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A new antimalarial agent; effect of extracts of *Artemisia diffusa* against *Plasmodium berghei*

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ABSTRACT
Malaria is one of the most serious health problems in many parts of the world, particularly in Africa and Latin America with a high mortality rate. The situation is further complicated by the spread of drug-resistant parasites in many parts where *plasmodium falciparum* is endemic. A few alternative drugs are under development, necessitating urgent efforts to identify new classes of antimalarial agents. There is therefore a need to find new, effective and affordable remedies for malaria, including those derived from plants. The clinical utility of the Chinese discovery of artemisinin from the herb *Artemisia annua* has stimulated much interest in traditional plants as potential sources of new antimalarial drugs. In this study, the antimalarial activity of *Artemisia diffusa* extracts and the fraction which contains sesquiterpene lactones including Tehranolide, on *Plasmodium berghei* in vivo on the mice model of malaria was investigated. We did our best to carry out the biological tests as well as the phytochemical investigations from the same collection. It demonstrates that crude extracts of *Artemisia diffusa* inhibit the growth of *Plasmodium berghei* in vivo in NMRI mice. The microscopic examination of Giemsa stained slides showed a virtual absence of all blood-stage of murine malaria treated with three concentrations of herbal extracts including 27, 2.7 and 0.27 mg/ml. These observations suggest that the active constituents in the extract may be cytotoxic for *P. berghei*, thereby inhibiting their development to the erythrocytic stage. The results specifically indicated the inhibitory effects of the *A.diffusa* crude extracts and the fraction which contains sesquiterpene lactones including Tehranolide, on the developmental stages of *P. berghei* by decreasing parasitaemia.
KEY WORDS: New antimalarial agent, *Artemisia diffusa, Plasmodium berghei,* Tehranolide.

INTRODUCTION

The multi drug resistance of *Plasmodium falciparum* is a major health problem in many countries and the number of drugs available, effective and affordable is very limited (1). The malaria situation is aggravated by the appearance of strains of *Plasmodium falciparum* resistant to antimalarial drugs as well as by the resistance of vector *Anopheles* mosquitoes to DDT and other insecticides. Nearly 300-500 million people are infected by malaria and the incidence of this disease is dramatically increasing, since many strains of *Plasmodium falciparum*, the parasite responsible for the majority of fatal malaria infections, have become resistant to chloroquine and other traditional antimalarial drugs (2). In Africa alone the disease is assumed to be responsible for the death of about 1 million children yearly (3). These are the principal factors that contribute to the difficulty of the malaria control and it is unrealistic to think about the eradication of this disease by means of destruction of the vector or use of vaccination. Studies in a number of African countries have shown that the emergence of the chloroquine-resistant malaria parasites is associated with a two-fold increase in malaria deaths, but in one study in Mlomp, Senegal, it was shown that

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malaria mortality in children under the age of four increased 11-fold within six years of the emergence of the chloroquine-resistance (4). This fact provides the reasons for research for new antimalarial drugs. Fortunately, these strains are still susceptible to the artimisinin derivatives. Artemisinin was originally isolated from *Artemisia annua*, an herb used as an ancient Chinese herbal remedy. Artimisinin is the most relevant advance in the treatment of the malaria disease in the last two and a half decades. Artemisinin is a sesquiterpene lactone with an endoperoxide group, which has been used for many centuries in traditional Chinese medicine as a treatment for fever and malaria, including those with both the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Due to their potent antimalarial activity, fast action and low toxicity, artemisinin and its derivatives have distinguished themselves as a new generation of antimalarial drugs (5-7). *A. annua* is presently being cultivated on a commercial scale in China and Vietnam for its antimalarial sesquiterpene lactone. The genus is of small herbs or shrubs found in Northern temperate regions. It belongs to the important family Compositae (Asteraceae), one of the most bulky vegetal groupings, which comprises about 1000 genera and over 20,000 species. Within this family, *Artemisia* is included into the tribe Anthemideae and comprises itself over 400 species. The 400 species of *Artemisia* are mainly found in Asia, Europe and North America. They are mostly perennial herbs and shrubs dominating the vast steppe communities of Asia (8) of which 150 were recorded for China, about 50 reported to occur in Japan and 35 species of the genus are found in Iran, of which two are endemic: *A. melanolepis* Boiss. and *A. kermanensis* Pold. (9).

The genus *Artemisia* has always been of great botanical and pharmaceutical interest and is useful in traditional medicines for a treatment of the variety of diseases and complaints. This genus including some Iranian species has been studied chemically and present of monoterpenes (10), sesquiterpenes (11,12), especially sesquiterpene lactones (10,13,14) and essential oils (15-19) were reported. This genus is not very uniform and the chemistry is somewhat diverse. However, most species contain sesquiterpene lactones, especially 11,13- dihydro derivatives.

The extract of the aerial parts of *A. diffusa* Krasch ex P. Poljakov collected in the Province of Khorassan (Iran) afforded, in addition to several eudesmanolides [1a,1ß,2a,2b,3a,3b and 4], a new type of sesquiterpene lactone (Tehranolide) (Fig. I) (13), with an endoperoxide group that probably has the same effect as the antimalarial agent artemisinin. We report here the antimalarial properties of the extract and the fraction which contains sesquiterpene lactones including Tehranolide of the same species (*Artemisia diffusa*) (20). The study especially examined the inhibitory effects of the extracts on the developmental stages of in vivo of *Plasmodium berghei* in mice. Since the endoperoxide group is an essential requirement for the antimalarial activity of artemisinin, we presume the antimalarial properties of the extracts of *A.diffusa* are attributed to Tehranolide. In the preliminary experiments, the toxicity of the ethanolic extract was tested and judging from the high doses that were tolerated without significant overt mortality or signs of toxicity, it was estimated that the plant ethanolic extract is of relating low toxicity.

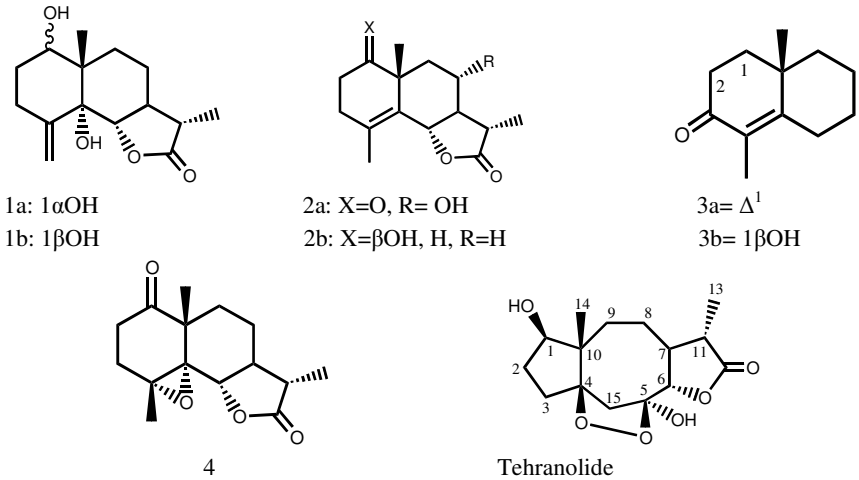

Fig.I. Eudesmanolides and a new type of sesquiterpene lactone (Tehranolide) from A.difussa

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MATERIAL AND METHODS

Equipment

NMR spectra were recorded at 500 MHz, with TMS as internal standard on a Bruker AM 500 instrument, under Aspect X32 control. IR spectra were taken on a BOMEM Canada FT-IR, MB-100 spectrometer. Silica gel 60 (70-230 and 230-400 mesh) and TLC was performed with Kieselgel 60 F_{254} (Merck aluminium support plates) and spot were detected after spraying with a 15% H_2SO_4 solution in MeOH. For separation of mixtures, a Knauer HPLC instrument with a 1001 pump detector was used and column C_{18} (120×8 mmID) was employed.

Plant material

Plants were identified and collected in September 2005 from their natural habitats in North East of Iran Province of Khorassan by Dr. V. Mozaffarian of the Research Institute of Forests and Rangelands (TARI).

Voucher specimens have been deposited at the Herbarium of the Research Institute of Forests and Rangelands, Tehran.

Extraction and Isolation

Ground aerial parts (850 g) were extracted with Et_2O/MeOH/Petrol (1:1:1) (2 x 6 L) at room temperature for 48h. Evaporation at reduced pressure furnished (36.5 g) of crude extract, which was suspended in EtOH (600 ml), diluted with H_2O (500 ml) and extracted successively with hexane (2x 650 ml) and $CHCl_3$ (2x 450 ml). Evaporation of the $CHCl_3$ extract at reduced pressure furnished (13.5g) of reside, which was column chromatographed over silical gel (340 mg, 70- 230 mesh) using $CHCl_3$ and increasing amounts of EtOAc (0-100%) and EtOAc/MeOH (9:1) to afford 32 fractions. These were grouped according to their TLC profiles and monitored by IR spectroscopy. Only fractions showing γ-lactone absorption in the 1780-1770 cm^{-1} range were processed. Fractions 7-8 (240 mg) were reunited and rechromatographed on silica gel (230-400 mesh) to give (45 mg) **1b**. Fractions 10-13 (250 mg) were combined and portion of (120 mg) was processed by HPLC using a C_{18}column (MeOH/H_2O 6:4) flow rate, 3 ml/min gave (35 mg) **2b** (R_t 8.5 min) and 11 mg **4** (R_t 13 min). Fractions 16-20 (230 mg) were reunited and rechromatographed on silica gel (230- 400 mesh) using Et_2O/MeOH (9:1) to yield (18 mg) of **1a**, (39 mg) **3a** and (9 mg) **2a**. Fractions 26-32 (250 mg) were combined and portion (120 mg) was processed by HPLC using a RP8 column (MeOH/ H_2O 5.5:4.5) flow rate, 3 ml/min to give (13 mg) **3b** (R_t 5.5 min) and (12 mg) **Tehranolide** (R_t 4 min). Compounds were identified by comparing the 500 MHz ^{1}H-NMR spectra with those of authentic material. The volatile constituents of hexane extract which does not contain any sesquiterpene lactone are under investigation.

Animals

Male outbred NMRI mice (supplied by the Karaj Laboratory Animal Unit, Pasteur Institute of Iran) were used in this study. The mice were housed at room temperature (20–23°C) on a 12-h light and 12 h dark cycle, with unlimited access to food and tap water. Experiments with animals were done according to the ethical standards formulated in the Declaration of Helsinki, and measures taken to protect animals from pain or discomfort. It has been approved by institutional ethical review board (*Ethical Committee of the Pasteur Institute of Iran*), in which the antimalarial test was done.

Malaria parasites

Plasmodium berghei NY kindly donated by Dr. M. J. Dascombe from the School of Life Sciences, University of Manchester, UK. Malaria parasite was maintained by blood passage in NMRI mice when active parasites were required; otherwise it was stored at -70°C in Alserver's solution (2.33% glucose, 0.525% NaCl and 1% sodium citrate in deionised water) and glycerol (9:1 parts by volume).

Inoculation of malaria parasites

Mice were inoculated (0.2 ml, i.v.) into a tail vein with blood from a donor mouse (41% parasitaemia *P. berghei*) diluted with 0.85% saline to contain 2×10^7 parasitised red blood cells (PRBC).

EXPERIMENTS AND GROUPS

Study on toxicity of herbal extracts on naive mice

Three different concentrations of herbal extracts including 27 mg/ml, 2.7 mg/ml and 0.27 mg/ml as test animals and a control group (vehicle) all were injected s.c. every day for 8 days with 100 µl solutions. Four groups of mice (n = 4) were investigated for assessment of pathology including body weight, physiological activities, hepatomegaly and splenomegaly.

Antimalarial effects of herbal extracts on murine malaria

Herbal extracts including low, average and high concentrations as test animals and a control group (vehicle) all were injected s.c. every day for 8 days with 100 µl solutions. Four groups of mice (n = 4) were investigated for antimalarial efficacy, degree of parasitaemia, assessment of pathology including body weight, physiological activities, hepatomegaly and splenomegaly.

Antimalarial effects of fractions which contains sesquiterpene lactones including Tehranolide

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Fractions of sesquiterpene lactones including Tehranolide in 100 mg/ml concentration (100 µl) were injected s.c. every day for 12 days after infection in malaria mice. Three groups of mice (n = 4) were investigated for antimalarial efficacy, degree of parasitaemia, assessment of pathology including body weight, physiological activities, hepatomegaly and splenomegaly. Parasitaemia was measured every other day by counting Geimsa-stained blood smears were taken from end tail cutting.

ASSESSMENT OF PATHOLOGY

Parasitaemia

The clinical diagnosis was confirmed by laboratory demonstration of the malaria parasite in the stained smears. In all animals parasitaemia was determined on different days after infection using blood smears stained with Geimsa stain (Sigma Chemical Co., USA). Parasitized red blood cells (PRBC) were counted in five different fields, each of approximately 200 cells. Results are expressed as the mean percentage (%) of erythrocytes containing Geimsa positive bodies. Experiments were licensed under the Animals (Scientific Procedures) Act 1986. In compliance with the conditions of this license, infected animals were humanely killed at the onset of the terminal phase of malaria (*P. berghei* NY) infection.

Assessment of degree of hepato / splenomegaly

Entire livers and spleens were removed *post mortem* at the end of the experimental period from mice after induction of terminal general anaesthesia by inhalation of diethyl ether (Sigma Co., Germany). Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly.

Measurement of survival rate

Survival rate was presented as the percentage of surviving experimental mice at every other week after inoculation; the significance of differences was determined by statistical test and compared with concurrent appropriate control groups.

Body weight

Body weight was measured initially and at different times of experiment using a top pan balance (OHAUS Scale Corp., USA).

Statistical analysis

Values are presented as the mean ± SEM for groups of n mice. The significance of differences were determined by Student's t-test using GraphPad Prism Software (GraphPad, San Diego, California, USA) ($*P<0.05$, $**P<0.01$, $***P<0.001$).

RESULT

The results specifically indicated the inhibitory effects of the *A. diffusa* extracts on the developmental stages of *P. berghei by decreasing parasitaemia*. The microscopic examination of Giemsa stained slides, Fig. II, showed a virtual absence of all blood-stage of the murine malaria treated with three concentrations of herbal extracts including 27, 2.7 and 0.27 mg/ml. These observations suggest that the active constituents in the extract may be cytotoxic for *P. berghei*, thereby inhibiting their development to the erythrocytic stage Fig. III.

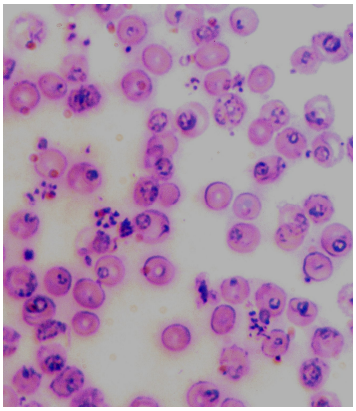

Fig. II. Plasmodium berghei blood-stage forms in Geimsa stained smears from NMRI mice

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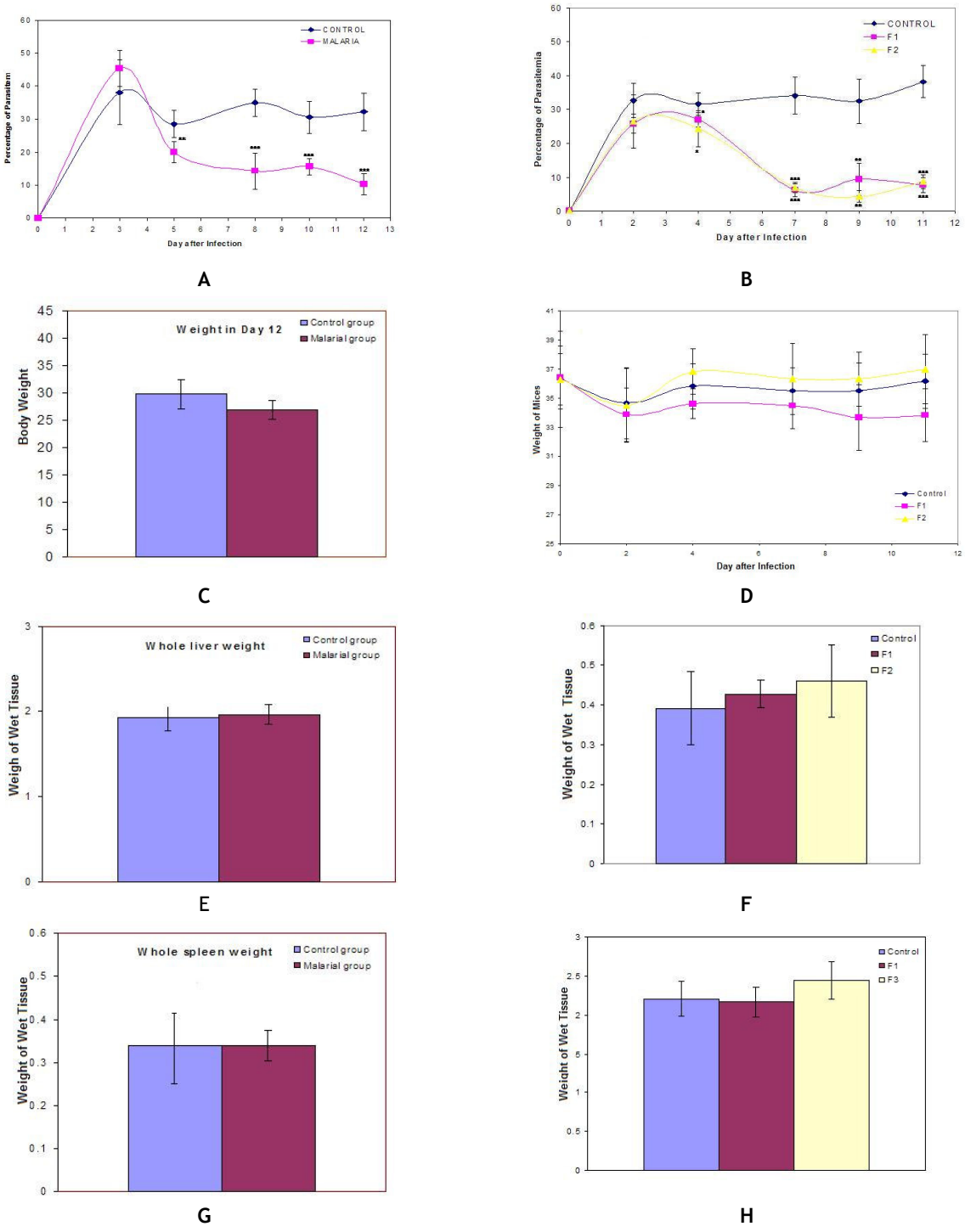

Fig.III. Antimalarial effects of herbal extracts and fractions (F_1 and F_2) containing sesquiterpene lactones including Tehranolide by measuring parasitaemia percentages in mice infected with P. berghei (A-B); The effects of herbal extracts on body weight in mice infected with P. berghei (C);The effects of fractions (F_1 and F_2) containing sesquiterpene lactones including Tehranolide on body weight in mice infected with P. berghei (D); The effects of herbal extracts (E) and F_1 and F_2 (F) on hepatomegaly in mice infected with P. berghei; The effects of herbal extract (G) and fractions F_1 anf F_2 (H) on splenomegaly in mice infected with P.berghei.

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DISCUSION AND CONCLUSION

Sesquiterpene lactones are found in *A. diffusa* with the peroxide functional group that probably has the same effect of Artemisinin as an antimalarial agent. The Artemisinin, as a sesquiterpene lactone endoproxide, is becoming an important plant-derived compound in the treatment of the resistant malaria.

Although there have been considerable scientific advances over the past hundred years, the overall burden of malaria is currently increasing, especially in sub-Saharan Africa. In the absence of a fully protective antimalarial vaccine, the control of malaria relies principally on the use of drugs for treatment or prophylaxis. Much of the increasing burden of malaria is due to the spread of the resistance of the major human malaria pathogen, *P. falciparum*, to most drugs presently available. Consequently, the World Health Organization and health authorities in malaria-endemic countries are recommending new therapies, based on the use of artemisinin derivatives, combined with other drugs, the so-called artemisinin combination therapy. Reports of the resistance of natural populations of *P.falciparum* to these drugs have not been forthcoming thus far, but most agree that resistant parasites will occur in the future, based upon previous experience with other antimalarial drugs. Further isolation and purification of Tehranolide is under investigation for antimalarial tests especially in vivo studies as well as in vitro activity against the multidrug resistant K_1 strain of *Plasmodium falciparum* and clinical trails to develop new antimalarial agents. Alternatively new therapies can be performed based on the use of combination therapy such as Tehranolide combination therapy.

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