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ORIGINAL PAPER

Growth-inhibitory effect of a fucoidan from brown seaweed *Undaria pinnatifida* on *Plasmodium* parasites

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Abstract The present study was undertaken to investigate the inhibitory effects of fucoidan, a sulfated polysaccharide isolated from the edible brown seaweed *Undaria pinnatifida*, on the growth of *Plasmodium* parasites. In order to assess the anti-malarial activity of fucoidan, growth inhibition activities were evaluated using cultured *Plasmodium falciparum* parasites in vitro and on *Plasmodium berghei*-infected mice in vivo. Fucoidan significantly inhibited the invasion of erythrocytes by *P. falciparum* merozoites, and its 50% inhibition concentration was similar to those for the chloroquine-sensitive *P. falciparum* 3D7 strain and the chloroquine-resistant K1 strain. Four-day suppressive testing in *P. berghei*-infected mice with fucoidan resulted in a 37% suppressive effect versus the control group and a delay in death associated with anemia ($P<0.05$). In addition, fucoidans had no toxic effect on RAW 264.7 cells. These findings indicate that fucoidans from the Korean brown algae *U. pinnatifida* inhibits the invasion of *Plasmodium* merozoites into erythrocytes in vitro and in vivo.

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Introduction

The asexual erythrocyte cycle of malaria begins with the growth and proliferation of intraerythrocytic parasites to individual merozoites, which, on erythrocyte rupture, invade other erythrocytes via multiple adhesive interactions involving with host cell surface molecules (Cowman and Crabb 2006). Therefore, an understanding of the basic molecular mechanisms of asexual growth cycle, particularly the process of merozoite invasion into host red blood cells (RBCs), may accelerate the development of an effective therapeutic method and method for preventing malaria.

Recently, efforts to identify new drug candidates have raised interest in sulfated fucans (Berteau and Mulloy 2003; Smit 2004), which are known to act as coagulation modulators. Like heparin, fucoidan is a sulfated polysaccharide, and hence a negatively charged polysaccharide, and is mainly composed of L-fucose and ester sulfate. Several biological activities, e.g., anti-coagulant, anti-thrombotic, anti-bacterial, anti-viral, anti-inflammatory, and immunomodulating activities, have been attributed to the fucoidans (Berteau and Mulloy 2003; Ponce et al. 2003; Smit 2004). Fucoidan has also been shown experimentally to inhibit the in vitro invasion of *Plasmodium falciparum* merozoites into erythrocytes and to block the cytoadhesion

of infected erythrocytes to various host receptors (Pancake et al. 1992; Xiao et al. 1996; Clark et al. 1997) or disrupt *P. falciparum* rosettes (Rowe et al. 1994). Furthermore, sulfate esters of fucoidan may have a biological effect on cellular recognition and adhesion via its specific binding to a cell surface ligand. Recently, it was reported that fucoidan extracted from the sporophylls of *Undaria pinnatifida* exhibits a potent inhibitory effect on cryptosporidiosis and viral diseases (Lee et al. 2004; Maruyama et al. 2007; Hayashi et al. 2008). Although fucoidan from *U. pinnatifida* has been studied in terms of its inhibitory effects on infections, the anti-malarial activity of fucoidan has not been previously investigated.

In the present study, we extracted three fractions of fucoidan (F1–F3) from the Korean brown seaweed *U. pinnatifida* and confirmed their inhibitory effects on the in vitro growth of *P. falciparum* parasites. In addition, the in vivo inhibitory effect of fucoidan was confirmed against the rodent malaria parasite *Plasmodium berghei* in mice.

Materials and methods

Isolation and purification of fucoidan from *U. pinnatifida*

Sporophylls of the brown algae *U. pinnatifida* were collected on the north east coast of the Republic of Korea (Gangwon Province) in February 2007. Seaweed samples were dried and milled to a powder. Seaweed samples were hydrolyzed with cellulose, pectinase, and arylsulfatase in the presence of $CaCl_2$ (to separate alginic acid) and partially purified using cetylpyridinum chloride and ethanol precipitation as previously described (Vieira et al. 1991). The fucoidan so obtained was fractionated by anion-exchange chromatography using DEAE-Sephadex A-25 (Cl^- form, Pharmacia, Uppsala, Sweden) using gradually increasing concentrations of sodium chloride (0–3.5 M). The three fractions obtained were dialyzed against pyridine acetate (0.1 M, pH 5.0–5.4) for 3 days, and carbohydrate and sulfate analyses by high performance anion-exchange chromatography were used to determine the fucose, monosaccharide, and uronic acid contents of the three fucoidan fractions. Three fractions of fucoidan (F-1, F-2, and F-3) were extracted from *U. pinnatifida* by hydrolytic enzymatic method.

Parasite culture and in vitro growth inhibition assays

P. falciparum parasites [3D7, chloroquine-sensitive strain (CQS) and K1, chloroquine-resistant strain (CQR)] were cultured in O^+ human erythrocytes and maintained at a hematocrit level of 1% in complete medium at 37°C in an incubator as previously described (Trager and Jensen 1976). This complete medium contained RPMI 1640 medium containing L-glutamine and 25 mM HEPS buffer (Gibco, NY, USA), 0.225% $NaHCO_3$ (Gibco), 0.5% AlbuMAX I (Gibco), 10 μg/ml of gentamicin (Gibco), and 50 mg/l of hypoxanthine (Sigma, St. Louis, USA). The parasite ring stage was synchronized using 5% sorbitol, as previously described (Lambros and Vanderberg 1979), and the late trophozoite and schizont stages were induced by incubating for additional incubation of 24 h.

The effects of the three fucoidan fractions on the invasion of erythrocytes with merozoites were assessed using growth inhibition assays. Flat-bottom 96-well cell culture plates (Costar, NY, USA) were first seeded with 190 μl of parasites (0.3% in final parasitemia) in the parasitized RBCs (pRBCs, trophozoite and schizont stages-rich) in 1.0% hematocrit. Different concentrations of fucoidan fractions were prepared with complete medium and chloroquine diphosphate (Sigma) and quinine hydrochloride (Sigma) were regarded as positive controls. Fucoidan and anti-malarial drugs serially diluted were transferred in triplicate (10 μl each) to pRBC suspensions in 96-well cell culture plates. Two hundred microliters of pRBCs and fresh RBCs (fRBCs) in suspension (1% hematocrit) were used as drug-free and negative controls, respectively. Culture plates were incubated for 40 h at 37°C in a modular chamber (Ipd/Appco, Vista, USA) in a 5% CO_2, 5% O_2, and 90% N_2 atmosphere. Percent parasitemia was determined by counting under a microscope after incubation, and cultured parasites were keep in a freezer (−20°C) at least 3 h. Parasite lactate dehydrogenase (pLDH) enzymatic assays were performed as follows.

pLDH enzymatic assays

Fifty microliters of culture parasite samples were thawed completely and dissolved in 100 μl of pLDH assay buffer for 30 min at room temperature and samples in microtiter plate covered with aluminum foil. The assay buffer was composed of 100 mM Tris (Sigma, pH 8.0), 50 mM sodium L-lactate (Sigma), 0.25% Triton X-100 (Bio-Rad), 20 μg of nitro blue tetrazolium (Sigma), 5 μg of 3-acetylpyridine adenine dinucleotide (Sigma), and 0.1 U of diaphorase (Sigma). The absorbance at 620 nm was measured using a 96-well Spectra II spectrophotometer (Tecan, Grödig, Austria). Percent inhibition by each drug sample was defined as $100-[(OD_{620}$ of drug treatment sample $- OD_{620}$ of fresh RBCs only$)/(OD_{620}$ of drug free sample $- OD_{620}$ of fresh RBCs only$)\times 100]$.

Cytotoxicity testing

The cellular viabilities of RAW 264.7 cells (a murine macrophage cell line) that had been treated with fucoidan

fractions were determined using MTT assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as previously described (Habtemariam 2003). Briefly, cells were cultured in RPMI 1640 medium and harvested in the log phase of growth and seeded (5×10^3 cells/well in 100 μl volume) in 96-well microtitre plates. After 24 h of incubation at 37°C in 5% CO_2 gas to allow cell attachment, cultures were treated with fucoidans (0, 1, 10, 25, 50, and 100 μg/ml, diluted in RPMI 1640 medium) for 24 h, and cell viabilities were assessed by measuring OD_{540} values using a 96-well Spectra II spectrophotometer.

Effect of fucoidan on the disease course of *P. berghei* ANKA in mice

Based on growth inhibition results obtained in vitro, the activity of fucoidan F-3 was evaluated in vivo by treating mice infected with *P. berghei* ANKA, a model of cerebral malaria. BALB/c mice (6–8 weeks old) were divided into three groups [phosphate-buffered saline (PBS)-, chloroquine-, and fucoidan-treated groups; $n=7$ mice in each group) and were intraperitoneally inoculated with *P. berghei* ANKA strain pRBCs. The test protocol was based on the 4-day suppressive test (Peters 1975). In brief, pRBCs were collected from an infected mouse and dissolved in PBS and test animals were infected by injecting a 0.2-ml suspension (1×10^6 parasites) intraperitoneally. From days 1 to 4 post-infection, 100 mg/kg of fucoidan (dissolved in 0.2 ml PBS) was administered to each mouse, whereas control group animals received 0.2 ml of PBS only, and animals in the standard group were treated with chloroquine (5 mg/kg). At 4 days post-infection, blood samples from all animals were smeared and stained with Giemsa; parasitemia was determined microscopically. Percentage inhibition was calculated as 100−[(mean parasitemia treated/mean parasitemia control)×100]. Survival times (in days) were recorded.

Statistical analysis

The 50% inhibitory concentrations (IC_{50}) values of the compounds against malaria in vitro were analyzed by nonlinear dose–response curve fitting using SigmaPlot program version 10.0 (Systat Software, Point Richmond, USA). Percentage inhibitions and survival times in vivo were compared using the Student's *t* test. Values of $P\leq0.05$ were considered to be statistically significant.

Results

Although the chemical structures of fucoidan and de-sulfated fucoidan have not yet been determined, the chemical properties of these natural compounds have been characterized.

The yields and compositions of the three fucoidan fractions purified from *U. pinnatifida* are shown in Table 1 and Fig. 1. All fucoidan fractions (molecular weight, about 15,000 kDa) contained fucose as the major component along with galactose and minor quantities of arabinose, rhamnose, glucose, xylose, and mannose, indicating that they were fucogalactans, with variable proportions of these sugar components. The fucoidan fraction F-1 that was eluted at the lowest NaCl concentration was rich in uronic acid and poor in sulfate groups. On the other hand, the fucoidan fraction F-3 that eluted at a higher concentration of NaCl had higher sulfate and lower uronic acid contents. The first fraction, F-1, was characterized by high percentage of uronic acid (21.1%) and a low sulfate content (11.4%). The second, F-2, and third, F-3, fractions were characterized by lower uronic acid levels (6.8% and 7.9%, respectively) and higher sulfate contents (20.6% and 31.2%, respectively).

Experimental results are presented in Fig. 1. All three fucoidan fractions demonstrated activity against *P. falciparum*, and fraction F-3 (with highest sulfate content) showed most anti-malarial activity, suggesting that sulfate content may play a pivotal role in the anti-malarial activity of fucoidans. The fact that fraction F-2 has less anti-invasion activity than F-3, although its fucose content was much higher, might be due to its uronic acid concentration, which also possesses anti-invasion properties (uronic acid content of fraction F-3 was 7.9%, while that of fraction F-2 was 6.8%). The mechanistic relations between anti-invasion

Table 1 Composition and yield of crude fucoidan fractionation by ion-exchange chromatography from hydrolysate in sporophylls of *U. pinnatifida* by enzymatic hydrolysis

Fraction	NaCl conc. eluted (M)	Yield (%)	SO_3Na (%)	Uronic acid (%)	Neutral monosaccharide (% mol)						
					Fucose	Galactose	Arabinose	Rhamnose	Glucose	Xylose	Manose
F-1	0.0–1.0	20.2±1.5	11.4±0.2	21.1±0.6	43.8±0.1	35.3±0.8	7.4±0.3	2.6±0.3	3.6±0.2	2.1±0.1	5.2±0.2
F-2	1.0–2.0	31.6±1.9	20.6±0.6	6.8±0.2	58.0±0.8	28.7±1.0	4.6±0.3	1.5±0.2	3.5±0.6	1.4±0.1	2.3±0.3
F-3	2.0–2.5	25.1±2.1	31.2±0.5	7.9±0.4	56.4±0.5	26.3±0.4	3.8±0.5	3.2±0.1	2.7±0.6	2.5±0.2	5.1±0.4

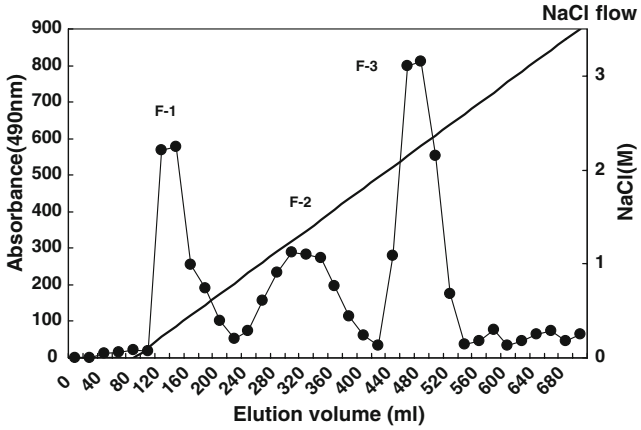

Fig. 1 Fractionation of crude fucoidan isolated from the brown alga *U. pinnatifida* on a DEAE-Sephadex A-25 column

activity and molecular weight and the ratios of fucose, uronic acid, and the sulfate group are not known.

To access anti-malarial potency of fucoidan, its effects on the growth inhibition of *P. falciparum* parasite strains were examined (Table 2). The effects of fucoidan (F-1, F-2, and F-3) on the invasion of erythrocytes by merozoites (3D7 and K1 strain) were assessed using pLDH enzymatic assay. We found that all three fractions had a range of effects on the invasion of erythrocytes by merozoites (Table 2), and their inhibitions were concentration-dependent with a sigmoidal curve (Fig. 2). These findings concur with similar observed decreases in parasitemia in Fig. 2.

These studies determined that the IC_{50} values of fractions F-1, F-2, and F-3 were 9.17, 7.28, and 1.95 μg/ml in *P. falciparum* 3D7 stain and 7.03, 4.74, and 2.21 μg/ml in *P. falciparum* K1 strain, respectively (Table 2). The anti-malarial efficacy of F-3 was higher than those of F-1 and F-2.

Because in vitro studies demonstrated that fucoidan inhibits the invasion of erythrocytes by *P. falciparum*, we evaluated the efficacy of the third fraction on parasitemia and survival in mice infected with *P. berghei* ANKA. After a 4-day suppressive test in vivo, fucoidan F-3 had a suppressive effect of 37.12±0.37% in *P. berghei*-infected mice at 100 mg/kg, while chloroquine (the positive control) had a suppressive effect of 94.37±0.94% at 5 mg/kg at fourth days (Table 3). Fucoidan F-3 was found to have lower anti-malarial activity than chloroquine, but it inhibited parasitemia significantly versus the PBS-treated controls ($P<0.05$). Furthermore, the survival times of mice treated with fucoidan F-3 were significant longer about 4 days than those of the control groups ($P<0.05$; Table 3).

Discussion

The growth inhibitory effects of fucoidan derived from *U. pinnatifida* seaweed were investigated on malaria infection in vitro and in vivo in this study. The in vitro anti-malarial efficacy of fucoidan described here was tenfold higher than that reported earlier, i.e., the IC_{50} value for fucoidan (molecular weight, 18,000 kDa) from *Fucus vesiculosus* against *P. falciparum* FCR-3 parasite was 22 μg/ml with 42.3% inhibition (Clark et al. 1997). An in vivo animal experiment performed during a previous study reported found that fucoidan does not significantly reduce parasitemia (Xiao et al. 1996). However, in the present study, fucoidan was found to have a significant effect.

The amount of fucose in fucoidans extracted using hydrolytic enzymes was 2.8- to 3.7-fold higher than that obtained from mekabu (15.6%) by acidic hydrolysis (Maruyama et al. 2003). Shimizu et al (2005) reported that high-molecular-weight fucoidan extracted from Okinawa mozuku changed the ratio of $CD4^+/CD8^+$ and increased the ratio of cytotoxic T cells in mice splenocytes. In the present study, all fractions had higher molecular weights than those previously reported (Lee et al. 2004) from the same source; the molecular weight of fucoidan determined by their study was 9,000 kDa. In addition, its sulfur content was estimated at 31.2% in fucoidan fraction F-3; this value was higher than the 10.4% sulfur content described by Lee et al. (2004).

Our in vitro experiments showed that fucoidan has no dose-dependent inhibitory effect on parasite growth from

Table 2 In vitro growth inhibition activity and cytotoxicity of extracts obtained both chloroquine sensitive (3D7) and resistant (K1) strain of *P. falciparum*

Extract/drug	IC_{50} (μg/ml)		EC_{50} (μg/ml)
	3D7	K1	RAW 264.7
Fucoidans			
Fraction 1 (F-1)	9.17±0.12	7.03±0.35	>100
Fraction 2 (F-2)	7.28±0.33	4.74±0.15	>100
Fraction 3 (F-3)	1.95±0.24	2.21±0.05	>100
Chloroquine	0.024±0.001	0.071±0.005	N.D.
Quinine	0.042±0.005	0.037±0.004	N.D.

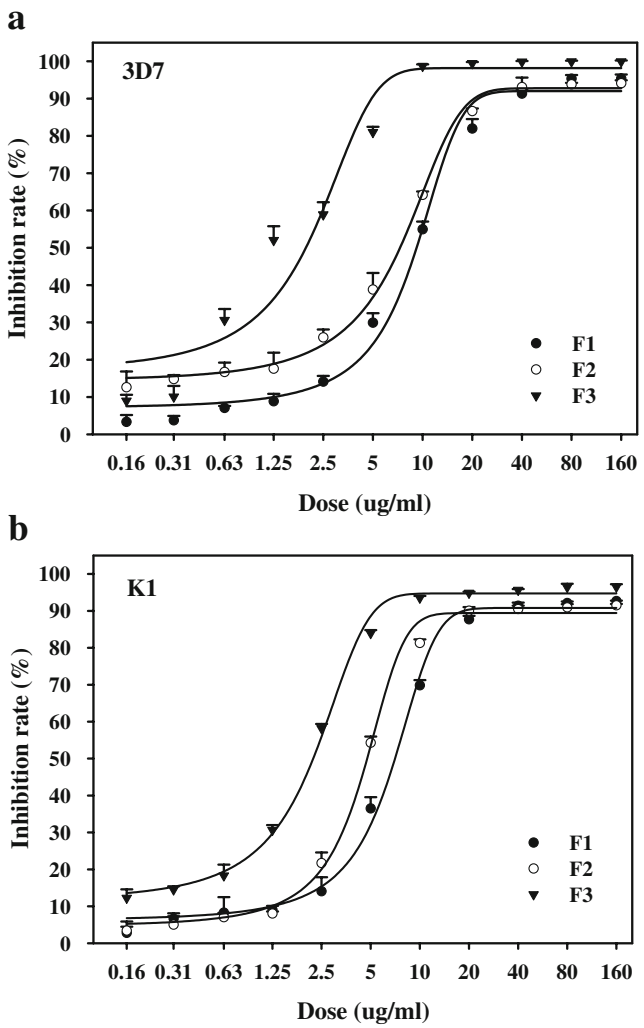

Fig. 2 In vitro growth inhibition rate of fucoidan in chloroquine sensitive (3D7) (**a**) and resistant (K1) (**b**) strain of *P. falciparum* at different concentrations

the ring to the schizont stage. However, in growth inhibition assay for whether fucoidan interfere invasion of the new merozoite to fRBCs, it was effectively prevented by fucoidan within a few days.

The results of our in vivo experiments (Table 3) showed that fucoidan suppressed the invasion of erythrocytes by *P. berghei* merozoites. Moreover, infected mice treated with fucoidan for 4 days had lower parasitemia during the entire course of infections than untreated mice. These treated mice died later during the anemia phase of the infection. The above effect can be explained by reduced parasitemia due to treatment. Thus, fucoidan may inhibit the adhesive interaction between merozoites and RBCs and direct binding of the sulfate of fucoidan to cell surface ligands of host cells, thereby inhibiting parasite invasion.

In this study, the IC_{50} of fucoidan in a mouse model was determined to be 100 mg/kg per mouse. However, eye hemorrhages and death occurred in five mice during the treatment period on either 250 or 500 mg/kg of fucoidan. These adverse reactions might have been caused by the anti-coagulatory effect of fucoidan. Therefore, for anti-malarial therapy, the application of fucoidan at doses as high as those mentioned above is not recommended, even though these doses suppress parasite growth.

In a different study, fucoidan could inhibit the invasion of human Duffy-positive and rhesus erythrocytes by *Plasmodium knowlesi*, analogous to the results reported in this study (Dalton et al. 1991). This finding is consistent with that described in earlier reports which demonstrate that the inhibitory effect of fucoidan on the invasion can be simply considered as a result of nonspecific electrostatic interactions rather than a consequence of specific cytoadhesion receptors (Xiao et al. 1996). But another experiment suggests that the inhibition of invasion is not due only to nonspecific ionic interactions between fucoidan and a parasite protein but, instead, a particular conformation of the anion(s) is required for effective inhibition (Clark et al. 1997).

In present study, the growth-inhibitory activity of fucoidan from the brown seaweed *U. pinnatifida* was evaluated on *P. falciparum* parasite strains by pLDH enzymatic assay and was found to highly inhibit the invasion of erythrocytes by merozoites. In addition, fucoidan was found to slightly inhibit parasitemia in *P. berghei*-infected mice. No report has been issued on a molecule with an affinity for fucoidan on the surfaces of free merozoites of *Plamodium* parasites. Further investigations of growth inhibition caused by fucoidan would be helpful in providing an understanding of the process of merozoite invasion. In particular, the identity of the molecule with an affinity for fucoidan is of interest.

Table 3 Effect of fucoidan on the parasitemia and survival of BALB/c mice infected with *P. berghei* ANKA

Compound	Dose (mg/kg) per day	Parasite density	% inhibition	Survival time (days)
		Mean ± S.D.		
Fucoidan (F-3)	100	4.98±0.42	37.12±0.37*	11.60±2.30*
Chloroquine	5	0.45±0.10	94.37±0.94*	>30*
Control (PBS)		7.93±0.93	0.00±0.00	7.00±1.00

*$P<0.05$ when compared to control

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