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Article

Antimalarial Activity of Aqueous Extracts of Nasturtium (*Tropaeolum majus* L.) and Benzyl Isothiocyanate

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Abstract: Malaria remains an important and challenging infectious disease, and novel antimalarials are required. Benzyl isothiocyanate (BITC), the main breakdown product of benzyl glucosinolate, is present in all parts of *Tropaeolum majus* L. (*T. majus*) and has antibacterial and antiparasitic activities. To our knowledge, there is no information on the effects of BITC against malaria. The present study evaluates the antimalarial activity of BITC and *T. majus* seeds, leaves, and stems aqueous extracts. We used flow cytometry to calculate the growth inhibition (GI) percentage of the extracts and BITC against unsynchronized cultures of chloroquine-susceptible *Plasmodium falciparum* (*P. falciparum*) 3D7-GFP strain. Extracts and/or compounds with at least 70% GI were validated by IC50 estimation against *P. falciparum* 3D7-GFP and Dd2 (chloroquine-resistant strain) unsynchronized cultures by flow cytometry, and the resistance index (RI) was determined. *T. majus* aqueous extracts showed some antimalarial activity higher in seeds than in leaves or stems. BITC's GI was comparable to chloroquine's. BITC's IC50 was similar in both strains, thus a cross-resistance absence with aminoquinolines was found (RI < 1). BITC presented features that could open new avenues for malaria drug discovery.

Keywords: antimalarial; *Plasmodium falciparum*.; *Tropaeolum majus* L.; Benzyl isothiocyanate

1. Introduction

Antimalarial drug development remains strongly linked to plant-based pharmaceuticals, as some of the most important therapeutics are based on their chemical scaffolds, such as aminoquinolines (e.g., chloroquine) and endoperoxides, i.e., artemisinin-based drugs [1,2]. The main causative agent of malaria, *Plasmodium falciparum* (*P. falciparum*), has developed resistance to all antimalarial drugs in clinical use, including aminoquinolines and endoperoxides [3–6]. Furthermore, the development of these antimalarials involves unaffordable environmental and economic costs for most malaria-endemic countries, hence the WHO's encouragement of applying natural extracts or plant-based pharmaceuticals based on traditional medicines [7,8]. Therefore, we propose a strategy that aims to repurpose traditional medicinal plants for antimalarial applications.

Tropaeolum majus L. (*T. majus*), an herbaceous plant commonly known as garden nasturtium, belongs to the family *Tropaeolaceae* and it is native to Peru [9,10] It was first introduced in Europe in

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the sixteenth century and then spread to other parts of the world, including malaria-endemic countries, such as Angola, Rwanda, and Vietnam [9–11]. *T. majus* was selected as a potential candidate for antimalarial drug development not only because of its widespread distribution, but also because of its traditional usages against bacterial infections, such as bronchitis, sinusitis, and urinary tract infections, as well as for its antifungal and antiviral activities [12–14].

The broad therapeutic spectrum of *T. majus* can be linked to a group of compounds known as glucosinolates [15–18]. Benzyl glucosinolate is a metabolite found in every part of the *T. majus* plant, especially in the seeds [15–19]. When hydrolyzed by the endogenous enzyme myrosinase (Thioglucoside hydrolase, EC 3.2.3.1), it generates a variety of breakdown products, including benzyl isothiocyanate (BITC) [16,17,19], which has been reported to have antimicrobial [20–23], larvicidal [24], anthelmintic [25], and anticancer [26] activities.

To our knowledge, there is no information on *T. majus* and BITC usages as antimalarials. However, a recent study by Hashimoto et al. (2023) demonstrated the *in vivo* antimalarial activity of another isothiocyanate, allyl isothiocyanate (AITC), and its metabolite, N-acetyl-S-(N-allyl thiocarbamoyl)-l-cysteine (NAC-AITC), by *in vitro* and *in vivo* assays, both extracted from *Wasabia japonica* [27]. Arianie et al. (2021) designed novel isothiocyanates based on eugenol and cinnamaldehyde derivatives and rhamnosyloxy benzyl isothiocyanate from *Moringa oleifera* leaves, used in traditional medicine to treat malaria, by molecular docking and demonstrated their potential as antimalarials through *in silico* approaches [28,29].

Hence, this study aimed to evaluate the antimalarial activity by flow cytometry of *T. majus* seeds, leaves, and stems aqueous extracts, traditionally applied as antimicrobials, and BITC, the major biologically active compound derived from the *T. majus* parts. This assessment prompted a subsequent investigation of BITC's cross-resistance with aminoquinolines, also using flow cytometry, and BITC did not demonstrate the same resistance mechanism as this antimalarial drug class.

2. Results

2.1. Antimalarial Screening Assessment

Flow cytometry was first used to evaluate the aqueous extracts and BITC for antimalarial activity against the asexual blood stage GFP-expressing *P. falciparum* (3D7-GFP), a chloroquine-sensitive strain. Using the 3D7-GFP strain obviates any staining procedure with a fluorescent dye since the parasites are auto-fluorescent, simplifying culture procedures [30,31]. The number of fluorescent events after drug exposure detected by flow cytometry, i.e., the percentage of surviving GFP parasites, allows the determination of the growth inhibition percentage, as described in Teixeira de Morais Gomes et al. (2020). The results were obtained from at least two experiments, each in triplicate, and are presented in Table 1.

Table 1. *P. falciparum* antimalarial screening of *T. majus* extracts, BITC, and growth controls. In bold are the compounds that displayed more than 70% growth inhibition.

Extracts, Compounds, and Growth Controls	Concentrations	*P. falciparum* Inhibition % ± SD
T. majus seed extract	132 µg/ml	38.62 ± 22.89*
	13.2 µg/ml	30.18 ± 13.47#
T. majus leaf extract	2510 µg/ml	6.54 ± 5.32*
	251 µg/ml	3.44 ± 2.67##
T. majus stem extract	1320 µg/ml	7.68 ± 3.15*
	132 µg/ml	NI [4]
Water (extracts solvent)	1%	NI [4]
	0.1%	NI [4]
BITC [1]	**0.50 µg/ml**	**97.13 ± 0.62Δ**
	0.050 µg/ml	14.26 ± 3.31#γ
DMSO [2] (BITC solvent)	0.4%	50.64 ± 3.61
	0.04%	4.89 ± 3.91

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CQ [3] (reference drug)	**5.17 µg/ml**	**96.57 ± 0.57**
	0.517 µg/ml	**94.71 ± 2.78**

[1] BITC, benzyl isothiocyanate; [2] DMSO, dimethyl sulfoxide; [3] CQ, chloroquine; [4] NI, no inhibition observed; SD, standard deviation. Statistical analysis (Mann-Whitney and unpaired t-test) of results: CQ at 5.17 µg/ml (*p<0.05); CQ at 0.517 µg/ml (#p<0.05; ##p<0.0001); DMSO at 0.4% ($^{\Delta}$ p<0.0001) and DMSO at 0.04% ($^{\gamma}$p<0.05). .

T. majus seed extract displayed a similar growth inhibition percentage for the tested concentrations (38.62 ± 22.89% at 132 µg/ml and 30.18 ± 13.47% at 13.2 µg/ml) and a higher growth inhibition percentage than the remaining extracts in all concentrations. The extract solvent had no antiplasmodial action at any concentration. Despite the better performance of the *T. majus* seed extract, none of the extracts presented more than 70% growth inhibition.

Benzyl isothiocyanate (BITC) at 0.50 µg/ml demonstrated a growth inhibition percentage above 70% (97.13 ± 0.62%) and was considered for antiplasmodial activity refinement. BITC solvent (DMSO) did not show a meaningful growth inhibition percentage in both concentrations (0.4% and 0.04%) and did not influence the inhibitory effect of BITC.

The reference antimalarial drug chloroquine (CQ) at 5.17 µg/ml and at 0.517 µg/ml exhibited growth inhibition percentages of 96.57 ± 0.57% and 94.71 ± 2.78%, respectively. BITC at the highest concentration (0.50 µg/ml) and CQ at a similar concentration (0.517 µg/ml) and ten times more concentrated (5.17 µg/ml) had comparable growth inhibitions against *P. falciparum*.

2.2. Dose-Response Evaluation

Since BITC was considered for further analysis, dose-response curves to determine the half-maximal inhibitory concentrations (IC50) against *P. falciparum* strains 3D7-GFP and Dd2, a chloroquine-resistant strain, were also made based on the assessment of the parasite growth by flow cytometry. The results are demonstrated in Figure 1 and were obtained from at least two experiments, each in triplicate.

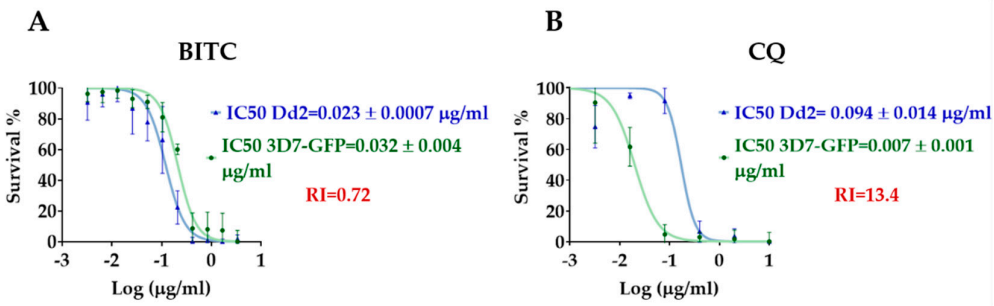

Figure 1. BITC biological antimalarial activity against resistant (Dd2) and susceptible (3D7-GFP) *P. falciparum* strains. (**A**) Dose-response curves of BITC with the respective IC50 values and resistance index (RI=IC50 Dd2/IC50 3D7-GFP); (**B**) Dose-response curves of the reference drug CQ with the respective IC50 values and resistance index. The green curves correspond to the susceptible strain (3D7-GFP), while the blue curves represent the resistant strain (Dd2). BITC, benzyl isothiocyanate; CQ, chloroquine; RI, resistance index.

Both BITC and the reference drug CQ displayed sigmoidal dose-response curves compatible with biological activity (Figure 1). The IC50 values and the resistance index calculated for CQ were consistent with previous results in similar laboratory conditions [32–34], hence validating the assay conditions. The resistance index provides a quantitative measure of activity against a resistant strain in comparison to a susceptible strain (RI=IC50 Dd2/IC50 3D7-GFP) [35,36].

The biological activity of BITC was similar against both susceptible and resistant-strains (p>0.05; Unpaired t-test), hence the absence of a right shift of the *P. falciparum* Dd2 dose-response curve (Figure 1) and a low resistance index (RI=0.72). As expected, the reference drug CQ, displayed lower biological activity against the resistant-strain, which was significantly different (p<0.05; Unpaired t-

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test), thus the right shift of the *P. falciparum* Dd2 dose-response curve (Figure 1), i.e., an increase of the IC50 value, and a higher resistance index (RI=13.4).

3. Discussion

When it comes to *T. majus* aqueous extracts, the differences in the growth inhibition percentages between the seed extract and the others can be attributable to a variety of factors. *T. majus* seeds contain a higher BITC content than the leaves or stems, and while benzyl glucosinolate is soluble in water, BITC, the active metabolite, is poorly soluble, volatile, and non-stable [37], which can account for the differences in *P. falciparum* growth inhibition.

The dose-response evaluation and the RI of BITC revealed that its phenotypic response was different than the reference drug CQ, i.e., if a compound exhibits a resistance mechanism similar to that of other antimalarials [35,36]. Since CQ is an aminoquinoline, our results suggest that BITC does not share the same resistance mechanism as aminoquinolines, which can be considered an advantage for antimalarial drug development.

The mechanism of action of CQ is linked to its diffusion through biological membranes and concentration inside the parasite's the food vacuole, which has an acidic pH (in contrast with the neutral pH of the cytosol) [38,39]. Resistance to aminoquinolines is related to mutations in various proteins, including the transporters *P. falciparum* chloroquine resistance transporter (*Pf*CRT) and the *P. falciparum* multidrug resistance 1 protein (*Pf*MDR1) [40,41]. Since *P. falciparum* 3D7-GFP is a CQ-sensitive strain and *P. falciparum* Dd2 a CQ-resistant strain and considering that the IC50 of BITC for both strains was identical, this strongly suggests that BITC does not have the same mechanism of action and resistance as CQ.

Isothiocyanates have been shown to interact mainly with thiol groups, forming labile dithiocarbamate derivatives, and with amine groups, which may result in increased oxidation, i.e., production of reactive oxygen species (ROS), and inhibition of key enzymes and/or proteins in microorganisms [42–44].

The electrophilic properties of BITC can render a high affinity for cellular sulfhydryl groups, such as enzymes and/or proteins with functional or structural cysteine residues [22,42]. One of *P. falciparum* most important enzymes involved in the redox equilibrium is glutathione reductase (GR), which has cysteine residues [45–47].GR is an antioxidant enzyme that catalyzes the regeneration of reduced glutathione (GSH), the active form of glutathione, from oxidized glutathione using NADPH as the source of reducing equivalents [46,47]. This reaction helps to avoid the synthesis of hydroxyl radical •OH from H_2O_2 produced due to hemoglobin digestion (inside the parasite's food vacuole) and mitochondrial electron chain reactions [45,47]. A study conducted by Li et al. (2020) characterized BITC as a potential GR inhibitor in human cancer cells and demonstrated that BITC was evaluated as a competitive and irreversible GR inhibitor in a time- and concentration-dependent mode and this reaction depended on the presence of NADPH [48].

Also, BITC might interfere with the GSH *de novo* synthesis *in P. falciparum*. It is known that in *P. falciparum*, GSH can be *de novo* biosynthesized by two enzymes, glutamylcysteine synthetase and glutathione synthetase, respectively, that require a source of amino acid precursors from the inactive form of glutathione (glutamate, cysteine, and glycine) [45,46]. In biological systems, a reaction between the inactive form of glutathione and BITC, catalyzed by glutathione-S-transferases, allows the formation of a dithiocarbamate derivative, which is then effluxed from the cell [42]. This reaction may increase the interference of BITC with *P. falciparum* redox equilibrium.

T. majus is characterized as a low toxicity plant, with a LD50 above 5000 mg/kg in a *in vivo* mice acute toxicity study of oral administration by Zanetti et al. (2003). The absence of toxicity signs in oral administration, up to 2 weeks, can be due to the low concentrations of glucosinolates presented in the extracts or due to their metabolization in the organism [49]. For the compound BITC the results of *in vivo* mice toxicology experiments [26] showed that animals had no evidence of major drug induced toxicity at doses up to 100 mg/kg, being the LD50 of 140 mg/kg.

The complexation of BITC from cyclodextrins performed by Li et al. (2015) improved the stability and the aqueous solubility of this compound [37]. Hence, the antimalarial activity of the aqueous

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crude extracts could be improved by the enhancement of the hydrolysis of benzyl glucosinolate and solubility of BITC in water, in particular the seed extracts.

4. Materials and Methods

4.1. Plant Material

Tropaeolum majus L. plant material was collected in November of 2022 in a cultivated field at Parque Bensaúde, Lisbon. Stems, leaves, and seeds were cleansed of residues, and the stems and leaves were also cut into small pieces. Stems, leaves, and seeds were weighted separately and kept at -20◦C.

4.2. Tropaeolum majus L. Extraction

T. majus seeds (6.37 g *dw*), leaves (18.74g *dw*), and stems (20 g *dw),* previously lyophilized for 72h, were powdered with a mill, and macerated in 50 ml phosphate buffer (pH 7.4) for 12h with occasional stirring, to allow endogenous myrosinase to promote glucosinolate degradation and filtered obtaining aqueous extracts.

Afterward, the aqueous extracts were filtrated and lyophilized for 96h, in previously tared volumetric flasks, obtaining the following 52.8 mg seed extract, 1.002 mg of leaves extract, and 527.2 mg of stem extract. Dry extracts were then dissolved in 4 ml of distilled water, obtaining the following final concentrations for the antimalarial assays: 13.2 mg/ml seed extract; 250.6 mg/ml leaves extract; 131.8 mg/ml stem extract.

4.3. Antimalarial Assays

4.3.1. *P. falciparum* In Vitro Culture

Laboratory-adapted *P. falciparum* lines 3D7-GFP (MRA-1029, MR4, ATCC® Manassas Virginia), a chloroquine-sensitive strain, and Dd2 (cryopreserved collection from IHMT), a chloroquine-resistant strain, were continuously cultured using a modified method of Trager and Jensen [50,51]. Parasites were cultivated in 5% hematocrit, 37°C, and an atmosphere with 5% of CO_2 and supplemented with complete culture medium (cRPMI), as previously described [51].

4.3.2. Sample Preparation

The stock solutions were made in compliance with the maximum solvent limits that can be used in antimalarial assays [52]. Keeping this in mind, a stock solution of BITC (Sigma-Aldrich®) with 112.5 µg/ml (754 µM) containing 90% dimethyl sulfoxide (DMSO; Sigma-Aldrich®) was diluted in sterile PBS (VWR™) to achieve a DMSO percentage in the assays ≤ 0.4%. The aqueous extracts were diluted in cRPMI to attain a water percentage ≤ 1% in the assays and previously filtrated with a 0.45-micron filter. The stock solution of the reference drug chloroquine (Sigma-Aldrich®) with 2584,3 µg/ml (5 mM) containing 100% of DMSO was diluted in cRPMI to also achieve a DMSO percentage in the assays ≤0.4%. The extract solvent (water) previously filtrated with a 0.45-micron filter, and BITC solvent (DMSO) were also diluted in cRPMI or sterile PBS, respectively, following the respective percentages used in the assays.

4.3.3. Extracts and Compounds Screening Assessment

All extracts and compounds were screened for their *in vitro* antimalarial activity against *P. falciparum* 3D7-GFP in at least two independent experiments in triplicate, as previously described with modifications [53]. In brief, unsynchronized culture with 2% hematocrit and 1% parasitemia was incubated in a 96-well flat-bottom plate with the following concentrations for 72h (37 °C and 5% CO2):

- *T. majus* seed extract: 132 µg/ml (1% water) and 13.2 µg/ml (0.1% water);
- *T. majus* leaf extract: 2510 µg/ml (1% water) and 251 µg/ml (0.1% water);
- *T. majus* stem extract: 1320 µg/ml (1% water) and 132 µg/ml (0.1% water);

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- BITC: 0.50 μg/ml (3.32 μM; 0.4% DMSO) and 0.050 μg/ml (0.332 μM; 0.04% DMSO).

Each plate also included growth control wells: untreated culture, 1%, and 0.1% water, 0.4% and 0.04% DMSO, 5.17 μg/ml (10 μM), and 0.517 μg/ml (1 μM) of chloroquine (reference drug). After the incubation period, cells were diluted to achieve a 0,7% hematocrit and the parasite growth was assessed by flow cytometry (Beckman Coulter, Cytoflex) with a 96-well plate reader, using Fl-1 (green fluorescent protein [GFP]; excitation wavelength, 488 nm). Typically, 100.000 RBCs were counted for each well. Samples were analyzed using FlowJo software (Tree Star Inc.). The growth inhibition percentage was then determined by the following formula:

$$\text{Growth inhibition (\%)} = 100 - \left(\frac{Parasitemia\ treated\ culture}{\bar{X} Parasitemia\ untreated\ culture} \times 100\right), \quad (1)$$

The extracts and/or compounds that displayed at least 70% of growth inhibition were selected as potential candidates and confirmed by IC50 estimation and resistance index determination [53].

4.3.4. Dose-Response Evaluation

The antimalarial activity was estimated by previously described protocols with adjustments in at least two experiments, each in triplicate [53,54]. In short, unsynchronized cultures with 2% hematocrit and 1% parasitemia of *P. falciparum* 3D7-GFP and Dd2 strains were incubated for 72h (37 °C and 5% CO_2) in a 96-well flat-bottom plate with BITC in 2-fold serial dilutions ranging from 0.50 μg/ml to 0.0005 μg/ml (3.32 μM to 0.0032 μM). Additionally, each plate included growth control wells with no drug added and chloroquine as a reference drug in a 5-fold serial dilution with concentrations ranging from 5.17 μg/ml to 0.0003 μg/ml (10 μM to 0.00064 μM). After 72 hours, cells were diluted to achieve a 0,7% hematocrit and the parasite growth was assessed by flow cytometry (Beckman Coulter, Cytoflex) in a 96-well plate reader, using FI-1 (green; excitation wavelength, 488 nm). Before the flow cytometry reading, *P. falciparum* Dd2 strain was stained with a mixture of SYBR™ Green I (Invitrogen, Thermo Fisher Scientific) 0.5X in PBS 30 minutes in the dark at standard culture conditions. Typically, 100.000 RBCs were counted for each well. Samples were analyzed using FlowJo software (Tree Star Inc.). The IC50 was estimated through a nonlinear regression by using the GraphPad Prism 9 software (trial version) and the resistance index calculated by the following formula:

$$\text{Resistance index} = \frac{IC50\ Dd2}{IC50\ 3D7-GFP}, \quad (2)$$

A resistance index above 10 predicts a high level of resistance, whereas a resistance index below 10 might indicate an intermediate resistance level and a resistance index close to or below 1 could reveal an absence of resistance [35].

4.3.5. Statistical Analysis

GraphPad Prism 9 software (trial version) was used for the non-parametric Mann-Whitney test and parametric Unpaired t-test. A significant difference was assumed when $p<0.05$.

5. Conclusions

BITC similar activity against both chloroquine -susceptible and resistant *Plasmodium falciparum* strains suggests a different mechanism of action. Hence, BITC and T*ropaeolum majus* L. extracts have a good potential to develop new antimalarial medicines.

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for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Sample Availability: Samples of the compounds are available from Ana Pintão (apintao@egasmoniz.edu.pt).

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