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ORIGINAL PAPER

Antiplasmodial activity of flavonol quercetin and its analogues in *Plasmodium falciparum*: evidence from clinical isolates in Bangladesh and standardized parasite clones

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Abstract Malaria is still a major threat in many parts of the world with resistance spreading to almost all classes of antimalarials. The limited arsenal of available antimalarial drugs emphasizes the urgent need for novel antimalarial compounds. Owing to the fact that novel leads from nature have traditionally played a pivotal role in the development of various classes of antimalarials, we investigated a set of eight naturally occurring dietary flavonoids and their analogues for their antiplasmodial activity on clinical field isolates in southeastern Bangladesh and culture-adapted chloroquine-sensitive and chloroquine-resistant parasite clones. Except for taxifolin, all the other flavonoids had 50% inhibitory concentrations below 14 μM, both in the field and laboratory-adapted parasites. Neither of the flavonoids showed any activity correlation with chloroquine. The quercetin analogue rutin (7.10±10.32 μM) was the most active substance in field isolates as well as laboratory-adapted cultures (3.53±13.34 μM in 3D7 and 10.38± 15.08 μM in K1), providing the first evidence of its activity against *Plasmodium falciparum* parasites. Thus, our results provide important evidence of the antimalarial activity of flavonoids in traditional use and thus warrant further investigation of these compounds as potential antiplasmodial agents.

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Introduction

Malaria remains a major threat causing almost 800,000 deaths in the year 2009 (WHO 2010), and resistance to antimalarial drugs is further aggravating the situation. Novel leads obtained from nature have always played a pivotal role as the basis of various antimalarials that have been developed into safe drugs by semisynthetic strategies. The best example for a novel lead from nature and the most potent antimalarial drug available for more than three centuries is a plant alkaloid, namely quinine from *Cinchona succirubra*. Certain other plant-derived compounds like terpenes, quassinoids, flavonoids, and chalcones are also known to have antiplasmodial activity (Kaur et al. 2009; Saxena et al. 2003). Artemisinin or qinghaosu is another good example of a plant-derived antimalarial compound. Artemisinin is a

sesquiterpene lactone endoperoxide (Saxena et al. 2003) originally developed in the 1970s in China by extraction from *Artemisia annua*, a plant that has been known for its medicinal properties for centuries (Bruce-Chwatt 1982). In the past decade, artemisinin-based combination therapies have become the backbone of the treatment of uncomplicated falciparum malaria. With the emergence of first cases of artemisinin resistance along the Thai–Cambodian border, there is an immediate need for novel compounds to treat falciparum malaria (Noedl et al. 2008, 2009).

Source of dietary flavonoids and their therapeutic importance

Flavonoids are a group of polyphenolic compounds that are ubiquitously found in plants and hence provide a rich dietary source. Quercetin is abundantly found in *Allium cepa* (onion), *Avena sativa* (oats), *Brassica* spp., tea (in the form of tannins), *Allium sativum* (garlic), pear, and spinach (Havsteen 2002). The flavonolignan silymarin is an active component of the plant *Silybum marianum* (milk thistle) and extracted from its seeds (Kren and Walterova 2005). A study conducted in the USA and the Netherlands estimated that the average dietary intake of flavonoids ranges between 23 mg/day (mainly aglycones) in The Netherlands and 170 mg/day in the USA. Quercetin was the major dietary flavonoid in the Netherlands (intake being 16 mg/day) (Cook and Samman 1996). Flavonoid preparations have been in use to treat disorders of peripheral circulation for more than 40 years and are known to be active against various human ailments such as inflammation, allergy, headache, fever, viral infections, and duodenal ulcers (Havsteen 2002; Lehane and Saliba 2008). These phytonutrients were also found to have an anticancer potential (DeWeerdt 2011). Anthocyanin from cranberries inhibited proliferation of eight different cancer cell lines. Lycopene in tomatoes has been reported to reduce prostate cancer risk by 10–20% (DeWeerdt 2011). However, high doses (1–1.5 g/day) of certain flavonoids have been reported to be associated with adverse effects like anemia, skin reactions, fever, and even hepatitis or acute renal failure. Nevertheless, flavonoid toxicity is unlikely to play a major role in a diversified flavonoid diet. In the recent years, flavonoids are also emerging as potential antiplasmodials. Isoflavones from *Andira inermis* and root bark of *Erythrina abyssincia* showed moderate in vitro antiplasmodial activities (Kren and Walterova 2005; Yenesew et al. 2003). These findings raise the question whether a diet rich in certain phytonutrients could potentially play a role in malaria treatment or prevention. In this study, we have focused on the potential of certain flavonoids and their derivatives for inhibiting the growth of *Plasmodium falciparum* in field isolates as compared to culture-adapted parasite clones.

Material and methods

Study location

The study was carried out at the Medical University of Vienna and at the Malaria Research Initiative Bandarban field site at the Bandarban Sardar Hospital, Bandarban, located in the Chittagong Hill tracts of southeastern Bangladesh. Written informed consent was obtained from all study participants or their legal representatives, and the study protocol was approved by the Ethical Review Committees of the Medical University of Vienna and the International Center for Diarrhoeal Disease Research, Bangladesh.

Substances tested for antiplasmodial activity and coating of the culture plates

Culture plates were coated as described previously (Noedl et al. 2004). In brief, 1 mg/ml stock solutions were prepared in 70% ethanol from quercetin hydrate (MW=302.240), taxifolin hydrate (MW=304.250), quercetin-3ß-glucoside (MW=464.380), quercitrin hydrate (MW=448.380), rutin (MW=664.560), quercetin-3-D-galactoside (MW=464.380), rhamnetin (MW=316.260), silymarin (MW=482.440), and dihydroartemisinin (DHA) (MW=284.352). These were diluted with distilled water to obtain the desired test concentrations: quercetin (0.453 to 330.800 μM), taxifolin (0.450 to 328.670 μM), quercetin-3ß-glucoside (0.295 to 215.340 μM), quercitrin (0.305 to 223 μM), rutin (0.206 to 150.470 μM), quercetin-3-D-galactoside (0.295 to 215.340 μM), rhamnetin (0.433 to 316.190 μM), silymarin (0.284 to 207.270 μM), and DHA (0.120 to 87.920 μM). Serial threefold dilutions (seven concentrations and one drug-free control well) of the drugs (25 μL/well) were dispensed in duplicate into standard 96-well microculture plates by a microdilution technique. The plates were dried overnight in an incubator and stored at 4°C before further use. All flavonoids were purchased from Sigma-Aldrich, Austria.

Sample collection and cultivation of *P. falciparum*: antiplasmodial activity assay in the field

Parasitized blood samples were obtained from male and nonpregnant female patients between 8 and 65 years who presented with acute, uncomplicated, microscopically confirmed *P. falciparum* monoinfections with a parasite density of 1,000 to 100,000 asexual parasites

per microliter. Parasite isolates with more than 1% parasitemia were diluted with uninfected red blood cells to an equivalent of 0.2% initial parasite density. Pregnant or breastfeeding women and patients with malaria drug therapy in the preceding 30 days were excluded from the study. Culture was maintained on 96-well culture plates precoated with the compounds at 1.5% hematocrit in complete RPMI 1640 (Sigma-Aldrich, Austria) medium containing 0.5% albumax (Albumax I; Gibco Bangkok, Thailand), 25 mg/l of gentamycin, and $NaHCO_3$ (2.8 ml of 7.5% $NaHCO_3$ per 100 ml of medium). The culture was maintained in candle jars, regased, and carefully shaken after 24 and 48 h. After a cultivation period of 72 h, the culture plates were frozen and stored at −20°C until further processing.

Continuous culture of laboratory-adapted *P. falciparum* clones

The 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) clones were obtained from MR4/American Type Culture Collection, Manassas, VA, USA. Parasites were cultured in RPMI 1640 medium containing 10% human serum at a hematocrit of 5% (blood group 0 negative) at 37°C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The medium was changed every 24 to 48 h. The culture was diluted, and fresh erythrocytes were added whenever the parasite density reached >1%.

Synchronization

After reaching a parasitemia of 5% or higher, 5 mL of cell medium mixture was centrifuged at 700×*g* for 5 min at room temperature (RT). Packed red cells were resuspended in 3 mL of 5% D-sorbitol in water at RT and immediately centrifuged again at 700×*g* for 5 min at RT followed by three washes with 3 mL of RPMI 1640 medium.

Antiplasmodial activity assay

Samples were diluted with complete RPMI 1640 medium and by adding uninfected red blood cells to 1.5% hematocrit and 0.05% parasitemia. 150 μL of this cell medium mixture was then added to each well of 96-well plates precoated with serial dilutions of flavonoids and incubated at 37°C for 72 h in a gas mixture containing 5% CO_2, 5% O_2, and 90% N_2.

Investigation of growth inhibition

Plates were thawed and parasite growth inhibition was quantified by using a histidine-rich protein 2 ELISA. Optical density was measured at 450 nm using a standard ELISA plate reader. The resulting readings were used for estimating inhibitory concentrations by nonlinear regression analysis (Noedl et al. 2002).

Table 1 Geometric mean inhibitory concentrations for flavonoid analogues against fresh *P. falciparum* field isolates from Bangladesh

Substances	*n*	50% inhibitory (IC_{50}) concentration in μM	90% inhibitory (IC_{90}) concentration in μM
Quercetin	9	14.7±12.62	113.02±92.372
Silymarin	17	13.16±10.76	89.37±44.765
Quercetin-3-glucoside	14	21.54±16.74	91.898±40.256
Quercetrin	13	20.39±14.49	83.862±42.434
Quercetin-3-galactoside	12	24.38±13.26	113.4494±35.633
Taxifolin	9	31.05±20.49	112.452±75.855
Rhamnetin	13	27.04±30.19	132.883±79.788
Rutin	7	7.10±10.32	60.624±45.598
DHA	14	0.006±0.012	0.018±0.028

n number of samples, *DHA* dihydroartemisinin

Results

In the present study, the flavonoid quercetin and its analogues were tested against 25 *P. falciparum* field isolates in Bangladesh. At the same time, this set of flavonoids was also investigated for growth inhibition in 3D7 and K1 clones.

Table 2 Geometric mean inhibitory concentrations for flavonoid analogues against lab-adapted *P. falciparum* 3D7 and K1 clones

Substances	50% inhibitory concentration (IC_{50}) in μM	
	3D7 (*n*=3)	K1 (*n*=3)
Quercetin	4.11±2.05	2.94±2.41
Silymarin	15.90±6.13	9.71±5.46
Quercetin-3-glucoside	4.55±13.80	7.02±14.46
Quercetin	35.37±6.44	11.67±27.24
Quercetin-3-galactoside	15.78±25.70	16.10±25.84
Taxifolin	58.31±28.39	67.34±9.8
Rhamnetin	55.56±51.77	11.76±12.65
Rutin	3.53±13.34	10.38±15.08
DHA	0.001±0.003	0.001±0.002

n number of repeats, *DHA* dihydroartemisinin

Effect of flavonoid derivatives on *P. falciparum* growth in field isolates

Eight flavonoids were investigated for their growth inhibitory activity. The substances tested showed a 50% inhibition of parasite growth in concentrations ranging from 7 to 31 μM with quercetin, silymarin, and rutin being the most active substances resulting in IC_{50}s below 14 μM. Detailed results are presented in Table 1.

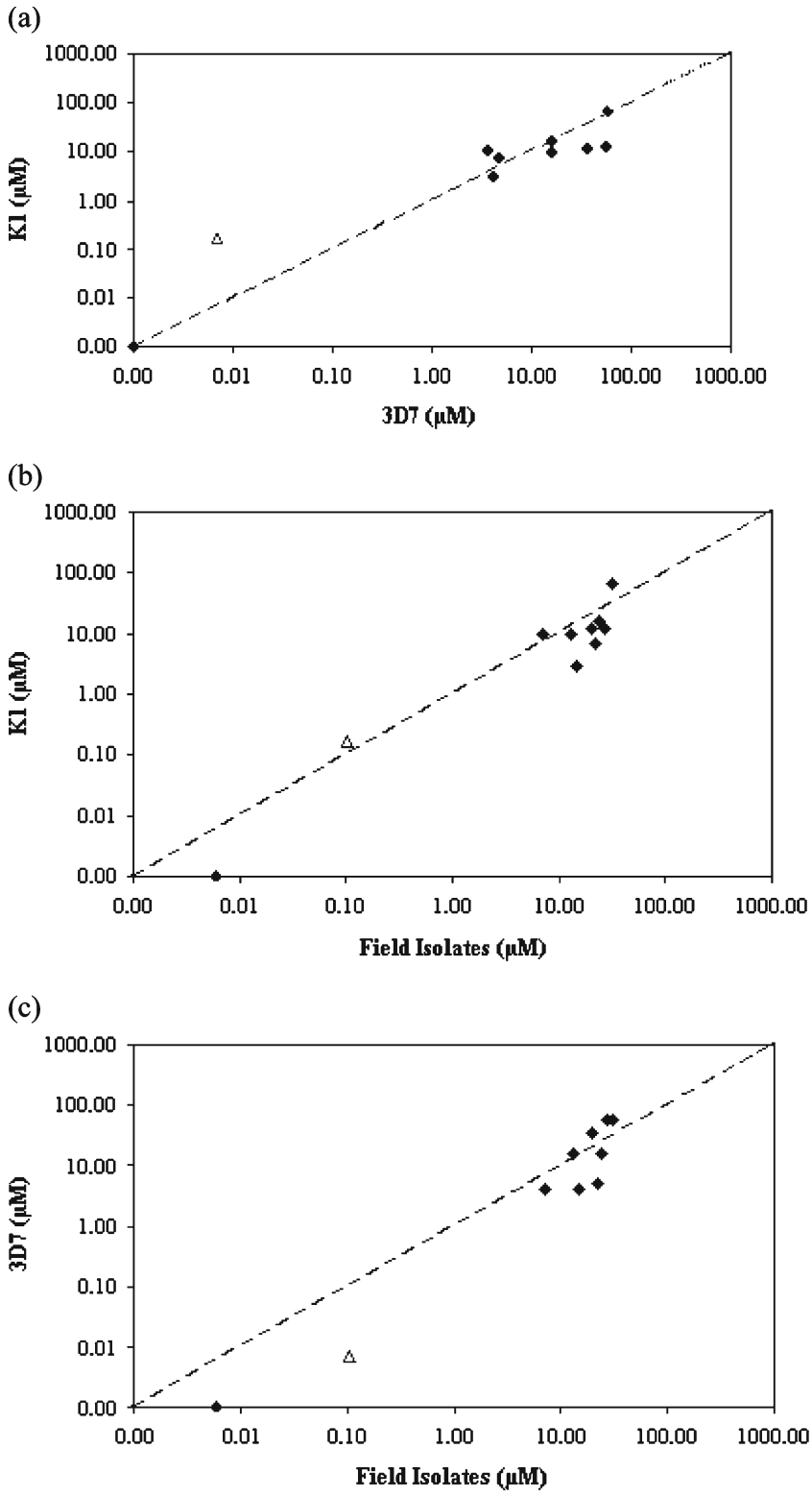

Fig. 1 Correlation IC_{50} values in **a** 3D7 and K1: in general, some data points (*black diamond*) are located close to the ideal 1:1 correlation (represented by similar IC_{50} values and hence on the line). Chloroquine (see the "Results" section for IC_{50}) is represented by a *white triangle* that deviated away from the line towards the other side of the line. **b** K1 and field isolates: Both the field isolates and culture-adapted K1 show resistance to chloroquine (*white triangle*), and hence, all the flavonoids tested (*black diamond*) show an inverse relation with chloroquine **c** 3D7 and field isolates: Culture-adapted 3D7 is sensitive to chloroquine (*white triangle*). All data points (*black diamond*) are located either close to ideal 1:1 correlation, indicating similar IC_{50} values for both 3D7 and field isolates or below the line showing higher sensitivity of these flavonoids in 3D7

Growth inhibition in culture-adapted *P. falciparum* clones

Two *P. falciparum* clones were used to test their susceptibility to flavonoids and to provide a reference for the field isolates. All substances tested inhibited both the culture-adapted clones in concentrations ranging from 4 to 58 μM for 3D7 and 2 to 67 μM for K1. Inhibitory concentrations are listed in Table 2.

Activity correlation between laboratory clones and field isolates

Chloroquine was used as a reference control in order to investigate the sensitivity of culture-adapted clones and field isolates to flavonoids tested. The IC_{50} of chloroquine was 0.104±0.189 μM against the field isolates, 0.007± 0.003 μM against 3D7 and 0.171±0.0025 μM against K1, respectively. All the flavonoids inhibited both 3D7 and K1 parasites with similar potencies. Comparison with the field isolates suggested that flavonoids generally showed higher activity in culture-adapted clones. However, rutin was found to have similar potencies both in the field and in the laboratory clones (Fig. 1).

Discussion

Flavonoids are polyphenolic entities in plants with properties favorable to human health. Most flavonoids have been reported to exert a moderate antiplasmodial activity in a number of different *P. falciparum* strains (de Monbrison et al. 2006; Gopiesh Khanna et al. 2011; Kraft et al. 2000; Lehane and Saliba 2008). In this study, we confirm that quercetin and its analogues certainly have an antiplasmodial potential as confirmed by our in vitro/ex vivo results. Quercetin, quercetin-3ß-glucoside, and rutin inhibited both the clones in low micromolar concentrations and were the most active analogues in both clones (Table 2). Activity of all the flavonoids was in sub-micromolar range against the field isolates, quercetin, silymarin, and rutin being the most

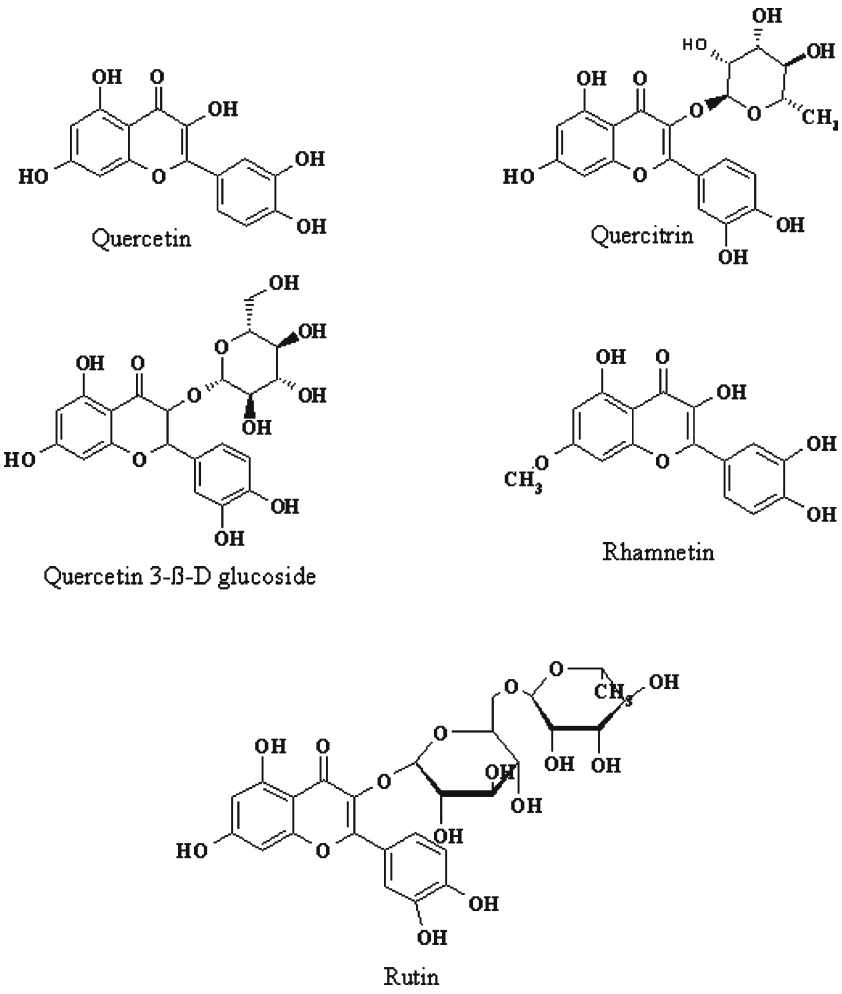

Fig. 2 Chemical structures of flavonoid quercetin and its glycosides discussed

active. Quercetin exhibited an IC_{50} value comparable to that of rutin but was tenfold more active in 3D7 and sevenfold more active in K1 as compared with the earlier published results (Lehane and Saliba 2008). Correlation studies (K1 and field isolates, K1 and 3D7) revealed chloroquine as an outlier that would suggest an inverse activity correlation of the flavonoids with chloroquine thus eliminating the chances of cross-resistance (Fig. 1a, b).

Except for silymarin and taxifolin, all the other analogues tested here are the glycosides of quercetin that are linked to a sugar moiety (Fig. 2). Characteristic feature of a sugar–flavonol is a β-glycosidic linkage that is resistant to hydrolysis by pancreatic enzymes and hence was believed to have a poor absorption in humans (Kuhnau 1976). However, a more recent study suggests that glycosides are also well absorbed by humans as volunteers fed with quercetin β-glucoside-rich diet actively absorbed the flavonoid from the small intestine (Hollman et al. 1999). All the glycosides showed activity in the low micromolar range against the clones and the field isolates. Rutin was the only glycoside which inhibited both the field isolates and laboratory-adapted cultures with similar potencies (Tables 1 and 2). Interestingly, it has been reported that rutin was ineffective in inhibiting the growth of *Plasmodium juxtanucleare*, a *Plasmodium* species causing avian malaria (Silveira et al. 2009). Our results provide the first evidence of antiplasmodial activity of rutin against *P. falciparum*.

Although the main active compound in *A. annua* is artemisinin, a sesquiterpene lactone, recent reports have also suggested a broad antioxidant activity of *A. annua* due to its high phenolic content. Phenolics present in this plant are coumarins, flavones, flavonols, and phenolic acids, the most abundant components being flavones and flavonols, particularly quercetin glucoside and rhamnetin (Ferreira et al. 2010). Beyond the activity of the main active compound artemisinin, the high content of flavonoids may therefore have played a major role in the traditional use of *A. annua* in Chinese medicine.

The recent surge in antimalarial drug resistance has emerged as a major threat to the ability to treat and control malaria in the future. Plant-derived products in combination with synthetic antimalarials have earlier proven to provide potent combination therapies for the fight against malaria. Although the activities of flavonoids tested here are much lower when compared with that of DHA (more than 1,000-fold more active than rutin and 2,000-fold more active than other analogues), yet they stand the potential to synergize the effects of artemisinin against malaria and thus might enhance the effect of this class of drugs if supplemented to the existing combination therapies as a potential third partner or as triple combination. Thus, we report a strong evidence for antiplasmodial activity of certain essential dietary flavonoids tested here, against *P. falciparum* clone and field isolates. These analogues with IC_{50}s in the low micromolar ranges and potent activity against chloroquine-resistant parasites warrant further investigation as candidates hopefully to be added to the limited armamentarium in the fight against malaria.

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