



## Anti-plasmodial activity of *Ailanthus excelsa* <sup>☆</sup>

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### Abstract

The anti-plasmodial activity of *Ailanthus excelsa* stem bark was investigated. The methanolic extract inhibited *in vitro* growth of chloroquine-sensitive (D10) and resistant strains (W2) of *Plasmodium falciparum* (IC<sub>50</sub> 4.6 and 2.8 µg/ml, respectively). The effect was retained in the chloroform fraction (3.1 and 2.1 µg/ml, respectively). The anti-plasmodial activity could be ascribed to the impairment of haemoglobin degradation through the inhibition of plasmepsin II activity (IC<sub>50</sub> of 13.43 ± 1.74 µg/ml) and of the haem detoxification to haemozoin.

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### 1. Introduction

*Ailanthus excelsa* is a fast-growing and large tree, up to 25 m tall, commonly known as Indian Tree of Heaven. In traditional medicine the bark is being used as anti-spasmodic and expectorant [1]. *A. excelsa* has been shown to possess anti-bacterial [2], anti-leukemic [3] and anti-fungal activities [4]. As for the other *Ailanthus* species *A. excelsa* contains the bitter principles known as quassinoids. Quassinoids isolated from *Ailanthus* spp. possess several biological activities including anti-tumor [5], anti-viral [6,7], anti-tuberculosis [8] properties reported in a Review [9]. Quassinoids were also shown to be the active principles responsible for the anti-malarial activity of *Ailanthus altissima*. The chloroform extract obtained from the stem and root bark of this plant inhibited *in vitro* the growth of *Plasmodium falciparum* and was active against *P. berghei* infections in mice [10]. The anti-plasmodial activity has been mainly referred to the presence of ailanthone, a quassinoid responsible for the anti-malarial activity recently observed also for the seedlings of *A. altissima* [11].

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Malaria represents one of the key priorities for the WHO due to the unavailability of a vaccine and the spread and intensification of drug resistance over the last 15–20 years.

Modern pharmaceuticals are unavailable and/or unaffordable for the populations living in those areas affected by malaria. In such conditions non-industrialized countries rely on traditional medicine and locally grown plants for the cure of malaria. Research Initiative on Traditional Antimalarial Methods (RITAM) is promoting the use of traditional remedies based on the concept of evidence-based medicine. While *A. altissima* and other plants of the Simaroubaceae have been investigated for their efficacy as anti-malarials [12], *A. excelsa*, which is used as anti-pyretic [1], was never explored at this regard. The present work was undertaken to evaluate *in vitro* the anti-plasmodial activity of extracts from *A. excelsa* and to investigate the mechanism of action. At this aim the extracts were assayed on chloroquine-sensitive and resistant strains of *P. falciparum* at the intra-erythrocytic stage. The survival of malaria parasites in the host cells relies on the degradation of haemoglobin in the food vacuole with the involvement of several proteinases, including plasmepsins (PLM), a family of aspartyl proteinases specific to *Pf*. The inhibition of PLM leads to the starvation of the parasite [13], then it represents a feasible mechanism of action for the anti-plasmodial effect. In addition, once haemoglobin is degraded, the parasite detoxifies the excess of released haem by converting it into the crystalline pigment haemozoin [14]. A synthetic compound, called  $\beta$ -haematin spectroscopically identical to the native pigment can be obtained *in vitro*. Quinoline anti-malarials have been shown to inhibit  $\beta$ -haematin formation [15]. Then we evaluated the ability of *A. excelsa* to inhibit PLM II and IV and the formation of  $\beta$ -haematin *in vitro*.

## 2. Experimental

### 2.1. Plant material

*A. excelsa* Roxb (Simaroubaceae), stem bark collected from Zoo garden, Giza, Egypt, in April 2002, was identified by Prof. Dr. Kamal El Batany, Professor of Taxonomy and Botany, Faculty of Science, Cairo University. A voucher was deposited in National Research Centre Herbarium.

### 2.2. Extraction

Dried powdered stem bark (1 kg) was Soxhlet-extracted with petroleum ether, Et<sub>2</sub>O, CHCl<sub>3</sub>, and MeOH (70%) and concentrated *in vacuo*. MeOH extract (67.5 g) was added with 500 ml of distilled water and extracted with CHCl<sub>3</sub>. The CHCl<sub>3</sub> solution was shaken with 10% HCl. The acid layer was made alkaline with NH<sub>4</sub>OH to pH 9, then it was extracted with CHCl<sub>3</sub>. The CHCl<sub>3</sub> layer dried was evaporated giving 1.7 g of crude alkaloid chromatographed over silica gel column using CHCl<sub>3</sub> and increasing polarity with MeOH. Elution with chloroform gave canthin-6-one identified by comparison with the reported spectral data [16,17].

### 2.3. Drug susceptibility assay on *P. falciparum*

The CQ-sensitive (D10) and the CQ-resistant (W2) strains of *P. falciparum* were sustained *in vitro* as described by Trager and Jensen [18]. Parasites were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (Gibco BRL, NaHCO<sub>3</sub> 24 mM) medium with the addition of 10% heat-inactivated A-positive human plasma, 20 mM Hepes (Euroclone), 2 mM glutamine (Euroclone). Compounds were dissolved in either water (chloroquine) or DMSO and then diluted with the medium to achieve the required concentrations (final DMSO concentration <1%). Asynchronous cultures with parasitemia of 1–1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD<sub>650</sub>) by measuring the activity of the parasite lactate dehydrogenase (LDH), according to a modified version of Makler's method in control and drug-treated cultures [19]. Anti-plasmodial activity is expressed as the 50% inhibitory concentrations (IC<sub>50</sub>), each IC<sub>50</sub> value is the mean  $\pm$  SD of at least three separate experiments performed in duplicate.

### 2.4. PLMs inhibition assays

Recombinant pro-PLM II and IV were purified from BL21-(DE3) pLysS *E. coli* (Invitrogen, Milano) according to Hill J. et al. [20], with slight modifications [21]. Protein concentration was determined according to the method of

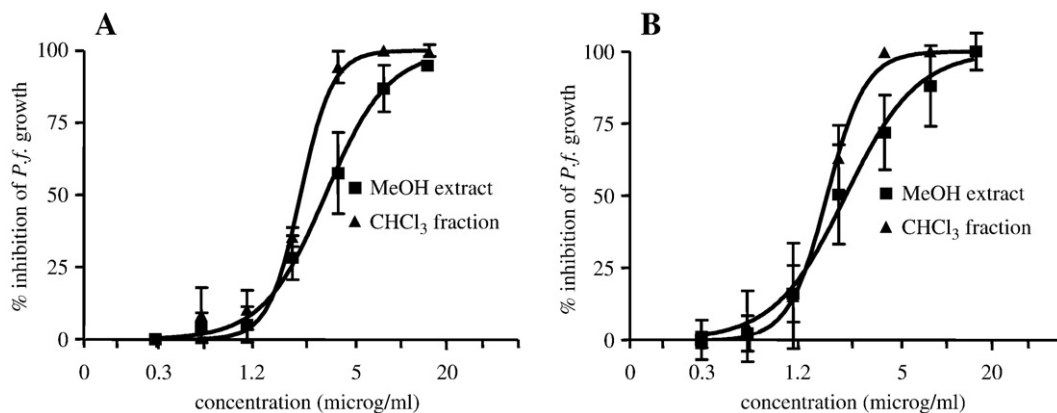


Fig. 1. Anti-plasmodial activity of MeOH and CHCl<sub>3</sub> extracts against *P. falciparum* D10 (A) and W2 (B) strains.

Bradford [22]. Proteins were diluted to the final concentration of 0.5 mg/ml in 50% glycerol and stored at  $-20^{\circ}\text{C}$ . Pro-PLM II and IV were activated by addition of one tenth volume of 100 mM sodium acetate buffer pH 4.7 by incubation at  $37^{\circ}\text{C}$  for 90 min. The enzyme activity of PLM II and IV was evaluated spectrophotometrically at 300 nm as described [20].

The CHCl<sub>3</sub> fraction from *A. excelsa* dissolved in DMSO was tested at 5–50  $\mu\text{g/ml}$  (final solvent concentration 1% of the sample volume). PS777621 [23] was used as the reference compound. The assays were conducted twice in triplicate. Inhibition curves and IC<sub>50</sub> values were calculated by a non-linear regression for sigmoidal curves using Graph Pad Prism 4.

### 2.5. $\beta$ -Haematin inhibitory assay

$\beta$ -Haematin formation was assayed by a spectrophotometric microassay BHIA ( $\beta$ -Haematin inhibitory assay) previously reported [24]. Haemin dissolved in DMSO (0.4  $\mu\text{mol/well}$ ) was distributed in 96-well microplates. CHCl<sub>3</sub> fraction dissolved in DMSO was added in doses ranging from 0.3 to 5 mg/ml. Chloroquine was used as reference compound and showed an IC<sub>50</sub> of  $0.9 \pm 0.05$  mg/ml (mean  $\pm$  SD).

### 2.6. Cytotoxicity assay

Cytotoxicity was evaluated in human fibroblasts from skin biopsies. Fibroblasts ( $8 \times 10^4/\text{well}$ ) were grown in 24 well plates with DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine as previously described [25]. Cell proliferation was followed by the MTT (3-[4,5-

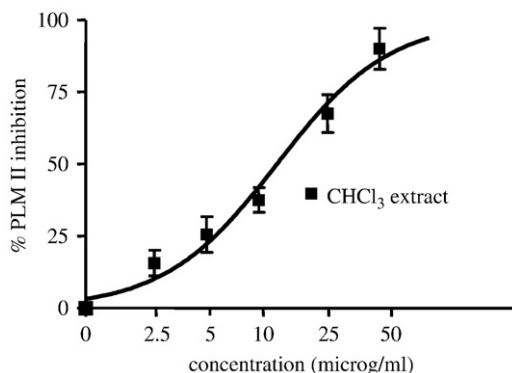


Fig. 2. Effect of the CHCl<sub>3</sub> fraction on PLM II. IC<sub>50</sub> value, expressed as the mean  $\pm$  SD of two experiments in triplicate, was  $13.4 \pm 1.7$   $\mu\text{g/ml}$ .

Table 1  
Cytotoxicity vs anti-plasmodial activity of the *A. excelsa* stem bark methanolic extract and CHCl<sub>3</sub> fraction

Material	HF	D10	W2
	LC <sub>50</sub> ±SD (µg/ml)	IC <sub>50</sub> ±SD (µg/ml)	IC <sub>50</sub> ±SD (µg/ml)
MeOH extract	116±19	4.6±1.4	2.8±0.88
CHCl <sub>3</sub> fraction	46±7.5	3.1±0.15	2.1±0.58

dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test [26], in the presence of the MeOH extract and CHCl<sub>3</sub> fraction (0–100 µg/ml) and LC<sub>50</sub> were calculated.

### 3. Results

The MeOH extract from the stem bark of *A. excelsa* inhibited parasite growth in a dose dependent manner, with IC<sub>50</sub> of 4.6 and 2.8 µg/ml on chloroquine-sensitive and -resistant strains, respectively (Fig. 1). After fractionation, the anti-plasmodial activity was retained by the CHCl<sub>3</sub> fraction (IC<sub>50</sub> of 3.1 and 2.1 µg/ml on D10 and W2, respectively). To investigate the mechanism of action, we evaluated whether the CHCl<sub>3</sub> fraction inhibited recombinant PLM II (Fig. 2), PLM IV, and the formation of β-haematin. CHCl<sub>3</sub> fraction reduced PLM II activity in a concentration dependent manner (IC<sub>50</sub> of 13.4±1.7 µg/ml, mean±SD), while was inactive against PLM IV. The CHCl<sub>3</sub> fraction inhibited also β-hematin formation dose dependently. At the highest dose tested (5 mg/ml) the inhibition was 37.7%.

As shown in Table 1, the MeOH extract and the CHCl<sub>3</sub> fraction were not devoid of cytotoxicity, but the MeOH extract was less cytotoxic than the CHCl<sub>3</sub> fraction (LC<sub>50</sub> of 116 µg/ml vs 45.5 µg/ml).

### 4. Discussion

In the present study it is reported that *A. excelsa* stem bark methanol extract is active *in vitro* against both chloroquine-sensitive and -resistant strains of *P. falciparum*, similar to *A. altissima* stem bark [10]. Our results suggest that extracts from *A. excelsa* stem bark exert anti-plasmodial activity through the inhibition of PLM II and by halting the haem detoxification process. Surprisingly, *A. excelsa* CHCl<sub>3</sub> extract was selective against PLM II, being inactive on PLM IV.

*A. excelsa* contains quassinoids [27], and β-carboline alkaloids, including canthin-6-one [28]. Canthin-6-one exhibited anti-plasmodial activity against W2 *Pf.* clone with IC<sub>50</sub> of 2.2 µg/ml, a value very close to that of the CHCl<sub>3</sub> fraction, as reported in the present work (Table 1). In a previous study, quassinoids were shown to exhibit anti-malarial effect through the inhibition of protein synthesis [9]. It cannot be excluded however that quassinoids and canthin-6-one and/or other β-carboline alkaloids act also against other targets such as the breakdown of haemoglobin and the haem detoxification to haemozoin. Either, other constituents of *A. excelsa* stem bark may act as the active principles.

Although the cytotoxicity studies revealed an anti-proliferative effect on mammalian cells by the MeOH extract, the LC<sub>50</sub> on human fibroblasts was 25 and 42 times higher than the IC<sub>50</sub> against *P. falciparum* D10 and W2 strains, respectively. Cytotoxicity increased when the crude MeOH extract was fractionated to give the CHCl<sub>3</sub> fraction, likely for the presence of higher concentrations of alkaloids which were found cytotoxic against tumor cell lines [29,30]. In conclusion *A. excelsa*, as other species of *Ailanthus*, possesses anti-plasmodial activity. The effect might be ascribable to the inhibition of haemoglobin cleavage and/or haem detoxification.

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