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ScienceDirect
Journal of Ethnopharmacology 112 (2007) 71–76
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Isolation and identification of antiplasmodial sesquiterpene lactones from *Oncosiphon piluliferum*

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Received 19 December 2006; accepted 5 February 2007
Available online 9 February 2007

Abstract

Oncosiphon piluliferum (Asteraceae) is used traditionally to treat a variety of ailments, mainly fevers. This prompted the screening of this plant for antiplasmodial properties. The dichloromethane extract of the aerial parts of the plant showed activity *in vitro* against the chloroquine-sensitive (IC_{50} 2.6 μg/ml) and the chloroquine-resistant (IC_{50} 3.1 μg/ml) strains of *Plasmodium falciparum*. Through conventional chromatographic techniques and bioassay-guided fractionation, sesquiterpene lactones of the germacranolide and eudesmanolide type displaying significant *in vitro* antiplasmodial activity (IC_{50} values ranging from 0.4 to 4.4 μg/ml) were isolated and identified by spectroscopic data. In addition, the cytotoxic effects of the active compounds against Chinese Hamster Ovarian (CHO) cells were evaluated and the compounds were found to be toxic to mammalian cells at similar concentrations. Structure–activity relationships were assessed.

Keywords: Antiplasmodial; Sesquiterpene lactones; *Oncosiphon piluliferum*; Cytotoxicity

1. Introduction

Malaria, caused by parasites of the genus *Plasmodium*, is one of the leading public health problems in Sub-Saharan Africa. It is estimated that malaria kills over a million annually and some 3.2 billion people living in 107 countries or territories are at risk (WHO, 2005). One the main factors contributing to the escalating prevalence and distribution of malaria is the emergence and spread of drug-resistant parasites. Efforts are subsequently being directed towards the discovery and development of novel, affordable antimalarial treatments.

Historically, medicinal plants have proven to be a major source of antimalarial drugs and novel template compounds. In light of this and the imperative need for new antimalarial agents, a collaborative initiative was undertaken by a consortium of South African institutions aimed at evaluating indigenous medicinal plants for antimalarial properties (Clarkson et al., 2004). Subsequent to the screening of a selection of South African medicinal plants used to treat malaria and/or fever, the dichloromethane extract of the aerial parts of *Oncosiphon piluliferum* (L.f) Källersjö (Asteraceae) showed promising antiplasmodial activity and was selected for further investigation.

The *Oncosiphon piluliferum* plant is very bitter and has a "heavy smell" from the volatile oil which it contains; hence its common name is stinkruid. The historical name of the plant is *Pentzia globifera*. It is a bushy annual herb with stalkless leaves deeply dissected with two stipule-like lobes at the base and the flower heads sit on solitary long erect leafless stalks.

The Europeans administered an infusion of the plant for convulsions and the Hottentots used an infusion of the flower and leaf for typhoid and other fevers. A decoction of the plant is an old fashioned "Dutch" remedy to bring out the rash in measles and both the Xhosa and Mfengu use it as an antifebrile. Extracts of the plant are reported to have given "negative results in experimental malaria" (Watt and Breyer-Brandwyk, 1962).

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doi:10.1016/j.jep.2007.02.002

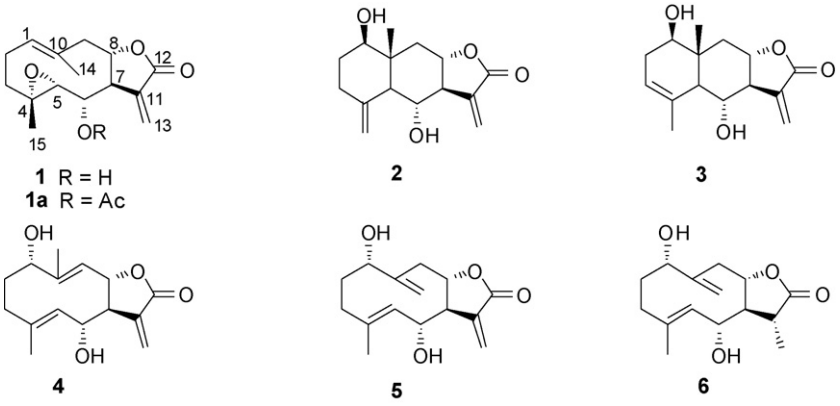

Fig. 1. Structures of compounds **1**–**6**.

As there are no previous reports on the chemical components of *Oncosiphon piluliferum*, the plant was studied further to isolate and characterise the major compounds responsible for its antiplasmodial properties. The bioassay-guided fractionation of the extract led to the isolation of compounds **1**–**5** (Fig. 1) identified as sesquiterpene lactones of the germacranolide and eudesmanolide type, previously isolated from other members of Asteraceae (Shafizadeh and Bhadane, 1973; Yunusov et al., 1976a,b, 1979; Bohlmann et al., 1982; Jakupovic et al., 1988; Sanz and Marco, 1991; Gören et al., 1992; Izbosarov et al., 2000). Structural elucidation was facilitated by spectroscopic data (NMR spectroscopy and mass spectrometry) and by direct comparison with published spectral data. This is the first account of the antiplasmodial properties of compounds **1**–**5**.

2. Materials and methods

2.1. Plant material

Aerial parts of *Oncosiphon piluliferum* (L.f) Källersjö were collected in October 1999 near Middleburg in the Eastern Cape, South Africa. A voucher specimen (EN00579) was deposited in the herbarium of the South African Biodiversity Institute (SANBI), Pretoria. Plant material was dried in an oven at 45 °C for 48 h, ground and stored at room temperature until extraction.

2.2. Isolation and identification of active compounds

Dried ground plant material (3.2 kg) was extracted with dichloromethane (20 l) at room temperature for 24 h with occasional stirring. The solvent was evaporated under reduced pressure to yield 70.5 g (2.2%) of crude extract. Fifteen grams was subjected to open column chromatography over Silica gel 60 (0.063–0.2 mm) and eluted using a gradient eluent of increasing polarity from 5% ethyl acetate/hexane to 100% ethyl acetate. Fractions were pooled according to their behaviour (R_f values) on TLC. Twenty pooled fractions were subsequently bioassayed against *Plasmodium falciparum* D10. Eight of these showed improved antiplasmodial activity (D10 IC_{50} values ranging from 0.4 to 1.5 μg/ml) relative to the crude extract (D10 IC_{50} 2.6 μg/ml).

Using *in vitro* antiplasmodial activity against the D10 *Plasmodium falciparum* strain as the biological indicator, the active fractions were subjected to repeated flash and column chromatographies to identify the active compounds. The fractionation process involved column and thin layer chromatographic techniques. Different sized columns, ranging from 1.5 to 6 cm in diameter, were used depending on the amount of sample and the purification stage. Silica gel column chromatography was conducted using Silica gel 60 (0.063–0.2 mm) and flash silica gel chromatography was carried out using 35–75 μm flash silica gel. Thin layer chromatography was carried out on 0.20 mm pre-coated (SIL-25 UV_{254}) glass-backed plates. The plates were first viewed under UV, developed using a vanillin: concentrated H_2SO_4 (1 g: 100 ml) spray reagent and then heated.

The bioassay-guided fractionation process yielded compounds **1** (Shafizadeh and Bhadane, 1973; Bohlmann et al., 1982), **2** (Bohlmann et al., 1982; Jakupovic et al., 1988; Cardona et al., 1990), **3** (Gören et al., 1992), **4** (Shafizadeh and Bhadane, 1973; Yunusov et al., 1979), and **5** (Yunusov et al., 1976a,b; Sanz and Marco, 1991; Gören et al., 1992; Izbosarov et al., 2000). Compounds were identified using spectral data such as ^1H, ^{13}C, DEPT, HSQC, HMBC, COSY and NOESY NMR spectra, as well as EI-MS spectra, and by direct comparison with published spectral data and structures. NMR spectra were recorded in $CDCl_3$ and/or acetone-d_6 using a Varian 400 MHz Unity spectrophotometer. High-resolution mass spectra were recorded on a VG 70SEQ HRMS instrument.

2.3. Acetylation of compound *1*

Semi-pure compound **1** (30 mg) was acetylated using 0.5 ml (5 mmol) acetic anhydride in 3 ml anhydrous pyridine to yield 16 mg (48%) of the acetylated compound **1a** as a yellow gum; ^1H NMR (400 MHz, $CDCl_3$) δ 6.34 (1H, dd, J = 0.7, 2.4 Hz, H-13b), δ 5.78 (1H, dd, J = 0.7, 2.1 Hz, H-13a), δ 5.31 (1H, m, H-1), δ 5.23 (1H, dd, J = 3.7, 11.5 Hz, H-6), δ 4.48 (1H, br ddd, J = 11.3 Hz, H-8), δ 3.00 (1H, br dddd, J = 11.5, 2.1, 2.3 Hz, H-7), δ 2.82 (1H, dd, J = 12.6, 11.3 Hz, H-9b), δ 2.64 (1H, d, J 3.7 Hz, H-5), δ 2.28 (2H, m, H-2), δ 2.03 (1H, m, H-3b), δ 2.02 (3H, s, OAc), δ 1.92 (1H, br dd, J = 12.6 Hz, H-9a), δ 1.72 (3H,

s, H-14), δ 1.23 (3H, s, H-15), δ 1.17 (1H, ddd, J = 9.5, 12.8, 10.5 Hz, H-3a).

2.4. Reduction of compound **6**

Fifteen milligrams of compound **5** (0.06 mmol) was dissolved in 10 ml of anhydrous methanol. The solution was cooled to 0 °C, and 78 mg (2 mmol) of $NaBH_4$ was added. The mixture was stirred at 0 °C for 10 min, after which 10 ml of 20% acetic acid was added. The solution was diluted further with 20 ml of water and extracted twice with 50 ml of chloroform. Flash silica gel chromatography of the chloroform extract yielded 5 mg (34%) of product **6** as a yellow gum; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.10 (1H, d, J = 10.1 Hz, H-5), δ 5.07 (1H, d, J = 2.2, ∼1 Hz, H-14b), δ 5.03 (1H, br d, J = 2.2 Hz, H-14a), δ 4.26 (1H, dd, J = 10.1, 9.8 Hz, H-6), δ 3.92 (1H, dd, J = 4.9, 9.7 Hz, H-1), δ 3.89 (1H, ddd, J = 2.5, 8.3, 5.5 Hz, H-8), δ 2.80 (1H, dddd, J = 2.3, 2.2, 2.5, 15.3 Hz, H-9b), δ 2.63 (1H, dq, J = 7.2, 9.1 Hz, H-11), δ 2.41 (1H, dd, J = 15.3, 8.3 Hz, H-9a), δ 2.26 (1H, ddd, J = 5.5, 9.1, 9.8 Hz, H-7), δ 2.06 (1H, ddd, J = 5.5, 5.1, 8.8 Hz, H-3b), δ 2.00 (2H, m, H-2), δ 1.90 (1H, ddd, J = 5.5, 5.7, 13.5 Hz, H-3a), δ 1.65 (3H, d, J = 1.3 Hz, H-15), δ 1.39 (3H, d, J = 7.2 Hz, H-13).

2.5. In vitro antiplasmodial assay

The chloroquine-sensitive (D10) and chloroquine-resistant (K1) strains of *Plasmodium falciparum* were continuously cultured according to the methods described by Trager and Jensen (1976). The parasites were maintained at a 5% haematocrit with RPMI 1640 (Biowhittaker) medium supplemented with Albumax II (lipid rich bovine serum albumin) (GibcoBRL) (25 g/l), hypoxanthine (44 mg/l), HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethansulphonic acid]) (Sigma–Aldrich) (6 g/l), sodium bicarbonate (Sigma–Aldrich) (2.1 g/l) and gentamycin (Sigma–Aldrich) (50 mg/l). The cultures were incubated at 37 °C in an atmosphere of 93% N_2, 4% CO_2 and 3% O_2.

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the methods described by Makler et al. (1995). The *in vitro* assays were performed as previously described by Clarkson et al. (2003). A solution of chloroquine diphosphate (Sigma) in Millipore water served as a positive control in all experiments. The initial concentration of chloroquine was 100 ng/ml. Crude extracts were first dissolved in methanol or DMSO, depending on their solubility, sonicated for 10 min and then diluted in Millipore water to give a 2 mg/ml solution. This was further diluted in medium to give 200 μg/ml stock solutions. The highest concentration of solvent that the parasites were exposed to was 0.5%, which was shown to have no measurable effect on parasite viability (Clarkson et al., 2004). Extracts were tested in nine serial two-fold dilutions (final concentration range: 100–0.2 μg/ml) in 96-well microtitre plates. All tests were performed in duplicate.

Fractions and pure compounds were dissolved in 10% methanol and were further diluted in complete medium on the day of the experiment. The starting concentration for a full dose–response was 100 μg/ml, which was diluted two-fold in complete medium to give ten concentrations, with the lowest concentration being 0.195 μg/ml.

The IC_{50} values were obtained from the dose–response curves, using non-linear dose–response curve fitting analyses with GraphPad Prism v.4.00 software.

2.6. In vitro cytotoxicity assay

Compounds were tested for *in vitro* cytotoxicity against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The CHO cells were cultured in Dulbecos Modified Eagles Medium (DMEM):Hams F-12 medium (1:1) supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamycin (0.04 μg/ml). The medium reagents were obtained from Highveld Biological, South Africa.

Samples were dissolved in methanol:water (1:9). Stock solutions (2 mg/ml) were prepared and were stored at −20 °C until use. The highest concentration of methanol to which the cells were exposed to had no measurable effect on the cell viability. Emetine was used as the positive control in all cases. The initial concentration of emetine was 100 μg/ml, which was serially diluted in complete medium with 10-fold dilutions to give six concentrations, the lowest being 0.001 μg/ml. The same dilution technique was applied to all test samples with an initial concentration of 100 μg/ml to give five concentrations, with the lowest concentration being 0.01 μg/ml.

The concentration of the test sample that inhibits 50% of the cells (IC_{50} values) was obtained from dose–response curves, using a non-linear dose–response curve fitting analyses via GraphPad Prism v.2.01 software.

3. Results and discussion

The crude dichloromethane extract of the aerial parts of *Oncosiphon piluliferum* had an *in vitro* 50% inhibitory concentration (IC_{50}) of 2.6 μg/ml against the chloroquine-sensitive D10 strain and an IC_{50} of 3.1 μg/ml against the chloroquine-resistant K1 strain of *Plasmodium falciparum*.

Bioassay-guided fractionation led to the identification of five structurally related compounds responsible for the observed activity of this extract. It was evident by TLC and LC–MS/MS analysis that less active analogues of these compounds were present in the crude extract and that the overall antiplasmodial activity of the extract and several of the fractions generated were due to the synergistic effect of a number of compounds. Purification was, however, focused on those fractions showing significant enrichment of antiplasmodial activity upon fractionation so as to isolate the compounds primarily responsible for the observed biological activity. Also, isolation of additional compounds from active fractions proved unsuccessful due to low yields and marked instability.

Due to the observed instability of the germacranolide **1**, the acetate of this compound was prepared and structural elucidation and bioassaying was conducted on the acetate **1a**. Compound **1** was identified as 4,5α-epoxy-6α-hydroxy-1(10)*E*,11(13)-germacradien-12,8α-olide. This compound is reported to have

been isolated from *Artemisia arbuscula* and *Artemisia tridentate* (Shafizadeh and Bhadane, 1973) as well as *Mikania pohlii* (Bohlmann et al., 1982) although the authors depicted the epoxide moiety as being 4α,5β-orientated. These reports do, however, substantiate the observed instability of compound **1**.

Compound **2** was identified as desacetyl-β-cyclopyrethrosin which has been isolated from several other plant species which include *Mikania pohlii* (Bohlmann et al., 1982) and *Brocchia cinerea* (Jakupovic et al., 1988).

Compound **3** is a compound commonly called sivasinolide, and reported to have first been isolated from *Tanacetum densum* subsp. *sivasicum* (Gören et al., 1992).

The structure of **4** corresponded to that of the compound, commonly known as tatridin A or tavulin. This compound has been isolated from a variety of plant species which include *Artemesia tridentata* and *Artemisia arbuscula* (Shafizadeh and Bhadane, 1973) as well as *Tanacetum vulgare* (Yunusov et al., 1979).

Compound **5** was identified as tanachin, first isolated from *Tanacetum pseudoachillea* (Yunusov et al., 1976a,b). This compound has subsequently been isolated from a variety of plants of the Asteraceae family in Middle Asia (Sanz and Marco, 1991; Gören et al., 1992; Izbosarov et al., 2000).

Compounds **1**, **4** and **5** were identified as germacranolides by their lactone and cyclodecane rings, as well as the exocyclic double bond conjugated with the lactone carbonyl. They were characterised as germacrane lactones with a linear structure due to the α,β-unsaturated lactone being fused to the C-7,8 positions of the carbocyclic skeleton. Similarly, compounds **2** and **3** were identified as the linear germacranolide derivatives, known as eudesmanolides, distinguished by the cyclodecane ring being split into two six membered rings by a C-5,10 bond. Although compounds **1**–**5** are all known compounds, having been previously isolated from a variety of plant species, this is the first report of their isolation from *Oncosiphon piluliferum*.

Once it was established that compounds **1**–**5** all possess an α-methylene–γ-lactone functional group, which is typically responsible for the biological activity of sesquiterpene lactones (Rodriguez et al., 1976), an attempt was made to determine what effect the reduction of the C-11,13 exocyclic double bond would have on the antiplasmodial activity and cytotoxicity of these compounds. Reduction of compound **5** with $NaBH_4$/methanol yielded compound **6** (Fig. 1), which was subsequently evaluated for its antiplasmodial and cytotoxic activities. The ^{1}H NMR data of compound **6** (Section 2.4) clearly indicated that the α-methylene lactone function of compound **5** had been reduced to give the 11,13-dihydro derivative and that the reaction yielded only one of the possible stereoisomers.

The pair of doublets corresponding to those of the C-13 methylene group in compound **5** were absent from the ^{1}H NMR spectrum of compound **6** and were replaced by a methyl signal identified as the doublet (J = 1.3 Hz) resonating at δ_H 1.39. There was also an additional proton signal at δ_H 2.63, identified as H-11 which appeared as a *dq* due to vicinal coupling with H-7 (J = 9.1 Hz) and the methyl protons, H-13 (J = 7.2 Hz).

The stereochemistry at C-11 followed from the magnitude of the coupling constant between H-7 and H-11 (J = 9.1 Hz), which suggested an anti-relationship between them. This was confirmed by the strong NOE correlation observed between H-13 and H-7 in the NOESY spectrum of compound **6**. Therefore, reduction of compound **5** with $NaBH_4$/methanol yielded 1,6-dihydroxy-11,13-dihydro-4*E*,10(14)-germacradien-12,8-olide. The fact that the reduction resulted in only one of the possible stereoisomers was in accordance with previous reports on the reduction of related sesquiterpene lactones with $NaBH_4$/methanol (Cardona et al., 1990).

Testing of compounds **1a**, **2**, **3**, **4**, **5** and **6** for *in vitro* antiplasmodial activity against the D10 *Plasmodium falciparum* strains using the pLDH assay revealed that the germacranolides **1a**, **4** and **5** were significantly more active than the eudesmanolides, **2** and **3** (Table 1). This is most likely due to the flexibility and conformational features of the 10-membered ring as opposed to the bi-cyclohexane ring system but the effect of other structural features cannot be ruled out. For instance, the presence of a 4,15-exocyclic methylene group, such as that in compound **2**, has been reported to decrease antiplasmodial activity in structurally related eudesmanolides (Lang et al., 2002). This might explain why compound **2** was the least active of the isolated compounds and why it had a higher IC_{50} than **3** when these two compounds differ only in the position of one double bond.

No significant conclusions could be drawn from the structure–activity relationship between the three germacranolides. In the case of compounds **4** and **5** the difference in antiplasmodial activity was minimal yet they also differ in the position of one double bond. While in compound **4**, C-10 has an exocyclic double bond; in compound **5**, there is a C-9,10 endocyclic double bond. Compound **1a**, which has a 4,5-epoxide moiety, was equipotent to compounds **4** and **5** which both have a double bond in this position.

There are no previous reports of compounds **1** and **2** being investigated for any biological activity. Compounds **3**, **4** and **5** have been found to show antibacterial activity (Gören et al., 1992; Izbosarov et al., 2000). This is the first report of any of the compounds having antiplasmodial activity.

In order to determine the specificity of the antiplasmodial activity of the sesquiterpene lactones, it was considered necessary to obtain information about their general cytotoxicity. Therefore, the compounds were tested for cytotoxicity against a Chinese Hamster Ovarian (CHO) cell line, using the microculture tetrazolium (MTT) assay.

Table 1
In vitro antiplasmodial activity, cytotoxicity and selectivity index (SI) values for chloroquine and compounds **1a**–**6**

Compound	Antiplasmodial D10 (IC_{50}, μg/ml)	Cytotoxicity CHO (IC_{50}, μg/ml)	SI[a]
Chloroquine	11.1×10^{-3}	18.5	1666.7
1a	0.5	2.2	4.4
2	4.4	10.1	2.3
3	2.6	4.0	1.5
4	0.4	6.0	15.0
5	0.5	6.4	12.8
6	70.0	>100	>1.4

[a] SI = cytotoxicity CHO IC_{50}/antiplasmodial D10 IC_{50}.

The corresponding IC_{50} and SI (selectivity index) values of chloroquine and the six compounds are listed in Table 1.

In considering a recent publication (Pink et al., 2005) outlining criteria for antiparasitic drug discovery, a compound can be considered a hit if it is

- active *in vitro* against whole protozoa with an IC_{50} of $\leq$1 μg/ml;
- selective (at least 10-fold more active against the parasite than against a mammalian cell line).

Based on these criteria, only compounds **4** and **5** can be considered hits. Compounds **2** and **3** were not active enough and compound **1a** was not sufficiently selective to kill the parasites without damaging mammalian cells. Overall, the data suggests that the observed antiplasmodial activity might be due to general toxicity. The antiplasmodial and cytotoxicity assay results of compound **6** clearly show that the C-11,13 exocyclic double bond of **5** is primarily responsible for both the antiplasmodial activity and toxicity to CHO cells as both are significantly decreased when this double bond is reduced. This result is in accordance with previous findings that the presence of an α-methylene–γ-lactone functional group is an active centre for cytotoxicity (Picman, 1986) and antiplasmodial activity (Phillipson and Wright, 1991; Francois et al., 1996).

Since compounds **1a**, **2**, **3**, **4** and **5** all possess an α-methylene–γ-lactone moiety; one would expect that they would all show equipotent antiplasmodial activity and toxicity to CHO cells, which is clearly not the case. The fact that there are significant differences in the IC_{50} values of each compound in the two assays as well as between the various compounds suggests that there is more than just the cytotoxic effect of the α-methylene–γ-lactone group coming into play.

4. Conclusions

The identification of antiplasmodial sesquiterpene lactones from *Oncosiphon piluliferum* suggests that they may play a role in the medicinal properties of the plant, but their potential for the development of antimalarial drugs is limited due to inherent cytotoxicity and lack of selectivity. This is often the case with antimalarial compounds identified from plants (Schwikkard and van Heerden, 2002). Although their activity and SI values cannot be compared to that of chloroquine, compounds **4** and **5** are considered as hits that could potentially be subjected to more detailed analysis, involving accurate IC_{50} determinations against different strains of the parasite, measurement of general cytotoxicity (using a range of mammalian cell lines) and *in vivo* assessment in animal models.

These compounds could also be used as scaffolds to generate leads with enhanced antiplasmodial activity, reduced cytoxicity and improved bioavailabilty. One such medicinal chemistry approach would be to investigate how the addition of known biologically active moieties to the C-11,13 exocyclic double bond (the cytotoxic component) would affect the antiplasmodial activity. Further structure–activity relationship studies would also help draw a conclusion as to whether the antiplasmodial activity observed for sesquiterpene lactones such as **1**, **2**, **3**, **4** and **5** is indeed biological activity or just the result of general cytotoxicity.

Acknowledgement

This work was financially supported by the Department of Science and Technology which awarded an innovation fund to five South African institutions (The Medical Research Council, South African National Biodiversity Institute, Council for Scientific and Industrial Research, University of Cape Town and University of Pretoria) to scientifically validate South African medicinal plants for the treatment of malaria.

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