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In vitro antifungal activity of the acetone extract and two isolated compounds from the weed, *Pseudognaphalium luteoalbum*

M.A. Aderogba [a,b], L.J. McGaw [c,*,1], V.P. Bagla [c], J.N. Eloff [c], B.M. Abegaz [b]

[a] Department of Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria
[b] Department of Chemistry, University of Botswana, Gaborone, Botswana
[c] Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Onderstepoort 0110, South Africa

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ABSTRACT

Invasive and weedy species such as *Pseudognaphalium luteoalbum* may serve as a source of biologically active extracts or compounds with application in crop and ornamental plant protection, among other uses. The acetone crude extract of *P. luteoalbum* leaves had strong antifungal activity when tested against a selection of plant pathogenic fungi *in vitro*. Fractionation of the crude extract to isolate, characterize and evaluate the antifungal constituents afforded two compounds. Structure elucidation of the isolated compounds was carried out using spectroscopic techniques: mass spectrometry and NMR (1D and 2D). The compounds were identified as: 5,4′-dihydroxy-6-methoxy-7-*O*-β-glucopyranosideflavone (hispidulin-7-*O*-glucopyranoside) (**1**) and stigmasterol-3-O-β-glucopyranoside (**2**). These compounds are reported from *P. luteoalbum* for the first time. The crude extract and isolated compounds were moderately to highly active against a selection of phytopathogenic fungal organisms with MIC values ranging from 0.02 to 1.25 mg/mL. No cytotoxicity of the isolated compounds against Vero kidney cells was observed at 200 μg/mL, the highest concentration tested.

1. Introduction

Plant diseases caused by fungi are of considerable economic concern due to crop production losses and post-harvest damage to fruits and vegetables. In addition to causing various diseases, several species of fungi including those belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* are important sources of mycotoxins which impact on human and animal health (Robert and Richard, 1992; Eaton and Gallagher, 1994; Placinta et al., 1999). Fungicides currently used to control fungal infections may be toxic, and often have harmful effects on the environment. Additionally, resistance to commonly used synthetic fungicides is developing in many pathogenic fungal species, strengthening motivation to search for alternative antifungal agents. Many plant species contain antifungal compounds (Hostettman et al., 2000), and these may be a potential renewable source of antifungal agents useful on a commercial scale. Developing products from plants requires the availability of a sufficiently large quantity of raw material (Mdee et al., 2009), and invasive or weedy species may be a useful source to investigate with this in mind. If the presence of fungal pathogens is significant in the establishment and growth of plant species, invasive plant species may have increased resistance to these fungi, leading to their success under conditions where most other species would fail to thrive (Eloff et al., 2007a; Mdee et al., 2009). Weeds may therefore become a readily available source of raw material for plant-based fungicidal agents.

Plants that are known as weeds are adaptable, vigorous growers, and are able to withstand unfavourable conditions. They are easily spread and are difficult to control. In addition to competing with crops for nutrients and water, they interfere with biodiversity, change the natural indigenous ecology and act as hosts for crop diseases and insect pests (Bromilow, 2010). *Pseudognaphalium luteoalbum* (L.) Hilliard & B.L. Burtt (Asteraceae), known as Jersey Cudweed, is a common and widespread weed, found in all continents. It is an annual herb, branching from the base, with cream, yellow, white or pink flowers appearing in early summer. *P. luteoalbum* (synonym *Gnaphalium luteoalbum*) originates from Europe and was introduced by early settlers to South Africa where it is now common and widespread, and is known as a winter weed of maize lands (Bromilow, 2010).

The current study was initiated to investigate *P. luteoalbum* as a source of antifungal compounds active against plant pathogenic fungi of economic importance. New biomedical products from problem plants and weeds are being discovered (Dold and Cocks, 2000) and alternative uses for these species should be considered before expensive eradication is undertaken (Webber et al., 1999).

* Corresponding author at: Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa. Tel.: +27 12 529 8525; fax: +27 12 529 8304.
E-mail addresses: lyndy.mcgaw@up.ac.za, mcgaw@ukzn.ac.za (L.J. McGaw).
[1] Present address: Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa. Tel.: +27 33 260 5132; fax: +27 33 260 5897.

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2. Materials and methods

2.1. General

All solvents were laboratory grade reagents and were distilled prior to use. All thin layer chromatography analyses were performed at room temperature using pre-coated plates (Merck, silica gel 60 F_{254}). Detection of spots was performed by viewing under ultraviolet light using a Camag Universal UV lamp TL-600 (254 and 365 nm). Open column chromatography was carried out using silica gel 60 (Merck, Darmstadt, Germany). Nuclear Magnetic Resonance (NMR) ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were recorded on a Bruker UltraShield spectrometer (Bruker Analytik GmbH, Karlsruhe, Germany). Chemical shifts are expressed in parts per million (ppm). The Electron Spray Ionisation (ESI) mass spectrum was recorded on a Finnigan LCQ Deca spectrometer (Thermo Finnigan, San Jose, USA).

2.2. Plant material

The leaves of *P. luteoalbum* were collected in Gaborone in October 2007 (early summer). The plant was identified by Mr M. Muzila, University of Botswana Herbarium, Department of Biological Sciences. A voucher specimen (number 1668, collector's number 345) was deposited in the same herbarium. The collected leaves were air dried in the shade for two weeks and powdered.

2.3. Extraction

Powdered leaves (700 g) were extracted with acetone (Eloff, 1998a) for 24 h. The extract was filtered and concentrated *in vacuo*. The crude acetone extract (40 g) was subjected to vacuum liquid chromatography (VLC) using the following solvent systems in turn: hexane 100%, hexane/dichloromethane (1:1), dichloromethane 100%, dichloromethane/ethyl acetate (1:1), ethyl acetate 100% and ethyl acetate/methanol (7:3). A total of six fractions were collected and concentrated *in vacuo*. These were labelled fractions 1–6, accordingly. Fractions with promising (visible) spots from the TLC analysis (dichloromethane/methanol 9:1) and reaction with spraying reagent vanillin–H_2SO_4 were selected for fractionation and isolation of compounds.

2.4. Antifungal assay

The minimum inhibitory concentration (MIC) values for the plant extract and isolated compounds were determined using a serial microdilution assay (Eloff, 1998b) modified by Masoko et al. (2005). Aliquots of the crude acetone extract and the isolated compounds were dissolved in acetone to final concentrations of 10 mg/mL and 1 mg/mL, respectively. Water (100 μL) was added to each well of a 96-well microtitre plate, and the test substances (100 μL) were added to the first well of allocated columns of the microplate. A twofold serial dilution of the test substances was prepared by pipetting 100 μL from well A to well B, continuing successively down the columns until 100 μL was discarded from well H. Fungal cultures, grown overnight in fresh Sabouraud Dextrose (SD) broth, were added (100 μL) to each well. Acetone was included as a solvent blank, and amphotericin B (Sigma) was used as the reference antibiotic. To indicate fungal growth, 40 μL of a 0.2 mg/mL solution of p-iodonitrotetrazolium violet (INT, Sigma) in water was dispensed in each well. The microtitre plates were then covered and incubated at 100% relative humidity at 35 °C. The MIC values were recorded as the lowest concentration of the test substance that inhibited fungal growth after 24 and 48 h. Inhibition of fungal growth was read as a decrease in the intensity of the red colour produced by reduction of INT by actively growing fungi compared to the negative control.

2.5. Isolation of the constituents

2.5.1. Compound 1

Fraction 6 [(ethyl acetate/methanol 7:3)] (2.9 g) was chromatographed on a silica gel column by eluting with hexane followed by an increasing gradient of ethyl acetate up to 100% and followed in turn by an increasing gradient of methanol up to 100%. A total of 53 test tubes (20 mL each) were collected. This afforded three fractions, A1–C1 following TLC analysis using chloroform/methanol 4:1 solvent system. Fraction B1 (1.7 g) was fractionated on a silica gel column using hexane as eluent followed by an increasing gradient of ethyl acetate up to 100% and followed in turn with methanol up to 20%. A total of 55 test tubes were collected and this afforded two fractions, A2 and B2 upon TLC analysis using dichloromethane/methanol 8.5:1.5 solvent system. Fraction A2 was purified on Sephadex LH-20 column using chloroform/methanol (4:1). This gave four fractions (A3–D3) on analysis by TLC (thin layer chromatography) using dichloromethane/methanol (4:1) as the solvent system. B3 (30 mg) was further purified on a Sephadex LH-20 column using ethyl acetate/methanol (4:1). Twenty test tubes were collected and analysed by TLC using dichloromethane/methanol (6:1) as the solvent system. Test tubes 16–19 showed a single spot and were bulked together. This gave compound **1** (12 mg).

2.5.2. Compound 2

Fractions 4 [dichloromethane/ethyl acetate (1:1)] and 5 [ethyl acetate 100%] (0.77 g) had a similar TLC profile (dichloromethane/methanol 9:1) on spraying with vanillin–H_2SO_4 (1%) and were combined. Further fractionation was carried out using silica gel column chromatography. The column was eluted with hexane followed by an increasing gradient of chloroform up to 100%. This was in turn followed with an increasing gradient of methanol up to 100%. A total of 60 test tubes were collected. The TLC analysis of the collected fractions was carried out using a mixture of dichloromethane/methanol (9:1) as solvent system. Four fractions were obtained, A4–D4. Fraction B4 was purified on a Sephadex LH-20 column starting with chloroform/methanol (9:1) followed by an increasing gradient of methanol up to 50%. A total of 20 test tubes were collected and this afforded two fractions (A5 and B5) on TLC analysis using a dichloromethane/methanol (9:1) solvent system. Fraction B5 was further purified on a Sephadex LH-20 column starting with ethyl acetate followed by ethyl acetate/methanol (4:1). A total of 45 test tubes were collected and analysed on a TLC plate using chloroform/methanol (9:1) as solvent system. Tubes 15–18 showed a single spot on the TLC plate sprayed with vanillin–H_2SO_4 (1%) and were bulked together. This gave compound **2**, a white powder (10 mg).

2.6. Cytotoxicity assay

The pure compounds were tested for cytotoxicity against the Vero (African green monkey kidney) cell line. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983) was employed to test for cell viability following exposure of the cells to the test substances. This assay is based on the ability of the mitochondrial succinate dehydrogenase enzymes of metabolically active cells to reduce MTT to a water-insoluble purple formazan. The amount of coloured formazan produced is directly proportional to the number of viable cells present (Mosmann, 1983).

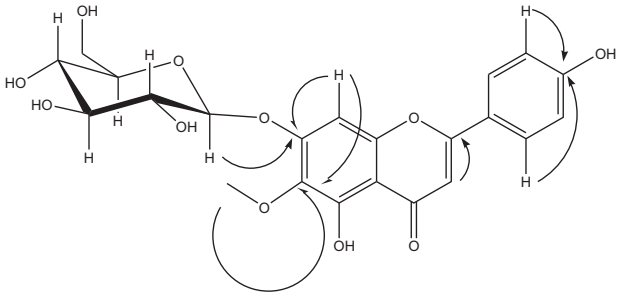
The cells were cultured in Minimal Essential Medium (MEM) Earle's Base (Sigma) with non-essential amino acids, L-glutamine and $NaHCO_3$ supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Confluent monolayer cultures were used to prepare cell suspensions which were plated into 96-well microtitre plates at a density of 0.5×10^3 cells per well. The microplates were incubated overnight at 37 °C (5% CO_2), and the attached, subconfluent cells were used in the cytotoxicity assay.

The purified compounds were resuspended in dimethyl sulfoxide (DMSO) to a concentration of 20 mg/mL before being diluted in fresh MEM to the required concentrations. The concentrations tested ranged from 10 to 200 μg/mL, and the compounds were exposed to the cells for 120 h. Berberine chloride (Sigma) was the positive control and solvent controls were also included. Cell-free controls were also incorporated to investigate the non-specific reduction of MTT by plant extracts. Tests were conducted in quadruplicate and each experiment was repeated at least twice. Following incubation with test compounds, the cells were examined using an inverted microscope to detect cytopathic effects (CPE). Each well was rinsed with phosphate buffered saline (PBS) after removal of the MEM containing plant compounds, and fresh MEM without test substance added to the wells. MTT (30 μL of a 5 mg/mL solution in PBS) was then added to each well and the plates were incubated for a further 4 h at 37 °C in 5% CO_2. The medium was aspirated from the wells after which 50 μL DMSO was added to each well to solubilise the formazan crystals. The absorbance was measured at a wavelength of 570 nm in a Versamax microplate reader (Molecular Devices).

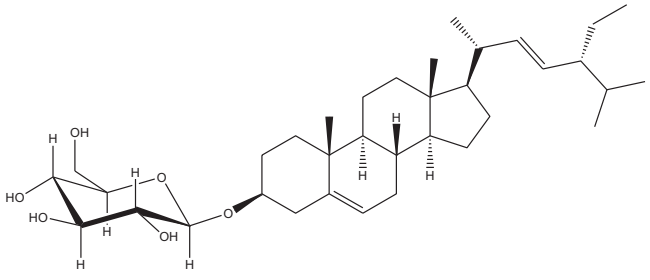
2.7. Structure elucidation of isolated compounds

2.7.1. Compound 1

The ESI mass spectrum (positive mode) showed the molecular ion peak as the base peak at m/z = 485.30 $[M + Na]^+$ that was consistent with the molecular formula $C_{22}H_{22}O_{11}$. This was confirmed with the base peak from the negative mode with m/z = 461.10 $[M^+-H]$. The ^{13}C NMR spectrum (CD_3OD, 75 MHz) indicated 14 signals along with 6 sugar signals. Five sugar signals appeared between δ 61.1 and 77.1 together with anomeric carbon at δ 100.5. The ^{13}C NMR signals were resolved by DEPT experiment and assigned as 1 methoxy, 4 methine and 9 quaternary carbons. Inspection of ^1H NMR spectrum (CD_3OD, 300 MHz) revealed the presence of AA′ and BB′ ring B while the remaining two singlets in the aromatic region present H-3 and H-8 protons. The presence of methoxy was obvious with the presence of a singlet at δ 3.90 integrating to three protons. The position of the methoxy was determined by HMBC correlations. Compound **1** (Fig. 1) was identified from the spectroscopic data as 5,4′-dihydroxy-6-methoxy-7-O-β-glucopyranosideflavone (hispidulin-7-O-glucopyranoside). ^1H NMR spectrum (CD_3OD, 300 MHz): δ 7.91 (2H, d, J = 9.0 Hz, H-2′ and 6′), 6.97 (1H, s, H-8), 6.95 (2H, d, J = 8.7 Hz, H-3′ and 5′), 6.66 (1H, s, H-3), 5.15 (1H, d, J = 7.2 Hz, glucosyl anomeric H), 3.90 (3H, S, $-OCH_3$), 3.40–4.10 (m, glucosyl Hs). The ^{13}C NMR spectrum (CD_3OD, 75 MHz): (Hispidulin nucleus) δ 165.5 (C-2), 102.3 (C-3), 182.9 (C-4), 152.9 (C-5), 132.8 (C-6), 156.5 (C-7), 94.4 (C-8), 152.7 (C-9), 106.1 (C-10), 121.6 (C-1′), 128.3 (C-2′6, ′), 115.6 (C-3′, 5′), 156.5 (C-4′), 60.1 (C-6-OCH_3), (glucosyl carbons) δ 100.5 (C-1″), 73.3 (C-2″), 77.1 (C-3″), 69.9 (C-4″), 76.6 (C-5″), 61.1 (C-6″). The spectroscopic data were in agreement with those previously published (Hase et al., 1995).



hispidulin-7-O-glucopyranoside **(1)**



stigmasterol-3-O-β-glucopyranoside **(2)**

Fig. 1. Isolated compounds from *Pseudognaphalium luteoalbum* leaf extract.

2.8. Compound 2

The ESI-mass spectrum (positive mode) had a molecular ion peak as the base peak at m/z = 597.48 $[M + Na]^+$ corresponding to the molecular formula $C_{35}H_{58}O_6$. The ^{13}C NMR spectrum ($CDCl_3$+ CD_3OD, 75 MHz) indicated 29 signals in addition to 6 sugar signals. Five sugar signals appeared between δ 61.9 and 76.6 together with anomeric carbon at δ 101.1. Two olefinic groups were identified as (C5-140.3, C6-122.2) and (C22-138.4, C23-129.3) among the 29 peaks in the ^{13}C NMR spectrum of the compound. The presence of sugar was confirmed in the ^1H NMR ($CDCl_3$+ CD_3OD, 300 MHz) by the presence of anomeric proton at δ 4.44. The spectroscopic data were compared with the literature and compound **2** (Fig. 1) was identified as stigmasterol-3-O-β-glucopyranoside (Alam et al., 1996). Stigmasterol-3-O-β-glucopyranoside is widely distributed in the plant kingdom.

3. Results and discussion

Hispidulin-7-O-glucopyranoside which was isolated in this study has previously been identified as a marker flavonoid in Algerian *Centaurea* species (family Asteraceae). It was identified in *Centaurea incana*, *Centaurea niceansis* and *Centaurea furfuracea* (Akkal et al., 2003). *P. luteoalbum* has been reported to contain two highly methylated flavonols as well as kaempferol 3-glucoside, quercetin 3-glucoside, 7-glucoside, apigenin 7-glucoside and luteolin 7-glucoside (Saleh et al., 1988). The two polymethylated flavonols were identified as calycopterin (5,4′-dihydroxy-3,6,7,8-tetramethoxyflavone) and 3′-methoxycalycopterin (5,4′-dihydroxy-3,6,7,8,3′-pentamethoxyflavone). The flavonoids apigenin, apigenin 7-*O*-β-D-glucopyranoside, luteolin, luteolin 4′-*O*-β-D-glucopyranoside, luteolin 7-*O*-β-D-glucopyranoside, jaceosidin and gnaphalin have also been isolated from *P.* (=*Gnaphalium*) *luteoalbum* (Mericli, 1980, cited by Zheng et al., 2013).

Prior to being screened in the antifungal assay, the leaf extract and pure compounds were redissolved in acetone because in the twofold serial dilution method the final concentration of acetone to which the microorganisms are subjected is 25%, which is non-toxic to fungal organisms (Eloff et al., 2007b). Additionally, acetone is miscible in water, making it ideal for use in the MIC microdilution assay. The antifungal activity of the acetone extract and the pure compounds is recorded in Table 1. Unfortunately there was insufficient quantity of the compounds isolated to test for antifungal activity against all the fungal species. Hence activity determinations were conducted against the organisms providing the most reproducible and reliable results. The acetone leaf extract of *P. luteoalbum* showed excellent antifungal efficacy against *Phytophthora nicotiana* and *Fusarium oxysporum*, with MIC values of 20 and 160 μg/mL respectively. The lowest antifungal activity obtained by Mdee et al. (2009) in a similar study investigating the antifungal nature of extracts of seven invasive plant species against plant pathogenic fungi was 80 μg/mL for *Lantana camara* leaf acetone extracts against *F. oxysporum*.

Where it was possible to screen the isolated compounds for activity, the compounds were generally only slightly more active than the crude

Table 1
Antifungal activity of *Pseudognaphalium luteoalbum* leaf acetone extracts and purified compounds.

Organism	MIC (mg/mL) after 24 h (and 48 h) incubation			
	Extract	Compound 1[a]	Compound 2[b]	Amp B[c] (μg/mL)
Aspergillus parasiticus	0.63 (1.25)[d]	0.63 (0.63)	0.63 (0.63)	0.32 (0.63)
Aspergillus niger	0.63 (1.25)	nt[e]	nt	1.25 (1.25)
Colletotrichum gloeosporioides	0.63 (0.63)	0.42 (0.42)	0.31 (0.31)	0.16 (0.42)
Fusarium oxysporum	0.16 (0.16)	nt	nt	0.16 (1.25)
Penicillium expansum	1.25 (1.25)	0.63 (0.63)	0.63 (0.63)	0.16 (0.32)
Penicillium janthinellum	1.25 (1.25)	nt	nt	0.08 (0.32)
Phytophthora nicotiana	0.02 (0.02)	nt	nt	1.25 (1.25)
Pythium ultimum	0.63 (0.63)	nt	nt	1.25 (2.50)
Trichoderma harzianum	1.25 (1.25)	nt	nt	0.63 (0.63)

[a] Hispidulin-7-O-glucopyranoside.
[b] Stigmasterol-3-O-β-glucopyranoside.
[c] Amp B = amphotericin B.
[d] Figures in brackets represent MIC values read after 48 h incubation.
[e] nt = not tested.

leaf extract. It would be expected that purified antifungal compounds would be a great deal more active than the crude extract, which contains a large number of other compounds. However the pure compounds may act additively or synergistically and other constituents enhancing the efficacy of antifungal compounds in the extract may add to the activity shown by the crude extract.

It has previously been noted that inhibition of fungal growth by plant extracts detected after 24 h may be overcome after further incubation of 48 h in total (Masoko et al., 2007). This may be attributed to decomposition of the active antifungal compounds, or ability of the fungus to gradually overcome the inhibitory effects of the antifungal principles. This phenomenon was noted only with regard to the activity of the crude acetone leaf extract against *Aspergillus parasiticus* and *Aspergillus niger* (Table 1). The activity of the purified compounds appeared to remain constant, indicating a fungicidal activity.

No cytotoxic effects to the cells were noted in the colorimetric MTT assay at concentrations up to 200 μg/mL, which was the highest concentration tested. It has been noted that certain chemical compounds, particularly those with antioxidant activity, may react with MTT in the absence of cells (Bruggisser et al., 2002; Shoemaker et al., 2004). In particular, flavonoids may non-specifically reduce MTT (Peng et al., 2005). In this study, the rinsing steps included in the MTT assay served to avoid this phenomenon, as did the inclusion of cell-free controls which showed no evidence of non-specific MTT reduction. To confirm the results obtained in the MTT assay, the cells were examined visually using an inverted microscope. No cytotoxic effects were observed. Flavonoids are generally considered to have low toxicity to animal cells (Havsteen, 2002), which reflects the results shown in this study.

In a recent review of the phytochemistry and biological activities of the genus *Gnaphalium* (*Pseudognaphalium*), it was noted that plants of this genus are widely distributed worldwide, but many of the 200 species belonging to the genus have received little or no attention (Zheng et al., 2013). It was recommended that further studies to exploit the chemistry and bioactivity of plants from this genus are necessary to develop more value-added products for use in the food and pharmaceutical industries (Zheng et al., 2013).

4. Conclusions

Following the discovery of antifungal activity in the acetone leaf extract of *P. luteoalbum*, two antifungal compounds, hispidulin-7-O-glucopyranoside and stigmasterol-3-O-β-glucopyranoside were isolated from a bulk collection of this weed species. These compounds were not cytotoxic at 200 μg/mL, the highest concentration tested, although antifungal MIC values reached as low as 20 μg/mL against one fungal phytopathogen. Invasive and weedy species may potentially find application as sources of fungicides, and constitute a ready source of raw material. Other bioactivities may also be investigated to discover further possible uses of these species. Future work may be focused on isolating more of the antifungal compounds and testing them for activity against other fungal phytopathogens as well as against fungi of clinical significance in human and animal diseases.

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