Microbial transformation of (+)-heraclenin by *Aspergillus niger* and evaluation of its antiplasmodial and antimicrobial activities

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Microbial transformation of (+)-heraclenin (1) by *Aspergillus niger* was studied in growth media to assess its antiplasmodial and antimicrobial activities. It was transformed to (–)-heraclenol (2) as the sole product in a stereospecific manner. The *in vitro* antiplasmodial activity of compounds 1 and 2 was tested with chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Further, the *in vitro* antibacterial activity of 1 and 2 against three Gram-positive bacteria, *Bacillus subtilis*, *Bacillus sphaericus* and *Staphylococcus aureus*, and three Gram-negative bacteria, *Pseudomonas aeruginosa*, *Escherichia coli* and *Chromobacterium violaceum* was analysed using agar-plate diffusion assay. The same method was employed for the evaluation of antifungal activity against five pathogenic strains of fungi, *A. niger*, *Rhizopus oryzae*, *Aspergillus flavus*, *Candida albicans* and *Saccharomyces cerevisiae*. Both furanocoumarins 1 and 2 displayed significant levels of antiplasmodial and moderate levels of antimicrobial activities against the tested pathogenic strains. Compound 2 exhibited two-fold less potent antiplasmodial activity (IC_{50} = 6.0 μg/ml) than the parent compound 1 (IC_{50} = 2.5 μg/ml), whereas no difference was observed in the antimicrobial activity of both furanocoumarins. The oxirane ring was found to be beneficial in terms of antiplasmodial activity.

Keywords: *Aspergillus niger*, antiplasmodial and antimicrobial activity, furanocoumarin, microbial transformation.

COUMARINS occur frequently in nature, and like flavonoids, which are also extensively represented in plants, are available as a part of everyday diet. Coumarins and their derivatives are reported to display numerous biological activities.

(+)-Heraclenin (**1**, Scheme 1) a naturally occurring furanocoumarin epoxide, isolated from fruits of *Aegle marmelos* Correa, is known to exhibit a broad spectrum of biological activities. It possesses cytotoxic[1], anti-platelet[2], anti-coagulant[3], anti-inflammatory[4,5] as well as mild phototoxic and photomutagenic activities[6]. It has been shown to significantly induce apoptosis in Jurkat leukaemia cells[7].

Epoxide hydrolases (EH) are ubiquitous enzymes – nature's catalysts, which are chemo[8], stereo[9] and regio-specific[10] for their substrates. EH from microorganisms have gained importance in microbial transformations. The first example of microbial EH found in the literature describes a *Flavobacterium*[11] and *Pseudomonas*[12] species involved in the conversion of 2,3-epoxysuccinate into tartaric acid, and dibromopropanol into glycerol. Since then, more than 100 EH have been identified or predicted from microbial sources such as bacteria and fungi. Recent reports on enantioselective EH from microorganisms[13,14] have stimulated interest in the potential use of these enzymes as tools for the resolution of racemic epoxides of interest. Also, interest in these enzymes is further fuelled by the fact that EH are co-factor independent, more abundant and accessible than their much-studied EH isolated from mammalian liver[15]. There is little information available on the biotransformation of a single enantiomeric epoxide to diol.

More recently, microbial metabolism is another exciting field of application of these microorganisms to natural products chemistry for structure activity relationship (SAR) studies. The strains of these organisms are capable of modifying molecular architecture unlike chemical methods to produce new secondary metabolites or analogues, which have been reported to yield more efficient drugs[16].

EH of fungus, *Aspergillus niger* (microtonal EH), is one of the most important microorganisms used in biotechnology for many decades to produce extracellular enzymes and citric acid. Chemical reactions performed by microorganisms have been used as modern tools in chemistry. The main objective of the present study is to investigate the ability of *A. niger* to modify the molecular architecture of furanocoumarin. Further, their *in vitro* antiplasmodial activity against chloroquine (CQ)-sensitive and CQ-resistant parasite strains of *Plasmodium falciparum* besides *in vitro* antibacterial activity against a panel of susceptible and resistant Gram-positive and Gram-negative organisms of **1** and **2** were evaluated in addition to *in vitro* antifungal activity.

A. niger (NCIM 620), *Aspergillus flavus* (NCIM 557) and *Gliocladium roseum* (NCIM 1037) were collected from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. *Aspergillus foetidus* (MTCC 508), *Beauveria bassiana* (MTCC 984), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 741), *Escherichia coli* (MTCC 1687), *Bacillus sphaericus* (MTCC 511), *Candida albicans* (MTCC 227), *Saccharomyces cerevisiae* (MTCC 170)

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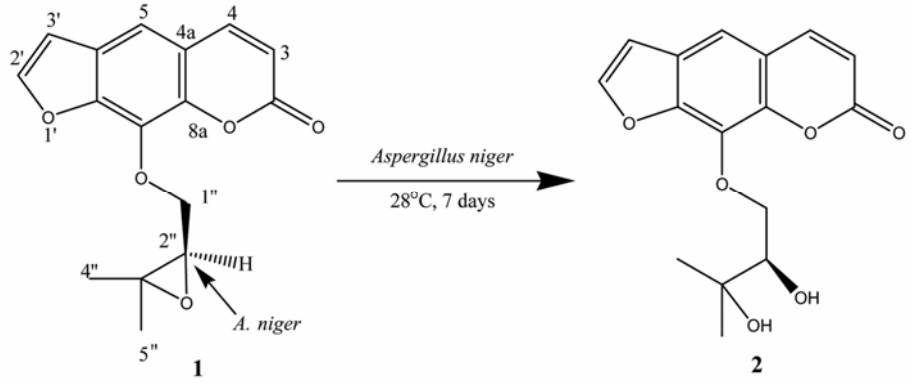

Scheme 1. Furanocoumarins (+)-heraclenin (**1**) and (–)-heraclenol (**2**).

and *Chromobacterium violaceum* (MTCC 2656) were obtained from the Institute of Microbial Technology, Chandigarah and NCIM. The bacteria were maintained on nutrient agar and fungi on potato dextrose agar (PDA) slants at 4°C. PDA, potato dextrose broth, Czapek-Dox agar and Czapek-Dox broth were obtained from Hi Media Laboratories, Mumbai.

For the microbial transformation of **1** by *A. niger*, 300 ml of Czapek-Dox medium inoculated with *A. niger* in a 500 ml Erlenmeyer flask was incubated, shaking (150 rpm) at 28°C. After 3 days of growth, heraclenin (180 mg) in acetone (2 ml) was added to the culture medium and the organism was cultivated for 7 days. The cultures were harvested and the broth and mycelia were separated using coarse filter paper in a Buchner funnel. The mycelia were washed with water and discarded. The culture broth was extracted with three equal volumes of ethyl acetate (EtOAc) and purified over a column of silica gel.

Thin layer chromatography (TLC) was performed on precoated silica gel GF_{254} plates (Merck) in EtOAc : hexane (40 : 60). The TLC plates were visualized under UV light or by spraying with 10% H_2SO_4 in methanol and heating at 110°C. The metabolites were subjected to liquid chromatography over a column of silica gel (60–120 mesh, Acme) using EtOAc/hexane solvent system in a gradient mode. Eluting from 10% to 30% EtOAc in hexane and collecting 25 ml fractions, identical fractions were pooled. The melting points were determined with Buchi apparatus.

The ^{1}H NMR spectra were recorded on Varian VXR Unity 200 MHz, and Bruker Avance 300 MHz, and the ^{13}C NMR recorded on Bruker Avance 75 MHz respectively. The chemical shifts were reported in parts per million, and *J* values in hertz. The IR spectra were recorded using a Perkin Elmer FTIR on a sodium chloride crystal plate. The electron spray ionization mass spectrum (ESI-MS) was recorded on LC-MSD-Trap-SL ion trap detector, Agilent-1100 series. The optical rotation was determined with a Horiba SFPA-200 high-sensitive polarimeter, a 5 ml sample tube length of 10 mm (λ = 589 nm).

Antiplasmodial activity of the compounds **1** and **2** was determined in CQ-sensitive and CQ-resistant parasite strains of *Plasmodium falciparum* using standard method[17]. The CQ-sensitive and CQ-resistant isolates were collected from patients reported with symptomatic malaria. They were adapted and maintained *in vitro* by using the candle-jar technique[18]. Parasites were cultured in human O^+ RBC in RPMI 1640 media enriched with 10% (v/v) AB^+ serum and supplemented with 25 mM HEPES buffer and 25 mM sodium bicarbonate. The assay was done in synchronous culture with ring form at 5% haematocrit containing 1% parasitaemia in 96-well flat-bottom tissue-culture plate. Compounds were dosed in wells in duplicate at concentrations of 125, 25, 5, 1, 0.2, 0.04 and 0.008 μg/well. The volume of culture per well was kept 200 μl, including media, drug and parasite inoculum. Parasite culture only in enriched media was taken as control. CQ was used as the reference antimalarial for comparison. To determine the activity of various compounds in total parasite growth, the assay was done for 48 h. Growth of the parasite from each well was monitored microscopically in JSB-stained smears by counting the total number of parasites per 5000 RBCs. Percentage of parasite growth inhibition was calculated using the formula: $(1 - Nt/Nc) \times 100$, where *Nt* and *Nc* represent the number of parasites in the test and control wells respectively. Inhibitory concentrations at 50% (IC_{50}) and 90% (IC_{90}) were calculated.

Agar cup bioassay was employed for testing antibacterial activity of the compounds following the standard protocol[19]. Commercially available nutrient agar medium (23 g) was suspended in distilled water (1000 ml) and heated to boil until it dissolved completely. The medium and the petri dishes were autoclaved at a pressure of 6.803 Pa for 20 min. Stock solutions were prepared by dissolving the compound in DMSO to obtain different concentrations (30 μg/ml and 100 μg/ml). The medium was poured into petri dishes under aseptic conditions in a laminar flow chamber. When the medium in the plates solidified, 0.5 ml of 24-h-old culture of test organism was inoculated. After inoculation, the cups were scooped out

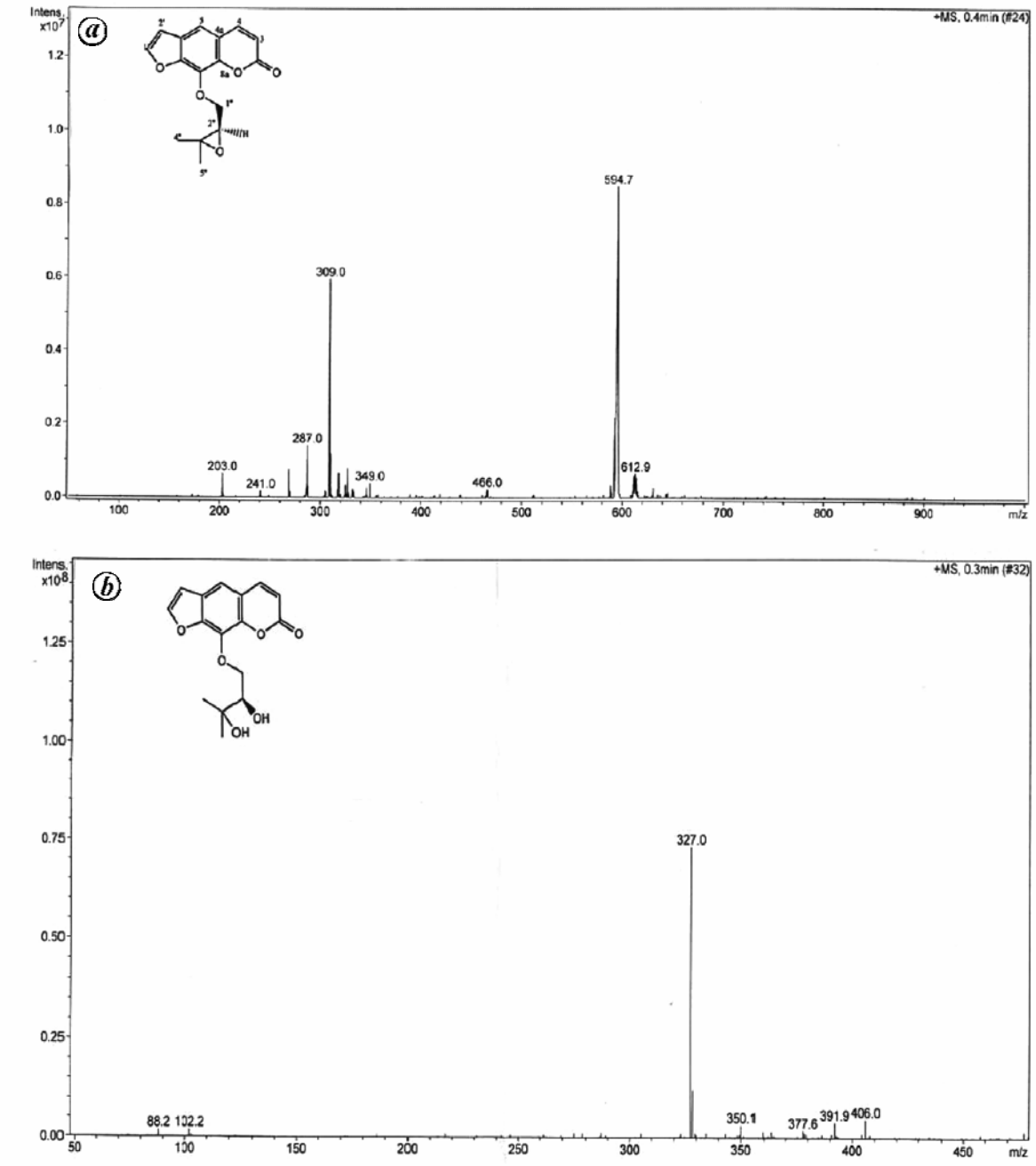

Figure 1. Mass spectrum of (***a***) (+)-heraclenin and (***b***) (–)-heraclenol.

with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup different concentrations of the test solution (30 and 100 µg/ml) were added. Controls were maintained with DMSO and penicillin G (for Gram-positive bacteria) and streptomycin (for Gram-negative bacteria). The treated samples and the controls were kept in an incubator at 37°C for 24–48 h. Inhibition zones were measured and diameter was calculated. Three replicates were maintained for each treatment.

The same agar cup method was adapted for antifungal assay, wherein the medium was PDA (39 g/l) and clotrimazole served as the positive control. Also, the treated samples and the controls were kept at room temperature for 48 h. The inhibition zones were measured and diameter was calculated.

A. niger efficiently transformed **1** (180 mg) into **2** (142 mg, 68% yield) as the sole product. This involves trans opening of the oxirane ring followed by the attack of water molecule from the less substituted oxirane carbon atom, and the rest of the molecule is unaltered by the fungus. The metabolite **2** was obtained as a colourless amorphous powder, m.p. 106–108°C, $[\alpha]_D^{27}$ –20° (c = 1.0,

Table 1. Comparison of ^{1}H NMR spectral data of compounds **1** and **2** with those of (+)-heraclenin and (–)-heraclenol

Proton no.	Compound **1** (δ), $CDCl_3$ 300 MHz	(+)-Heraclenin[20] (δ), $CDCl_3$	Compound **2** (δ), $CDCl_3$ 300 MHz	(–)-Heraclenol[20] (δ), $CDCl_3$
3	6.33 (1H, d, J = 9.8 Hz)	6.30 (1H, d, J = 9.0 Hz)	6.30 (1H, d, J = 10.1 Hz)	6.36 (1H, d, J = 9.3 Hz)
4	7.72 (1H, d, J = 9.8 Hz)	7.77 (1H, d, J = 9.0 Hz)	7.78 (1H, d, J = 10.1 Hz)	7.76 (1H, d, J = 9.3 Hz)
5	7.35 (1H, s)	7.40 (1H, s)	7.37 (1H, s)	7.36 (1H, s)
2′	7.68 (1H, d, J = 2.3 Hz)	7.76	7.72 (1H, d, J = 2.3 Hz)	7.72
3′	6.79 (1H, d, J = 2.3 Hz)	6.86	6.82 (1H, d, J = 2.1 Hz)	6.82
1″ a	4.49 (1H, dd, J = 11.3, 6.0 Hz)	4.56 (2H, m)	4.38 (1H, dd, J = 10.1, 8.5 Hz)	4.56 (2H, m)
1″ b	4.58 (1H, dd, J = 10.5, 5.3 Hz)		4.73 (1H, dd, J = 10.1, 2.3 Hz)	
2″	3.26 (1H, t, J = 5.3 Hz)	3.20 (1H, m)	3.86 (1H, dd, J = 8.5, 2.3 Hz)	3.90 (1H, m)
Me-4″	1.29 (3H, s)	1.30 (3H, s)	1.28 (3H, s)	1.30 (3H, s)
Me-5″	1.35 (3H, s),	1.36 (3H, s)	1.31 (3H, s)	1.32 (3H, s)

MeOH) and exhibited a broad band at 3465 cm^{-1}, indicating the presence of hydroxyl group in its IR spectrum. The ESI-MS data of **1** included molecular ion at *m*/*z* 309 [M^+ + Na], 594.7 [$2M^+$ + Na] (Figure 1 *a*) and compound **2** showed molecular ion at *m*/*z* 327 [M^+ + Na] (Figure 1 *b*). The ^{1}H NMR spectral data (Table 1) of metabolites **1** and **2** are compared with those of heraclenin[20] and heraclenol[20]. The physical and spectral data of **1** and **2** agreed well with those of reported data proving their identity[20].

Compound **1** and microbial transformed product **2** were evaluated for their *in vitro* antiplasmodial and antimicrobial assays. The results of preliminary screening tests are summarized in Figure 2 and Tables 2 and 3. Product **2** displayed lower antiplasmodial activity with IC_{50} = 6.0 μg/ml and IC_{90} = 29.0 μg/ml, compared to that of the parent compound **1** with IC_{50} = 2.85 μg/ml and IC_{90} = 31.0 μg/ml, against CQ-sensitive strains. Product **2** also exhibited IC_{50} = 6.0 μg/ml and IC_{50} = 25.5 μg/ml compared to that of parent compound **1** with IC_{90} = 2.5 μg/ml, and IC_{90} = 20.5 μg/ml against CQ-resistant parasite strains. Thus the microbial transformed product underwent oxirane ring cleavage followed by hydrolysis by *A. niger* and exhibited two-fold less potent antiplasmodial activity than the parent compound.

Based on the results of the disc diffusion assay, the antibacterial activity of compounds **1** and **2** was evaluated to determine the inhibitory zones for each of them. The results are given in Table 2. No significant difference between the antibacterial activity of **1** and **2** was observed. The former was found to be a good antibacterial agent with an inhibition zone diameter of 15 mm at 100 μg/ml test concentration against Gram-positive *B. sphaericus*. Both the compounds did not show zone of inhibition against Gram-negative *P. aeruginosa* and *Rhizopus oryza*s. In general, the compounds tested showed modest antibacterial activity compared to that of the reference compound, Penicillin-G for Gram-positive and streptomycin for Gram-negative bacteria.

The *in vitro* antifungal activity of **1** and **2** was evaluated against five pathogenic fungi, *A. niger*, *R. oryzae*, *A. flavus*, *C. albicans* and *S. cerevisiae*, which are often encountered clinically. The results are shown in Table 3. Compounds **1** and **2** were inactive against *R. oryzae*. The inhibition zones of the parent compound against *A. niger* and *A. flavus* at 150 μg/ml test concentration were 9 mm, whereas the metabolized product showed 8 mm under the similar condition. Compound **1** exhibited inhibitory zones of 10 and 11 mm against *C. albicans* and *S. cerevisiae* respectively, at 150 μg/ml test concentration whereas **2** showed 9 and 10 mm respectively to the reference compound clotrimazole. It is noteworthy that the parent compound and biotransformed product showed moderate levels of activity against the tested fungi.

Microorganisms are one of the most efficient biocatalytic agents known for their capacity to metabolize a wide range of substrates. The observed biotransformation of **1** suggested that the oxirane ring in this substrate was accepted by the fungal enzyme, and converted **1** to **2**. In contrast, the geminal methyls are not oxidized by the fungus unlike the furanocoumarin, imperatorin, in which one of the methyls is oxidized to alcohol and further to acid[21]. This is attributed to the presence of olefin moiety in place of the oxirane ring. Thus the oxirane ring was found to be the active site in the biotransformation. *A. niger*, which possesses EH, catalyses hydrolysis of **1** to **2** in a stereospecific manner, as the epoxides are highly reactive compounds that easily undergo ring-opening reactions with a variety of nucleophiles. It has been reported that *A. niger* behaves similarly to the mammalian microsomal and cytosolic EH. It involves a trans opening, implying nucleophilic attack of water molecule in the oxirane ring. It is often used for resolution of enantiomers of racemic epoxide in which the oxirane ring opens via attack of a water molecule at the less substituted oxirane carbon atom and *R* enantiomer reacts more rapidly than its *S* antipode[13]. Thus enantiopure **1** is transformed to **2** as the sole product. The present study reveals that EH from *A. niger* not only catalyses resolution of racemic epoxides but also enantiopure epoxides to produce enantiopure vicinal diols exclusively, whereas the rest of the molecule is unaltered.

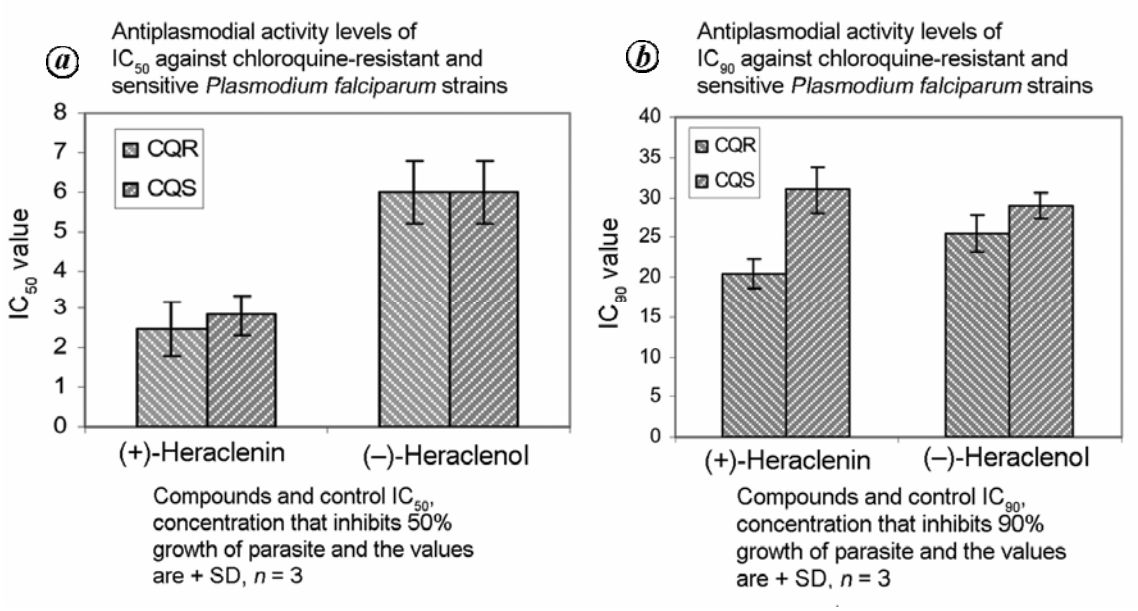

Figure 2. Antiplasmodial activity of heraclenin and heraclenol.

Table 2. Antibacterial activity levels of (+)-heraclenin and (–)-heraclenol

	Inhibitory zone diameters (mm)				
	Heraclenin (μg)		Heraclenol (μg)		
Microorganism	30	100	30	100	
Gram-positive					Control penicillin-G (30 μg)
Bacillus subtilis	7	11	6	9	16
Bacillus sphaericus	9	15	8	12	18
Staphylococcus aureus	7	10	6	8	15
Gram-negative					Streptomycin (30 μg)
Pseudomonas aeruginosa	–	–	–	–	26
Escherichia coli	8	12	6	9	28
Chromobacterium violaceum	8	11	7	10	30

Three replicates were maintained for each treatment.

Table 3. Antifungal activity levels of (+)-heraclenin and (–)-heraclenol

	Inhibitory zone diameter (mm)				
	Heraclenin (μg)		Heraclenol (μg)		
Microorganism	100	150	100	150	Control Clotrimazole (100 μg)
Aspergillus niger	7	9	6	8	26
Rhizopus oryzae	–	–	–	–	23
Aspergillus flavus	6	9	6	8	30
Candida albicans	7	10	6	9	28
Saccharomyces cerevisiae	8	11	7	10	26

Three replicates were maintained for each treatment.

The rising resistance of plasmodium species, especially *P. falciparum* to known antimalarials such as CQ makes the search for new antimalarial drugs increasingly important. The resistance of *P. falciparum* to CQ is a major health problem in many parts of the world, and the present study suggests the potential role of furanocoumarins

in the treatment of CQ-resistant *falciparum* malaria. There are no reports on the antiplasmodial and antimicrobial activities of **1** and **2**.

It was observed that **1** exhibited significant antiplasmodial activity against CQ-resistant and CQ-sensitive isolate of *P. falciparum*, which had IC_{50} values in the low micromolar range (2.5–2.8 μg/ml). This might be due to the presence of epoxide functionality that can result in the formation of adduct with nucleophiles in the biological system. However, **2** lacking oxirane ring showed moderate antiplasmodial activity. Therefore, it appears that the oxirane group is essential for antiplasmodial activity.

In general, the plant antibiotic substances appear to be more inhibitory to Gram-positive organisms than Gram-negative ones. But our results did not show the discrepancies in their antibacterial levels against the tested Gram-positive and Gram-negative bacteria.

Among these tested fungi, *C. albicans* was the most common infection-causing fungus; about 45% of clinical fungal infections were caused by it[22]. Based on our results and the framework of furanocoumarins, the structural changes in the side chain will not alter the antifungal activity. But further studies are required to confirm the relation of substituents with furanocoumarins and antifungal activity.

The results of the present study showed that furanocoumarins possess antiplasmodial activity, as evidenced by its significant levels of activity against CQ-resistant and CQ-sensitive strains of *P. falciparum*.

In conclusion, **1** and **2** with significant antiplasmodial and antimicrobial properties against a number of pathogens warrant further study in the light of developing new antifungal and malarial drugs. Further studies are also required to confirm SAR of furanocoumarins.

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