PERGAMON

Phytochemistry 62 (2003) 747–751

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Antiplasmodial and antifungal activities of iridal, a plant triterpenoid

Françoise Benoit-Vical[a,*], Christine Imbert[b], Jean-Paul Bonfils[c], Yves Sauvaire[c]

[a]*Laboratoire de Chimie de Coordination du CNRS, 205 Route de Narbonne, F-31077 Toulouse Cedex 4 and Service de Parasitologie-Mycologie, CHU Rangueil, TSA 50032, F-31059 Toulouse Cedex, France*
[b]*Laboratoire de Parasitologie et Mycologie Médicales, CHU la Milétrie, BP 577, F-86021 Poitiers Cedex, France*
[c]*Laboratoire de Recherche sur les Substances Naturelles Végétales UPRES EA 1677, Université Montpellier II, Place Eugène Bataillon cc 024, F-34095 Montpellier Cedex 5, France*

Received 7 May 2002; received in revised form 28 October 2002

Abstract

Iridal, a triterpenoidic compound extracted from *Iris germanica* L., was previously shown to have an interesting activity on two cultured human tumor cell lines (A2780 and K562). In the present work, this same product was tested in vitro on *Plasmodium falciparum* chloroquine-resistant and -sensitive strains, in vivo on *P. vinckei*, and on some *Candida albicans* and *C. parapsilosis* strains too. The IC_{50} obtained in vitro on human malaria strain ranged from 1.8 to 26.0 μg/ml and the ED_{50} in vivo is about 85 mg/kg/day by intraperitoneal route. The minimal inhibitory concentrations were higher than to 50 μg/ml, whatever the strain of yeast tested. This product presents an antiplasmodial activity similar to that obtained with extracts from the plant *Azadirachta indica* classically taken as reference in malaria phytomedicine. Conversely iridal shows no important antifungal activity. The specific activity of iridal on human malaria parasite and on tumor cell lines is discussed.

Keywords: *Iris germanica*; Iridaceae; Malaria; Yeasts; *Plasmodium falciparum*; Traditional medicine

1. Introduction

The increasing prevalence of malaria exhibiting resistance of *Plasmodium falciparum* to standard treatments has led to searches for new antimalarial compounds. Traditional plant-based medicines have several potential advantages. They are inexpensive and easily available, particularly if people grow them themselves. In developing countries, 80% of people are thought to rely on herbal remedies (Willcox and Bodeker, 2000). Results on cytotoxic effects obtained with a triterpenoid from *Iris missouriensis* Nutt. on murine P-388 lymphocytic leukemic cells (Wong et al., 1986) as well as results with *Iris germanica* L. showing the presence of triterpenoids with piscicidal (Ito et al., 1995, 1999) and cytotoxic effects on human tumor cell lines (Bonfils et al., 1996a) prompted us to investigate the potential antimalarial activity of the triterpenoid iridal on human *Plasmodium* strains.

The present report describes the biological activity of iridal on *P. falciparum* in vitro and on *P. vinckei* in vivo. The lack of important in vitro susceptibility of *Candida* spp. yeasts suggests specific antiplasmodial activity.

2. Results and discussion

Table 1 reports concentrations inhibiting 50% (IC_{50}) of parasite growth measured with iridal and *Azadirachta indica*, an antimalarial plant taken as control. In parallel, IC_{50} values of chloroquine on the three *P. falciparum* strains, routinely tested in the laboratory, show that these laboratory strains keep constant drug sensitivities over time (Benoit et al., 1996; Benoit-Vical et al., 1999). Iridal presents IC_{50} values which range from 1.8 to 26.0 μg/ml on *P. falciparum*. These values are close to the results obtained with aqueous extracts of the control

* Corresponding author. Tel.: +33-561-323446; fax: +33-561-55-3003.
E-mail address: francoise.vical@toulouse.inserm.fr (F. Benoit-Vical).

0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(02)00625-8

Table 1
IC_{50} of iridal and control plant against three *P. falciparum* strains in vitro

P. falciparum strains	Nigerian CQS[a]	FcB1 CQR[a]			FcM29 CQR + [a]
Plant extracts	48 h[b]	32 h[b]	48h[b]	72 h[b]	48 h[b]
Iridal μg/ml	26 (2)[c]	14 (8)	20 (14)	1.8 (1)	12 (2)
μM	56	30	43	4	26
Azadirachta indica[d] μg/ml	6.8 (4)	7.5 (5)	n.d.	6.3 (3)	n.d.
Chloroquine[e] μg/ml	40×10^{-3}	80×10^{-3}	75×10^{-3}	75×10^{-3}	100×10^{-3}
μM	0.07	0.15	0.14	0.14	0.19

[a] Chloroquine sensitivity: CQS: chloroquine-sensitive strain; CQR: chloroquine-resistant strain; CQR + : high chloroquine-resistant strain.
[b] Incubation time between the parasite culture and the plant extract tested.
[c] Values in parentheses are standard deviations determined from $n = 2$–5 experiments.
[d] IC_{50} in molar concentration cannot be calculated for crude vegetal extracts.
[e] Routinely tested in the laboratory.
n.d. Not determined

plant *A. indica* (6.3–7.5 μg/ml) commonly used in traditional antimalarial treatments (Tella, 1994; Gessler et al., 1995) and better than those found in the major part of the literature on medicinal plants, often > 50 μg/ml (Weniger et al., 2001). Different IC_{50} values obtained for three incubation times show a better activity for a longer period. The IC_{50} at 32 h, close to the end of the trophozoite stage, evaluates the influence of the drug on parasite maturation. An incubation time of 48 h, enables to test the activity of the extract over a complete cycle of malaria. With an incubation time of 72 h, 1.5 times longer than the parasite erythrocytic life cycle, information on a possible cumulative effect on the main metabolic pathways of the parasite and on a potential effect on erythrocyte reinvasion are obtained. Iridal IC_{50} values are lower after 72 h than after 32 or 48 h which suggests that this compound is probably more active in the reinvasion step than in the maturation step of *P. falciparum* in vitro or has an action during the first or the last hours of the parasite cycle. Iridal IC_{50} values obtained with the three strains of *P. falciparum* show no correlation between the chloroquine sensitivity and the iridal sensitivity. This observation indicates that the modes of action of iridal and chloroquine should be different.

The in vivo antimalarial activity of iridal on *P. vinckei petteri* is presented in Fig. 1. The parasite growth inhibition is correlated with the intraperitoneal dosage inoculated to the malarial mice. With 13, 40 and 80 mg/kg/day, parasitaemia is reduced by 38, 41 and 47% respectively. ED_{50} (efficient dose for 50% parasitemia inhibiting) close to 80 mg/kg/day and is by extrapolation about 85

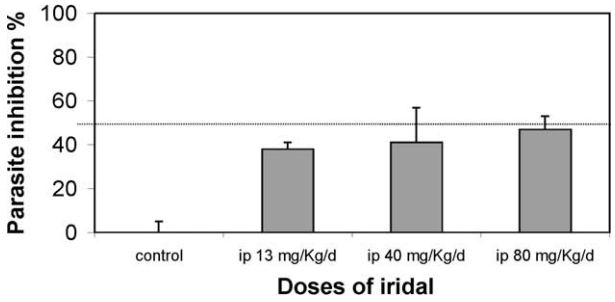

Fig. 1. In vivo antimalarial activity of iridal on mice infected by *P. vinckei petteri*. Data are shown as mean + S.D. of five mice per condition. The five mice control are infected by *P. vinckei* and treated every day with a 0.9% sodium chloride solution (0.2 ml) by intraperitoneal route.

mg/kg/day. However, even if iridal injections decrease parasitaemia and delay death, the 3 doses tested are not sufficient to cure the mice. Indeed, the mice control with 0.9% NaCl treatment died on day 9 whereas the mice treated by 13, 40 and 80 mg/kg/day, died on days 10, day 12 and 13, respectively. These results are encouraging because ED_{50} obtained from other plants tested for their antimalarial activity are often largely higher (Tona et al., 2001; Addae-Kyereme et al., 2001). Moreover, this in vivo test enables to conclude on the absence of side effects or toxicity of iridal for these doses on an animal model.

Studies on two yeast species (data not shown), *Candida albicans* (strains 1066, SR 75, SR 76, SR 78, 127) and *C. parapsilosis* (strains ATCC 22019, 28, 7) show that the Minimal Inhibitory Concentrations (MICs) are for all strains higher than to 50 μg/ml for both 24 and 48 h incubation times. Whatever the *Candida* strain used for this study, iridal had a very low antifungal efficiency. These results are in agreement with those presenting MICs of triterpenoids close to 60 μg/ml (Fabry et al., 1996).

Malaria is a worldwide problem and the increasing spread of *P. falciparum* strains which are resistant to standard treatments has initiated numerous studies aimed at identifying new antimalarials. Traditional medicine seems to be a very important source of active molecules (Bodeker, 2000). Indeed, for the moment, treatments from vegetal origin like quinine or its alkaloids (Bustos et al., 1994) and more recently artemisinin and its derivatives (Hoffman, 1996; Price et al., 1998) are the most efficient on serious malaria crises. In parallel, anti-cancer searches have enabled to isolate taxol from a common plant which shows a spectacular efficacy on MDR + and MDR- tumour cell lines, and a generalized use in anti-cancer treatment. Besides, taxol presents a strong antimalarial activity on the blood-stage of the parasite in vitro and in vivo (Pouvelle et al., 1994).

This work studies antimalarial activity of iridal, a compound isolated from *Iris* and purified by RP-HPLC. It has a basic structure in the iridals family and can be considered as one of the first bio-synthesized iridals. As such it could be used for the synthesis of all other iridal

family members. Other authors have also studied this plant family and a recent publication showed the isolation of seven iridalglycosides from rhizomes of *Iris spuria* (Marner et al., 2002). A first biological study showed a strong cytotoxic activity on the lines A2780 and K562 for 6 iridals (Bonfils et al., 1996a, 2001) and iridal had very interesting IC_{50} values on these human tumour cell lines. That is why we decided to investigate the antimalarial activity of iridal on human *Plasmodium* strains. As found with taxol, the cytotoxicity on tumour cell lines does not mean an in vivo toxicity (Sinou et al., 1996). Results obtained with yeasts confirm the specific efficacy of this compound on the intracellular parasite *Plasmodium* and on tumour cell lines. Moreover, iridal does not appear toxic on mice treated with ED_{50} doses.

Different hypothesis on the mode of action of iridal (mechanisms and period of inhibition) have been envisaged after varying incubation time and chloroquine-sensitivity of the strains. Iridals seem to play structural and functional roles in cell membranes comparable to those of sterols (Bonfils et al., 1996b). Maguire and Sherman (1990) have shown that changes in lipid composition and cholesterol exchange between uninfected cells and membranes of malaria-infected erythrocytes may be responsible for the increased fluidity and permeability of *P. falciparum*-infected cells. This supports the hypothesis that the effect of iridal is the strongest during the reinvasion step of *P. falciparum*. By testing iridal on yeasts, it could be considered as selective for cell cultures and *Plasmodium* over fungi.

In summary, iridal has an interesting antiplasmodial activity both in vitro on various laboratory strains and in vivo, without being toxic on mice.

3. Experimental

3.1. Plant material

Rhizomes of *Iris germanica* L. var. Rococo (Iridaceae) were obtained from the Iris producer ''Les Iris de Thau'', 14 rue des Logis, Loupian, F-34140 Mèze, France. A voucher specimen (IGR5) was deposited in the Botanical Garden of Montpellier (University Montpellier I, France). *Azadirachta indica* A. Juss (Meliaceae), used as positive control because of its interesting antimalarial properties were extracted and tested as described by Benoit et al. (1996).

3.2. Iridal purification and identification

Rhizomes were cleaned under water and ground (grinder Janke & Kunkel, model A10). The extraction was carried out at room temperature with EtOH–H_2O (7:3). Crude extracts were filtered through analypore EC membrane (0.45 μm) and the solvent concentrated under vacuum (40 °C). Hydrophilic compounds were removed after loading the crude extract onto Extrelut 20 (Merck) columns and eluting lipophilic fraction with diethylether. Iridal was isolated from this lipophilic fraction after RP HPLC separation using the columns indicated below with MeOH/H_2O mixtures used in gradient or isocratic condition. HPLC apparatus consists of 2 Gilson 305 and 302 pumps (25 SC heads), a Waters Lambda max 481 UV–vis detector. Columns: semi-preparative Hibar Lichrospher RP-18, 100 Å, 10 μm, 250 × 25 mm (Merck, Germany), Lichrospher RP-18, 100 Å, 40–63 μm, 300 × 39 mm (Merck, Germany). Iridal was identified by comparison of retention time and UV spectrum with those of authentic standard obtained from Dr. Marner (Cologne, Germany), and also by comparison of MS, ^1H and ^{13}C NMR data with those reported in the literature (Krick et al., 1983). Copies of the original spectra can be obtained from the corresponding author. The formula of iridal tested in this present work is given in Fig. 2.

3.3. P. falciparum strain and in vitro culture

Three strains of *P. falciparum* were cultured according to the method of Trager and Jensen (1976) in a 5% CO_2 atmosphere at 37 °C (Van Huyssen and Rieckmann, 1993). The chloroquine-sensitive strain, Nigerian ($IC_{50} = 40 \pm 5$ ng/ml) and two chloroquine-resistant strains, FcB1-Colombia ($IC_{50} = 75 \pm 5$ ng/ml) and FcM29-Cameroon ($IC_{50} = 100 \pm 5$ ng/ml) were chosen for this study. The parasites were maintained in vitro in human red blood cells (O±) diluted to 1% hematocrit in RPMI 1640 medium (GIBCO BRL, Paisley, Scotland) supplemented with 25 mM Hepes and 30 mM $NaHCO_3$ and complemented with 5% human AB^+ serum. Parasite cultures were synchronized by combination of gelatine (Plasmagel, Roger Bellon, Paris, France) according to Jensen (1978) and 5% D-sorbitol lysis (Merck, Darmstadt, Germany) as reported by Lambros and Vanderberg (1979).

3.4. In vitro antimalarial activity

The antiplasmodial activity of iridal was evaluated by the radioactive microdilution method described by Desjardins et al. (1979) and modified as follows. Extract dilutions were tested 3–5 times in triplicate in 96-well

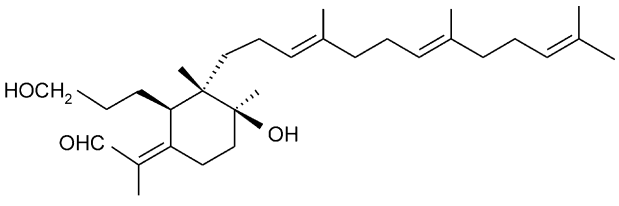

Fig. 2. Structure of iridal (21-desoxy-iridogermanal) (Krick et al., 1983).

plates (TPP, Switzerland) with cultures at ring stage (synchronization interval: 15 h) at 1% parasitaemia (hematocrit: 1%). For each test, the plates of parasite culture were incubated with compound at decreasing concentrations for 48 h or 72 h, radioactive hypoxanthine being added to the medium 24 h after the beginning of incubation (Benoit-Vical et al., 1999). The first dilution of the extract (5 mg/ml) was performed with dimethylsulfoxide (Merck, Darmstadt, Germany), and the following ones with RPMI 1640 (Bio Media, Boussens, France). Parasite growth was estimated by [^{3}H]-hypoxanthine (Amersham-France, Les Ulis, France) incorporation. Concentrations of iridal inhibiting 50% of the parasite growth (IC_{50}) were graphically determined in concentration versus percent inhibition curves for the three incubation times (Valentin et al., 1995). The chloroquine sensitivity of the three strains was routinely tested.

3.5. In vivo antimalarial activity

Cell line 106 WH of *P. vinckei petteri* was maintained in mice by seringue passage. Female Swiss (10 weeks of age, 25±2 g) were used in this study. The mice were kept at a temperature of 22±3 °C and provided with a standard diet and water. They were inoculated intraperitoneally with 2×10^7 infected erythrocytes on day 0. The treatment dose was given intraperitoneally 3 h after infection on day 0 and was repeated once daily for 3 days, as a "4-day-blood schizonticidal test" (Peters et al., 1975). For this study, 3 doses of iridal were tested: 13, 40 and 80 mg/kg/day dissolved first with DMSO then in 0.9% sodium chloride with a volume of injection of 0.2 ml. For each dose, 5 mice were studied, their parasitemia and mortality were followed. In parallel, 5 mice as control animals received only 0.9% sodium chloride (0.2 ml). On day 4, thin blood smears were made from mouse tail blood and stained by Giemsa. At least 1000 cells were counted to determine parasitaemia. Mortality of mice infected by *Plasmodium* and treated by iridal was studied in comparison with mice control.

3.6. Fungi strains

Five strains of *Candida albicans* (1066, 127, SR 75, 76 and 78) and three of *C. parapsilosis* (7, 28 and ATCC 22019) were used in this study. ATCC 22019 was purchased from the American Type Culture Collection and all other strains were clinical isolates. All organisms were maintained on slopes of Sabouraud Dextrose Agar (Difco) and subcultured monthly.

3.7. Minimal inhibition concentration (MIC) of iridal extract on yeasts

The extract was prepared as a stock solution of 5 mg/ml in dimethyl sulfoxide (Merck) and a broth microdilution method was used to evaluate the fungistatic activity.

A series of dilutions at 100 times the final concentration was prepared from the extract stock solution in the same solvent. Each intermediate solution was then further diluted to final strength in RPMI 1640 medium buffered with 0.165 mol/l MOPS (test medium).

3.8. Inoculum preparation

Yeasts were grown for 48 h at 28 °C onto Sabouraud Dextrose Agar (Sanofi Diagnostics Pasteur), inocula were then prepared by suspension of these yeasts in test medium and were adjusted to a final concentration of 10^4 yeasts/ml. Each well of microdilution plates was inoculated with 150 µl of the 10^4 yeasts/ml suspension and 150 µl of various concentrations of extract, for 24 and 48 h at 37 °C.

The MIC was the lowest concentration of the tested extract that substantially inhibited growth of the organism as detected visually (National Committee for Clinical Laboratory Standards, 1997).

Acknowledgements

Dr. I. Dixon, an English native-speaker, is gratefully acknowledged for her helpful discussions and linguistical assistance.

References

Addae-Kyereme, J., Croft, S.L., Kendrick, H., Wright, C.W., 2001. Antiplasmodial activities of some Ghanaian plants traditionally used for fever/malaria treatment and of some alkaloids isolated from *Pleicarpa mutica*; *in vivo* antimalarial activity of pleiocarpine. Journal of Ethnopharmacology 76, 99–103.

Benoit, F., Valentin, A., Pélissier, Y., Diafouka, F., Marion, C., Kone-Bamba, D., Mallié, M., Yapo, A., Bastide, J.M., 1996. Antimalarial activity *in vitro* of vegetal extracts used in West African traditional medicine. American Journal of Tropical Medicine and Hygiene 54, 67–71.

Benoit-Vical, F., Robert, A., Meunier, B., 1999. Potentiation of artemisinin activity against chloroquine-resistant *Plasmodium falciparum* strains by using heme models. Antimicrobial Agents and Chemotherapy 43, 2555–2558.

Bodeker, G., 2000. Searching for antimalarials in plants. Journal of Alternative Complement of Medicine 6, 127–129.

Bonfils, J.P., Pinguet, F., Culine, S., Sauvaire, Y., 1996a. Composition de triterpènes à activité anticancéreuse. Brevet Français No. 96 03 892, p. 21.

Bonfils, J.P., Pinguet, F., Culine, S., Sauvaire, Y., 2001. Cytotoxicity of iridals, plant triterpenoids from *Iris*, on human tumor cell lines A2780 and K562. Planta Medica 67, 79–81.

Bonfils, J.P., Sauvaire, Y., Maurin, L., 1996b. Evidence of cycloiridals as membrane constituents: effects on fluidity patterns compared to those of cholesterol. Planta 200, 353–357.

Bustos, M.D.G., Gay, F., Diquet, B., 1994. *In vitro* tests on Philippine isolates of *Plasmodium falciparum* against four standard antimalarials and four qinghaosu derivatives. Bulletin WHO 72, 729–735.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative assessment of antimalarial activity *in vitro* by a semi-automated microdilution technique. Antimicrobial Agents and Chemotherapy 16, 710–718.

Fabry, W., Okemo, P., Ansorg, R., 1996. Fungistatic and fungicidal activity of East African medicinal plants. Mycoses 39, 67–70.

Gessler, M.C., Tanner, M., Chollet, J., Nkunya, M.H.H., 1995. Tanzanian medicinal plants used traditionally for the treatment of malaria: *in vivo* antimalarial and *in vitro* cytotoxic activities. Phytotherapy Research 9, 504–508.

Hoffman, S.L., 1996. Artemether in severe malaria. Still too many deaths. New England Journal of Medicine 335, 124–126.

Ito, H., Onoue, S., Miyake, Y., Yohida, T., 1999. Iridal-type triterpenoids with ichthyotoxic activity from *Belamcanda chinensis*. Planta Medica 62, 89–93.

Ito, H., Miyake, Y., Yohida, T., 1995. New piscicidal triterpenes from *Iris germanica*. Chemical and Pharmaceutical Bulletin 43, 1260–1262.

Jensen, J.B., 1978. Concentration from continuous culture of erythocytes infected with trophozoites and schizonts of *Plasmodium falciparum*. American Journal of Tropical Medicine and Hygiene 27, 1274–1276.

Krick, W., Marner, F.J., Jaenicke, L., 1983. Isolation and structure determination of the precursors of α- and γ-irone and homologous compounds from *I. pallida* and *I. florentina*. Zeitschrift für Naturforschung 38, 179–184.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. Journal of Parasitology 65, 418–420.

Maguire, P.A., Sherman, I.W., 1990. Phospholipid composition, cholesterol content and cholesterol exchange in *Plasmodium falciparum*-infected red cells. Molecular Biochemistry and Parasitology 38, 105–112.

Marner, F.J., Singab, A.N., Al-Azizi, M.M., El-Emary, N.A., Schafer, M., 2002. Iridal glycosides from *Iris spuria* (Zeal), cultivated in Egypt. Phytochemistry 60, 301–307.

National Committee for Clinical Laboratory Standards, 1997. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard, M27-A, 17.

Peters, W., Portus, J., Robinson, B.L., 1975. The chemotherapy of rodent malaria XXII. The value of drug resistant strains of *P. berghei* in screening for blood schizonticidal activity. Annals of Tropical Medicine and Parasitology 69, 155–171.

Pouvelle, B., Farley, P.J., Long, C.A., Taraschi, T.F., 1994. Taxol arrests the development of blood-stage *Plasmodium falciparum in vitro* and *Plasmodium chabaudi adami* in malaria-infected mice. Journal of Clinical Investigations 94, 413–417.

Price, R., Luxemburger, C., Van Vught, M., Nosten, F., 1998. Artesunate and mefloquine in the treatment of uncomplicated multidrug-resistant hyperparasitaemic *falciparum* malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene 92, 207–211.

Sinou, V., Grellier, P., Schrevel, J., 1996. *In vitro* and *in vivo* inhibition of erythrocytic development of malarial parasites by docetaxel. Antimicrobial Agents and Chemotherapy 40, 358–361.

Tella, A., 1994. Studies on *Azadirachta indica* in malaria. British Journal of Pharmacology 58, 318.

Tona, L., Mesia, K., Ngimbi, N.P., Chrimwani, B., Okond'ahoka Cimanga, K., de Bruyne, T., Apers, S., Hermans, N., Totte, J., Pieters, L., Vlietinck, A.J., 2001. *In vivo* antimalarial activity of *Cassia occidentalis, Morinda morindoides* and *Phyllanthus niruni*. Annals of Tropical Medicine and Parasitology 95, 47–57.

Trager, W., Jensen, J., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Valentin, A., Pelissier, Y., Benoit, F., Marion, C., Kone, D., Mallie, M., Bastide, J.M., 1995. Composition and antimalarial activity *in vitro* of volatile components of *Lippia multiflora*. Phytochemistry 40, 1439–1442.

Van Huyssen, W., Rieckmann, K.H., 1993. Disposable environmental chamber for assessing the drug susceptibility of malaria parasites. Tropical Medicine and Parasitology 44, 329–330.

Weniger, B., Robledo, S., Arango, G.J., Deharo, E., Aragon, R., Munoz, V., Callapa, J., Lobstein, A., Anton, R., 2001. Antiprotozoal activities of Colombian plants. Journal of Ethnopharmacology 78, 193–200.

Willcox, M.L., Bodeker, G., 2000. Plant-based malaria control. Parasitology Today 16, 220–221.

Wong, S.M., Oshima, Y., Pezzuto, J.M., Fong, H.H.S., Farnsworth, N.R., 1986. Plant anticancer agents. XXXIX. Triterpenes from *Iris missouriensis*. Journal of Pharmaceutical Sciences 75, 317–320.