Isolation and characterization of two acaricidal compounds from *Calpurnia aurea* subsp. *aurea* (Fabaceae) leaf extract

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Abstract The menace caused by ticks and tick-borne diseases is a major limitation to the livestock industry in Africa. The high costs and non-availability of synthetic, chemical acaricides to resource-limited farmers, resistance of ticks to available acaricides and residue problems in meat and milk consumed by humans further complicate matters. The use of plant extracts as a possible source of new acaricides has received much interest in the last decade. In our endeavour to discover natural acaricidal compounds, tick toxicant bioassays were conducted and the chloroform fraction of *Calpurnia aurea* ethanol leaf extract had good acaricidal activity. Further purification of the fraction revealed two flavonoids, isolated from *Calpurnia aurea* for the first time. These flavonoids were characterized as apigenin-7-*O*-β-D-glycoside and isorhoifolin by means of NMR spectroscopic and mass spectrometry analysis. Isorhoifolin was the most potent compound (LC_{50} = 0.65 mg/ml), was not cytotoxic and should be further investigated for its potential as an acaricidal agent.

Keywords Acaricidal activity. Plant extracts. Flavonoids. Apigenin-7-*O*-β-D-glycoside. Isorhoifolin

Introduction

Ticks rank second only to mosquitoes as the most common vectors of diseases to animals and humans (Oberchain and Galun 2013). In addition, ticks are the direct causes of deadly tick paralysis, allergic reactions and toxicoses (Sonenshine and Roe 2013). Current tick control involves application of chemical acaricides, usually synthetic, but tick resistance and negative environmental impacts have become problematic from years of overuse. As such, the search for alternative control measures such as acaricidal or repellent medicinal plants is strongly advocated (Mondal et al. 2013).

Calpurnia aurea (Ait.) Benth. of the family Fabaceae, commonly called wild laburnum, is a small tree of about 4 m, reaching up to 15 m under forest conditions. The plant is widely distributed in Africa from southern Africa (Eastern Cape through to KwaZulu-Natal, Mpumalanga, Gauteng, Limpopo and Swaziland) to Eritrea (the horn of Africa), stretching northwards to as far as Ethiopia and southern India (Getiye et al. 2016). In South Africa, the juice of crushed leaves, ground bark and roots of *C. aurea* are used to kill ectoparasites and to relieve itching while unspecified parts are used to destroy maggots. The acaricidal and tick attractant activities of the plant have been reported (Zorloni et al. 2010; Nana et al. 2010; Nana et al. 2015).

Previous screening was conducted to evaluate the acaricidal properties of some plant species selected based on their ethnoveterinary use against ticks in South Africa (Fouche et al. 2016; Wellington et al. 2017). They found that extracts of *C. aurea* had good acaricidal activities against larvae of *Rhipicephalus* (*Boophilus*) *decoloratus* (85% tick mortality) and *Rhipicephalus* (*Boophilus*) *microplus* (100% tick mortality). However, the bioactive compounds responsible for these observations have not been identified. This study aimed to isolate and characterize the acaricidal compounds in *C. aurea* using chromatographic and spectroscopic techniques respectively.

Materials and Methods

Plant material

Calpurnia aurea leaves were collected from the Lowveld National Botanical Garden, Nelspruit, Mpumalanga (GPS coordinates 25.444^{o}S, 30.970^{o}E) on 29 January 2015 and a voucher specimen deposited at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (Specimen no: PRU 121520). The plant material was dried at room temperature (c. 25^{o}C) for two weeks in a well-ventilated room. Collection, drying and storage of the plant material were as outlined previously by McGaw and Eloff (2010).

Extraction and isolation procedure

Dried and pulverised leaves of *C. aurea* (423.48 g) were extracted with ethanol (4 L) as described by Mukandiwa et al. (2013) to give 44.9 g of dry ethanol extract. This extract was dissolved in a mixture of chloroform and water and fractionated by solvent-solvent extraction to yield hexane (8.7 g), chloroform (26.5 g), butanol (1.3 g), methanol/water (1.8 g) and water (1.2 g) (Eloff, 1998). The chloroform fraction was subjected to further separation via silica gel column chromatography. The column was eluted with chloroform:methanol (gradient 0 to 100% methanol) to afford four sub-fractions. Sub-fraction III showed interesting ultraviolet (UV) absorbing bands on thin layer chromatography (TLC), which turned to yellow with vanillin reagent. Accordingly, it was further refined using silica gel column chromatography. The process continued until two compounds were obtained in their pure forms.

***In vitro* acaricidal bioassay**

Ticks

Adult *Rhipicephalus turanicus* ticks (both sexes) were obtained from Clinvet International, Bloemfontein, South Africa. The ticks were kept at the Phytomedicine laboratory, Faculty of Veterinary Sciences, University of Pretoria in glass humidity chambers at an average temperature of 25±1^{o}C. Relative humidity was maintained at 75±10% by placing supersaturated sodium chloride solution in the glass chamber. The ticks were stored in vials covered with cotton mesh (to allow for air exchange) and set on a square glass plate placed at the base of the chamber on four small bearings. The four sides of the glass plate were 1.5 cm from the wall of the humidity chamber to prevent the ticks from reaching the walls.

Experimental protocol

Two-fold graded increasing concentrations (3-100 mg/ml diluted in acetone) of the more non-polar fractions (chloroform, hexane and butanol) were used for adult immersion tests (Al- Rajhy et al. 2003). Ten adult ticks were immersed in each test solution for one minute, dried on Whatman No. 1 filter paper and kept at room temperature (25±1^{o}C) and 85% relative humidity in 20 ml glass vials closed with a perforated stopper. Graded two-fold increasing concentrations of cypermethrin (0.2-5 mg/ml) served as the positive control while acetone was the negative control. Each extract concentration was tested in triplicate on each of three different occasions to yield nine replicates. Percentage mortality was determined 24 hours post treatment by viewing the ticks under a stereo microscope (American Optical Corporation). Ticks were confirmed dead based on signs of cuticle darkness, halted Malpighian tubule movement and haemorrhagic skin lesions. This bioassay was repeated in a

subsequent experiment using the isolated compounds (0.06-1 mg/ml). Each concentration was tested in duplicate and the experiment was done once due to the limited quantity of the isolated compounds available.

Cytotoxicity (*in vitro* safety) bioassay

Maintenance of cell lines

Vero monkey kidney (ATCC® CCL-81™) and HepG2 human liver cancer (ATCC® HB-8065™) cells were purchased from Cellonex, South Africa. Vero cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 1% gentamicin and 5% foetal calf serum (FCS, Highveld Biological, South Africa) while HepG2 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM, Whitehead Scientific, South Africa) containing 4.5 g/l glucose and 4 mM L-glutamine supplemented with 10% FCS. Both cell types were maintained at 37^{o}C in a 5% CO_2 incubator (Hera Cell 150, Germany). Cells were passaged three times weekly by trypsinization with trypsin/ethylenediaminetetraacetic acid solution (Invitrogen, Cergy-Pontoise, France) into 75 cm^2 culture flasks.

Experimental protocol

Viable cell growth after incubation of Vero and HepG2 cells with test samples was determined using the tetrazolium-based colorimetric MTT assay [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] described by Mosmann (1983). Cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 minutes and resuspended in growth medium (MEM for Vero cells and DMEM for HepG2 cells) to 10^5 cells/ml. One hundred microliters (100 µl) of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate, then 200 µl of MEM or DMEM was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated overnight at 37ºC in a 5% CO_2 incubator to allow cells to attach to the bottom of the plates.

Test samples were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mg/ml and then diluted 100-fold in culture medium for the concentration tested (1000 µg/ml). One hundred microliters (100 µl) of decreasing concentrations of the test samples (1000, 750, 500, 250, 100, 50 µg/ml) were added in the corresponding wells and the plates were again incubated at 37ºC for 48 hours in the 5% CO_2 incubator. Untreated cells served as the negative control while doxorubicin hydrochloride (Pfizer Laboratories, South Africa) served as the positive control. After incubation, the growth medium was aspirated from the cells which were washed with 200 µl phosphate buffered saline (PBS, Whitehead Scientific,) and replaced with 200 µl of fresh medium and 30 µl MTT (Sigma-Aldrich, South Africa; stock solution of 5 mg/ml in PBS). The plates were

incubated further for 4 hours at 37ºC in the 5% CO_2 incubator. After this, the growth medium in each well was carefully removed using a suction pump (Integra, USA), without disturbing the MTT formazan crystals. These crystals were dissolved by adding 50 µl DMSO to each well and shaken gently for 2 minutes. The MTT reduction was measured immediately by detecting absorbance in a spectrophotometer (Biotek Synergy, USA) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The lethal concentration of test samples resulting in a 50% reduction of absorbance (LC_{50}) compared to untreated cells was calculated. Each extract concentration was tested in quadruplicate and the assays repeated three times. This bioassay was repeated in a subsequent experiment using the isolated compounds (5-100 µg/ml).

Structure identification of compounds

Two compounds were identified by means of 1D and 2D NMR (spectroscopic and mass spectrometry analysis. ^{1}H NMR and 2D NMR experiments data were acquired on a 400 MHz NMR spectrometer (Bruker Avance III 400 MHz). HPLC-HR-ESI-MS was performed on Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument.

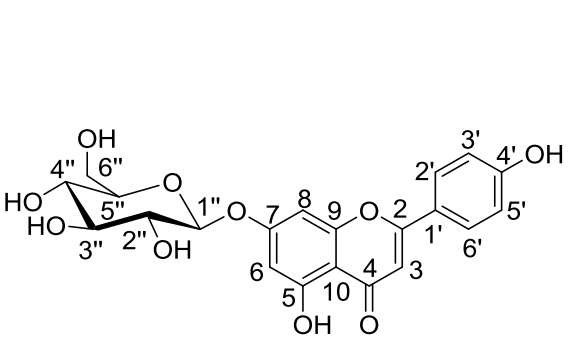
Data analysis

Data on acaricidal activity and cytotoxicity were presented as the arithmetic mean values ± standard error of mean. Significance was analysed using one-way analysis of variance followed by Tukey's multiple comparison test on GraphPad Prism 7.02 (GraphPad Software, San Diego-CA, USA). Values were considered to differ statistically when $p \leq 0.05$. The LC_{50} and LC_{90} values for acaricidal activity were determined using the linear regression model.

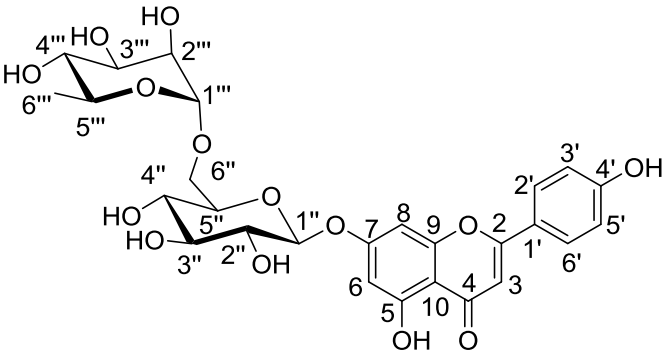
Results

Structure characterization of acaricidal compounds

Two compounds, apigenin-7-*O*-β-D-glycoside (molecular formula $C_{21}H_{20}O_{10}$) (Gulluce et al. 2015; Peng et al. 2016) and isorhoifolin (apigenin 7-O-rutinoside, 4',5,7-trihydroxyflavone 7-O-rutinoside; molecular formula $C_{27}H_{30}O_{14}$) (Kokotkiewicz et al. 2012) (Fig. 1), were isolated as yellow powders, which gave strongly UV absorbing bands on TLC at 254 nm and turned to yellow with vanillin spray reagent. Their spectroscopic data agreed with published data (Kokotkiewicz et al. 2012; Peng et al. 2016).



Apigenin-7-*O*-β-D-glycoside



Isorhoifolin

Fig. 1 Apigenin-7-*O*-β-D-glycoside (molecular formula: $C_{21}H_{20}O_{10}$) and isorhoifolin (molecular formula: $C_{27}H_{30}O_{14}$) isolated from *Calpurnia aurea*

The ^{1}H NMR and ^{13}C NMR data are presented in Table 1. A search in the Dictionary of Natural Products (Chapman and Hall, 2017), the Chemical Abstracts Services (SciFinder) and comparing the spectroscopic data with available literature confirmed the structures. With respect to the coupling constant of the anomeric proton (J = 7.3 Hz), the sugar is *β*-connected and should have (D)-configuration, according to the Klyne rule (Klyne, 1950).

Table 1 ^{1}H NMR and ^{13}C NMR data of apigenin-7-*O*-β-D-glycoside and isorhoifolin (in DMSO-d_6)

Position	Apigenin-7-*O*-β-D-glycoside		Isorhoifolin	
	H	C	H	C
2	-	165.5	-	165.5
3	6.87, s	103.5	6.87, s	104.0
4	-	182.0	-	182.0
5		162.5	-	161.5
6	6.45, s	100.0	6.45, s	100.0
7	-	163.5	-	163.5
8	6.83, s	95.8	6.77, s	95.5
9	-	157.5	-	157.5
10	-	106.0	-	106.0
1'	-	121.5	-	121.5
2', 6'	7.95, d, J = 8.7 Hz	129.5	7.95, d, J = 8.7 Hz	129.5
3', 5'	6.95, d, J = 8.7 Hz	116.5	6.95, d, J = 8.7 Hz	116.5
4'	-	162.7	-	162.0
1"	4.92, d, J = 7.3 Hz	100.2	5.06, d, J = 7.3 Hz	100.5
2"	3.31, m	73.6	3.30, m	74.0
3"	3.28, m	77.3	3.28, m	77.0
4"	3.20, m	70.0	3.15, m	70.5
5"	3.33, m	77.0	3.59, m	76.5
6"	3.71, d, J = 11.2 Hz, Ha-6"	61.0	3.84 , d, J = 10.9 Hz, Ha-6"	66.0
	3.51, d, J = 11.2 Hz, Hb-6"		3.41, m, Hb-6"	
1"'			4.54, s	101.0
2"'			3.64, brs	71.0
3"'			3.41, m	71.5
4"'			3.13, m	72.5
5"'			3.46, m	69.0
6"'			1.07, d, J = 6.1 Hz	17.8

***In vitro* acaricidal activity**

The chloroform fraction of *C. aurea* was the most active fraction followed by hexane fraction and butanol fraction. The LC_{50} and LC_{90} values were 28.64, 21.53, 65.09 mg/ml; 102.74, 81.23 and 173.45 mg/ml respectively (Table 2). Two flavonoid compounds, apigenin-7-*O*-β-D-glycoside and isorhoifolin, at a concentration of 1 mg/ml had LC_{50} and LC_{90} values of 1.35 and 0.65 mg/ml; 3.95 and 1.71 mg/ml respectively compared with the LC_{50} and LC_{90} values value of cypermethrin (0.6 and 1.09 mg/ml respectively).

Table 2 Acaricidal activity (LC_{50} and LC_{90} in mg/ml) of the fractions and isolated compounds from *Calpurnia aurea* against *Rhipicephalus turanicus* ticks after 24 hours

	LC_{50} (mg/ml)	**LC_{90} (mg/ml)**
	Mean ± SEM	**Mean ± SEM**
***Calpurnia aurea* plant**		
Hexane fraction	21.53±1	81.23±9
Butanol fraction	65.09±10	173.45±65
Chloroform fraction	28.64±13	102.74±59
Apigenin-7-O-β-D-glycoside	1.35±0.2	3.95±1
Isorhoifolin	0.65±0.1	1.71±0.1
Cypermethrin (positive control)	0.6	1.09±0.10

Cytotoxicity

For toxicity, we used a cut-off of 100 µg/ml as an indicator for toxicity (Nondo et al. 2015). Based on this criterion, most of the fractions and isolated compounds were non-toxic to Vero and HepG2 cells, with the exception of the chloroform fraction with LC_{50} of 76 µg/ml for HepG2 cells. In comparison, doxorubicin had LC_{50} of 5.03±6.39 and 0.18±0.05 µM against Vero and HepG2 cells respectively (Table 3).

Table 3 Cytotoxicity (LC_{50} in µg/ml) of the crude extract, fractions and isolated compounds from *Calpurnia aurea* against Vero and HepG2 cells

***Calpurnia aurea* plant**	**Cytotoxicity LC_{50} (µg/ml) ±SEM**	
	Vero cells	**HepG2 cells**
Crude extract	623±4	123±11
Chloroform fraction	428±9	76±13
Butanol fraction	>1000	>1000
Hexane fraction	499±10	337±5
Water fraction	>1000	>1000
Methanol/Water fraction	>1000	934±6
Apigenin-7-*O*-β-D-glycoside	>100	73±1
Isorhoifolin	>100	72±5
Doxorubicin (positive control, µM)	5.03±6.4	0.18±0.1

Discussion

Solvent-solvent fractionation of the crude ethanol extract of the leaves of *C. aurea* gave five fractions (hexane, chloroform, butanol, methanol/water and water) based on solubility characteristics of the constituents. The chloroform fraction afforded two acaricidal compounds, identified as apigenin-7-*O*-β-D-glycoside and isorhoifolin (apigenin-7-O-rutinoside). Apigenin-7-*O*-β-D-glycoside is a glycosyloxyflavone, that is, apigenin substituted by a β-D-glucopyranosyl moiety at position 7 via a glycosidic linkage. This compound, while identified for the first time from *C. aurea*, has been previously identified in other plants such as *Pteris multifida* Poir. (Pteridaceae), *Marrubium globosum* Montbret & Aucher ex Benth (Lamiaceae), *Elsholtzia splendens* Nakai ex F.Maek. (Lamiaceae), *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae), *Lonicera gracilipes* var glandulosa (Caprifoliaceae) and *Humulus scandens* L. (Cannabaceae) (Chen et al. 2011; Wölwer-Rieck, 2012; Peng et al. 2016). Isorhoifolin has been found in many *Citrus* species such as *Citrus paradisi* Macfad (Rutaceae), *Mentha* species (Lamiaceae), *Teurium polium* var. *gnapholodes* (Lamiaceae) and *Cynara scolymus* L. (Cynareae) (Nassar et al. 2013; Boghrati et al. 2016; Hawryl et al. 2016).

Flavonoids have been reported to have significant antiparasitic activities. Their effects on arthropods have been reported to involve repellency (Habeeb, 2010), inhibition of feeding, developmental disorders, deformation, infertility, inhibition of oviposition (Ghosh et al. 2015) or death (Dantas et al. 2015). The potential role of flavonoids in the modulation of reproductive activity of ticks was reported by Ravindran et al. (2011) and

Juliet et al. (2012), where 1.56 mg/ml of *Leucas aspera* L. and 50 mg/ml of *Jatropha curcas* L. produced 100 and 90% failure of eclosion of eggs respectively. This was attributed to acacetin and apigenin isolated from *L. aspera* and flavone apigenins (apigenin 7-O-β-D-neohesperidoside, apigenin 7-O-β-D-galactoside), orientin, vitexin, vicenin II and the biflavone di-C-β-Dglucopyranoside-methylene-(8,8')-biapigenin isolated from the leaves of *J. curcas* (Abd-Alla et al. 2009). The presence of apigenin and luteolin in *Lippia javanica* Burm F. Spreng tested at 50 mg/ml may have contributed to the 92% adult tick mortality reported by Madzimure et al. (2011). The LC_{50} value of 0.65 mg/ml for isorhoifolin compares favourably with some known acaricidal compounds isolated from plants. This makes flavonoids an interesting group of study molecules for acaricidal activity.

To verify the safe use of the fractions and isolated compounds from *C. aurea*, cytotoxicity tests were undertaken against two mammalian cell lines. These cell-line tests showed the two compounds to be toxicologically acceptable within the limits of concentration tested.

Conclusions

New classes of acaricides are urgently required and plant flavonoids represent a novel set of potential lead structures. Although the use of plant extracts to control ticks is common in impoverished communities, the possibility of discovering a compound that may become a new acaricide should not be ignored. Further work is required to characterize the true acaricidal activities of apigenin-7-*O*-β-D-glycoside and isorhoifolin.

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