgia salutaris* leaf and bark.

	Klebsiella pneumoniae ATCC 13883	*Moraxella catarrhalis* ATCC 23246	*Pseudomonas aeruginosa* ATCC 743971	*Staphylococcus aureus* ATCC 25923
W. salutaris bark extract (MeOH)	1.00	2.00	1.66	1.66
W. salutaris bark extract (Dichloromethane)	1.00	0.42	0.25	0.50
Essential oil from leaves (cultivated tree)	0.66	1.00	0.50	1.00
Essential oil from bark (cultivated tree)	0.50	1.00	0.50	1.00
Essential oil from bark (muthi market)	0.83	0.50	0.25	0.50
Drimenol (**1**)	0.208	0.062	0.312	0.062
E-nerolidol (**2**)	0.312	0.031	0.260	0.416
12α-Acetal-polygodial (**3**)	0.156	0.625	0.625	0.521
Polygodial (**4**)	0.025	˚0.25	˚0.25	˚0.25
Ugandensidial (**5**)	0.130	0.104	0.078	0.130
Warburganal (**6**)	0.130	0.208	0.104	0.156
Ciprofloxacin positive control (μg/ml)	0.078	0.078	0.065	0.065

on appropriate lung cell lines and epithelial or oesophagal cell lines will be necessary, as the therapy with *Warburgia* would most likely be through inhalation.

The antimicrobial and other biological activities of the remaining compounds isolated in the current study, namely drimenol, 12α-acetal-polygodial and ugandensidial (also known as cinnamodial), are not well known. The antimicrobial activities demonstrated in the current study are therefore relevant in adding to overall understanding of the potential efficacy of *W. salutaris* as a traditional medicine and potential phytomedicine.

Since *W. salutaris* is administered as a smoke inhalation therapy, it is necessary to consider the biological activities of metabolites stable in the gaseous phase. The essential oil components are therefore of relevance in this context. The dialdehyde molecules risk degradation at higher temperatures, but there is a chance that some will come into contact with the mucous membranes of the lungs in such smoke therapies. However, it may be of more relevance to consider drimenol in this scenario. In the current study, drimenol had somewhat good antimicrobial activity and so it is considered as one of the relevant compounds involved in therapeutic activity in smoke inhalation therapy. At high temperatures used in smoke inhalation therapies, polygodial and 12α-acetal polygodial can be made gaseous and will be present in the smoke. They are unlikely to appear in hydrodistilled essential oil as they require high temperatures to become gaseous.

In applications where poultices and lipophilic extracts are applied topically, or where ingestion of an extract is involved, the other compounds may be considered. For example, both polygodial and mukadial displayed significant anti-inflammatory activities *in vitro* by lipoxygenase inhibition (Frum et al., 2005). Such activity is of relevance where intestinal inflammation is a targeted symptom. In addition, where insecticidal activity is the objective it is self-evident that the insecticidal drimanes, such as warburganal, are the active compounds.

5. Conclusions

The essential oil of *W. salutaris* yielded two major components, drimenol and *E*-nerolidol, the latter here reported for both the genus *Warburgia* and *W. salutaris* for the first time. Furthermore, 12α-acetal-polygodial and ugandensidial are not only new reports for *W. salutaris*, but they were the major components in crude extracts. Together with demonstrated antimicrobial activity (especially for ugandensidial and the volatiles drimenol and *E*-nerolidol), these results provide some new perspectives on the traditional uses of *Warburgia* in treating skin and respiratory ailments.

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