

Anti-Malarial Activity of Leaf-Extract of *Hydrangea macrophylla*, a Common Japanese Plant

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To find a new anti-malarial medicine derived from natural resources, we examined the leaves of 13 common Japanese plants *in vitro*. Among them, a leaf-extract of *Hydrangea macrophylla*, a common Japanese flower, inhibited the parasitic growth of *Plasmodium falciparum*. The IC₅₀ of *Hydrangea macrophylla* leaf extract to *Plasmodium falciparum* was 0.18 µg/ml. The IC₅₀ to NIH 3T3-3 cells, from a normal mouse cell line, was 7.2 µg/ml. Thus, selective toxicity was 40. For the *in vivo* test, we inoculated *Plasmodium berghei*, a rodent malaria parasite, to ddY mice and administered the leaf-extract of *Hydrangea macrophylla* (3.6 mg/0.2 ml) orally 3 times a day for 3 days. Malaria parasites did not appear in the blood of in the treated mice, but they did appear in the control group on day 3 or 4 after inoculation with the parasites. When leaf extract was administered to 5 mice 2 times a day for 3 days, malaria parasites did not appear in 4 of the mice but did appear in 1 mouse. In addition, the leaf-extract was administered orally 3 times a day for 3 days to *Plasmodium berghei* infected mice with a parasitemia of 2.7%. In the latter group, malaria parasites disappeared on day 3 after initiating the treatment, but they appeared again after day 5 or 6. Although we could not cure the mice entirely, we confirmed that the *Hydrangea macrophylla* leaf extract did contain an anti-malarial substance that can be administered orally.

Key words: *Hydrangea macrophylla*, malaria, medicinal plant, *Plasmodium berghei*, *Plasmodium falciparum*

Malaria is a significant health problem for the human population, as it is a serious infectious disease. Many attempts have been made to develop an anti-malarial medicine from natural resources. In particular, medicinal plants have been the focus of many studies. Quinine is a classic anti-malarial medicine found in the bark of *Chinchona* species, which is a tree native to South America (1). An alternate anti-malarial medicine, artemisinin, was discovered in China; it is from a medicinal plant, *Artemisia annua*, and is currently used world-wide (2). Thus far, this medicine is effective against chloroquine and the other drug-resistant malaria parasites.

Another Chinese medicinal plant, *Dichroa febrifuga* (*D. febrifuga*), has been used as a traditional medicine against malaria. Ch'ang Shan, the roots of *D. febrifuga*, and Shuu Chi, the leaves of *D. febrifuga*, both are reported to be effective against malaria (3, 4). However this plant is rare in China, and it is not possible to obtain it even in amounts small enough for experimental use. *D. febrifuga*, on the other hand, belongs to the family of *Hydrangeaceae*. This family consists of 16 genus, including the genus *Hydrangea*. In Japan, *Hydrangea macrophylla* (*H. macrophylla*) is native and very common. Leaves from *H. macrophylla* are plentiful in summertime. We assumed that an anti-malarial substance similar to that in *D. febrifuga* would be found in the leaves of *H. macrophylla*. Therefore, we tested the anti-malarial activity of *H. macrophylla* leaf extract *in vitro* and *in vivo*.

Materials and Methods

Plant leaves tested. Leaves of the *Hydrangea macrophylla* plant were collected in Shakujii Park, Tokyo,

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in July 1998. Leaves of the other common plants (Table 1) were also collected in Tokyo.

Parasites. The human malaria parasite used for the *in vitro* test was *Plasmodium falciparum*, strain FCR-3. The rodent malaria parasite used for the *in vivo* test was *Plasmodium berghei*, strain NK-65.

Extraction of leaves. Leaves were washed once with tap water and then were washed with distilled water. After being washed, the leaves were wiped dry and weighed. Thirty grams of leaves were boiled in 500 ml of distilled water for about 60 min to a total volume of 50 ml. Whole extracted materials were filtered through gauze, filter paper, and Milipore filters of 0.45 μm and 0.20 μm pore size, in this order. We refer to this product as the "leaf extract". A portion of this extract was freeze-dried and weighed. The concentration of the *H. macrophylla* leaf extract was 18.3 mg/ml. The colour of the *H. macrophylla* extract was clear yellow and the pH was 4.7. When the extract was added to culture media in a 5% volume, the media retained original pH of 7.4–7.8.

Anti-malaria test in vitro. Human malaria parasite, *Plasmodium falciparum* (*P. falciparum*) strain FCR-3, was cultured by the method described by Trager and Jensen (5) in 96 flat-bottom micro-titer plates. The total volume of medium (RPMI1640 with 10% human serum) in each well was 200 μl , each of which contained 10% red blood cells and 0.76% parasitemia. 10 μl of leaf-extract samples were added (the final concentration of

H. macrophylla was 0.92 mg/ml) and were incubated at 37 °C in 5% CO₂, 10% O₂, and 85% N₂. Every 24 h, the medium and the samples were changed, up to 72 h after the starting point. Six wells were prepared for each sample and the level of parasitemia was followed at 24-hour intervals by harvesting the contents from 2 wells. Red blood cells were smeared on 2 glass slides, stained with 3% Giemsa solution. Then the level of parasitemia was measured. The number of infected red blood cells was counted in increments of 10,000 red blood cells. For the positive control, 10 ng/ml of artemisinin (Sigma A-5430, St. Louis, MO, USA) was prepared and added (final concentration of 0.5 ng/ml = 1.5×10^{-9} M). To determine the 50 percent inhibitory concentration (IC₅₀) of leaf-extract to *P. falciparum*, the leaf-extract samples were diluted in various concentrations and *P. falciparum* was cultured under the same conditions as those listed above. Seventy two hours later, the level of parasitemia was measured by counting infected RBCs on Giemsa stained glass slides. The IC₅₀ was determined by comparison of experimental samples to leaf extract free controls incubated under the same conditions.

Cytotoxicity to a normal mouse cell line. To determine the cytotoxicity of the leaf extract to mammalian cells, NIH 3T3-3, a cell line of mouse fibroblast, was purchased from Riken Cell Bank, Tsukuba, Japan. Dulbecco's Modified Eagle Medium (DMEM: Gibco-BRL, Grand Island, NY, USA) with 10% of

Table 1 Effect of leaf-extracts on the growth of *P. falciparum*

Scientific Name	English Name	Japanese Name	Parasitemia* (%)		
			24 h	48 h	72 h
<i>Artemisia princeps</i>	artemisia	yomogi	2.1	4.2	5.9
<i>Camellia sasanqua</i>	-	sazanka	2.0	4.1	7.1
<i>Cerasus yedoensis</i>	cherry	sakura	2.1	3.5	6.1
<i>Commelina communis</i>	dayflower	tsuyukusa	2.1	4.4	6.0
<i>Fagopyrum esculentum</i>	buckwheat	soba	3.6	4.5	6.3
<i>Ficus carica</i>	fig	ichijiku	2.5	4.7	6.1
<i>Gardenia jasminoides</i>	gardenia	kuchinashi	2.2	4.3	5.8
<i>Ginkgo biloba</i>	ginkgo	icho	1.9	4.3	6.3
<i>Hydrangea macrophylla</i>	hydrangea	ajisai	1.6	0.6	0.0
<i>Morus alba</i>	mulberry	kuwa	3.0	3.9	7.3
<i>Perilla frutescens var. crispa</i>	perilla	shiso	2.0	5.1	4.9
<i>Plantago asiatica</i>	plastain	oobako	2.3	3.7	3.9
<i>Rosa rugosa</i>	-	hamanasu	2.4	4.0	5.2
Positive control	artemisinin		0.2	0.0	0.0
Negative control	distilled water		1.8	3.9	6.9

*Parasitemia at 0 h was 0.76% in each sample.

fetal calf serum was used for the cell culture. Leaf extract was diluted in various concentrations and added to the medium. Samples (0.4 ml/well) were placed in a 24-well plate. Cells ($2 \times 10^3/0.1$ ml) were added to each well. Two wells were prepared for each sample concentration. The plate was incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. Cell numbers were counted using a blood cell counter. The IC₅₀ was determined by comparison of experimental samples with leaf extract-free controls that had been incubated under the same conditions.

Anti-malaria test in vivo. An anti-malaria *in vivo* test was performed according to the methods of Thurston (7) and Peters (8), with some modifications.

Experiment 1: One hundred *P. berghei*-infected red blood cells were intra-peritoneally injected into 7 ddY mice purchased from SLC Inc., Shizuoka, Japan. Three mice served as an experimental group and received *H. macrophylla* leaf extract (3.6 mg/0.2 ml) orally 3 times a day for 3 days, starting on the same day as the day of the parasite inoculation. Four mice, as a control group, received 0.2 ml of physiological saline orally 3 times a day for 3 days. We used a stomach tube to ensure that the mice ingested the extract or saline. Parasitemia in blood extracted from the tail vein was examined up to day 7 after the inoculation with the parasite.

Experiment 2: *P. berghei* parasites were inoculated into 20 ddY mice according to the same method as that used in Experiment 1. Mice were separated into 4 groups. Group 1 mice received *H. macrophylla* leaf extract (3.6 mg/0.2 ml) orally 3 times a day for 3 days, starting on the same day as that inoculation with the parasite. Group 2 mice received the extract (3.6 mg/0.2 ml) 2 times a day for 3 days. Group 3 mice received the extract (3.6 mg/0.2 ml) once a day for 3 days. Group 4 mice were not treated but did receive inoculation with the parasite. Parasitemia in blood extracted from the tail vein was examined up to day 7 after inoculation with the parasite.

Experiment 3: *P. berghei* parasites were inoculated into 8 ddY mice by the same method used in Experiment 1. When parasitemia reached 1.3–4.7%, 3 mice received *H. macrophylla* leaf extract (3.6 mg/0.2 ml) orally 3 times a day for 3 days, and 5 mice received 0.2 ml of a physiological saline solution. The level of parasitemia in blood extracted from the tail vein was measured every day by day 8 after the initiation of the treatment.

Results

Anti-malaria test in vitro (Table 1). Among 13 leaf-extract samples studied, an extract of *H. macrophylla* was effective against malaria parasites. Parasitemia in wells containing *H. macrophylla* increased slightly at 24 h but decreased at 48 h and disappeared by 72 h. The other leaf extracts were not effective against *P. falciparum* *in vitro*. The IC₅₀ of the *H. macrophylla* leaf extract against *P. falciparum* was determined to be 0.18 µg/ml, and the IC₅₀ of the extract in the study of NIH 3T3-3 cells was 7.2 µg/ml. The selective toxicity of *H. macrophylla* was calculated as 40.

Anti-malaria test in vivo. Experiment 1 (Fig. 1): In the control group, parasites appeared on day 3 or 4 after inoculation with the parasite and subsequently increased in number. All 4 mice died by day 10. In the *H. macrophylla* leaf extract treatment group, one mouse died on day 2, which occurred during the term of treatment with the extract. In the other 2 mice, no parasites appeared during the term of observation and the mice survived up to day 60.

Experiment 2: In Group 1 (Fig. 2-a), one mouse died on day 2, which occurred during treatment with *H.*

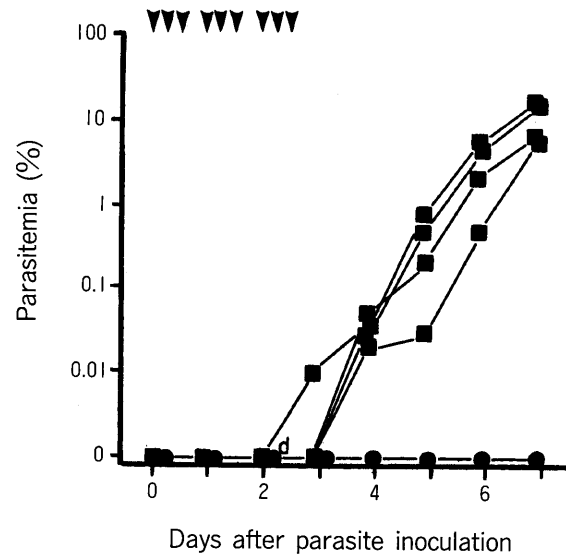


Fig. 1 *In vivo* experiment 1. Mice were inoculated with *P. berghei* intra-peritoneally, and orally given the *H. macrophylla* leaf extract (●—●, 3 mice) or physiological saline (■—■, 4 mice) for 3 days. A mark (▼) indicates the leaf-extracted treatment, and (d) indicates died.

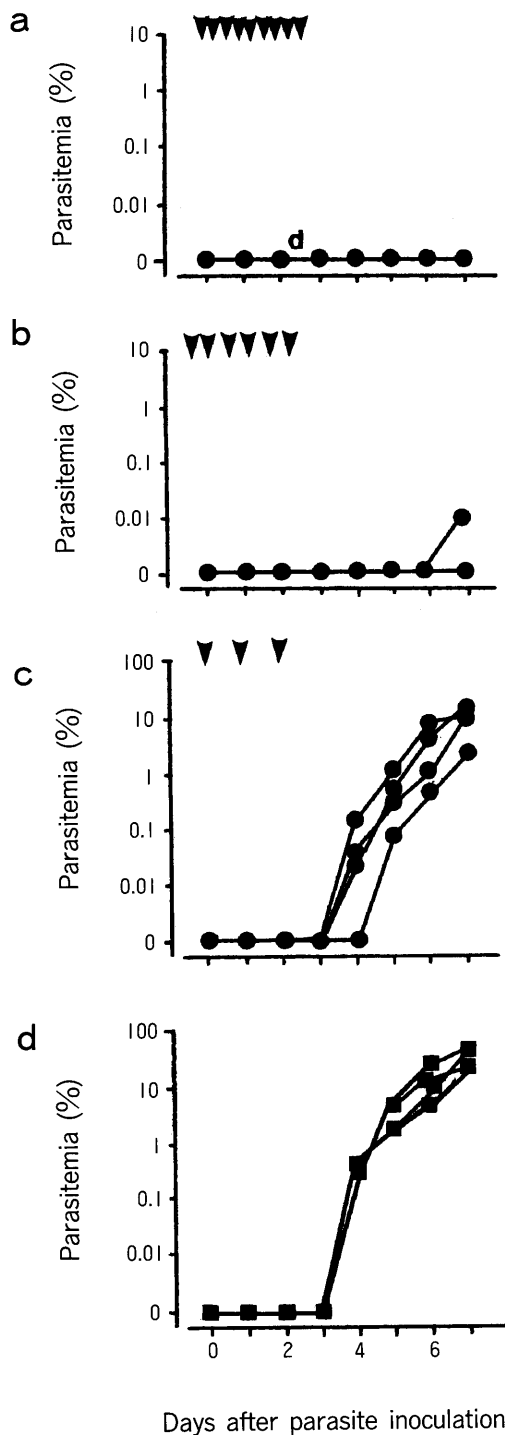


Fig. 2 *In vivo* experiment 2. Mice were inoculated with *P. berghei* intra-peritoneally, and orally given the *H. macrophylla* leaf extract for 3 days. a (group 1), 3 times a day; b (group 2), 2 times a day; c (group 3), once a day; d (group 4), no treatment. There were 5 mice in each group. A mark (▼) indicates the leaf-extract treatment, and (d) indicates died.

macrophylla. In the other 4 mice, no parasites appeared during the observation period and the mice survived up to day 60. In Group 2 (Fig. 2-b), no parasites appeared in 4 mice, which all survived up to day 60. However, parasites appeared in 1 mouse on day 7 after the parasite inoculation. That mouse died on day 13. In Group 3 (Fig. 2-c), parasites appeared on day 4 or 5 and subsequently increased in number. All 5 mice died by day 18. In Group 4 (Fig. 2-d), the group that was not treated, parasites appeared on day 4 after inoculation with parasite and the level of parasitemia subsequently increased. All of the group 4 mice died by day 17.

Experiment 3 (Fig. 3): In 3 experimental mice, parasitemia decreased on days 1 and 2 after treatment with *H. macrophylla* leaf extract had been initiated. On day 3, no parasites were found under an immersion lens (100×10) in 50 microscopical fields. Approximately 30,000 red blood cells were observed in order to determine an animal “parasite negative”. In the control group, parasitemia increased continuously. All 5 mice died by day 6 after the saline treatment was initiated. In the leaf-extract treatment group, parasites appeared again on day 5 or 6, and subsequently increased in number, which suggests that 3-day treatment with *H. macrophylla* leaf extract was somewhat effective against the parasites but it did not entirely eradicate them. One mouse in the treatment

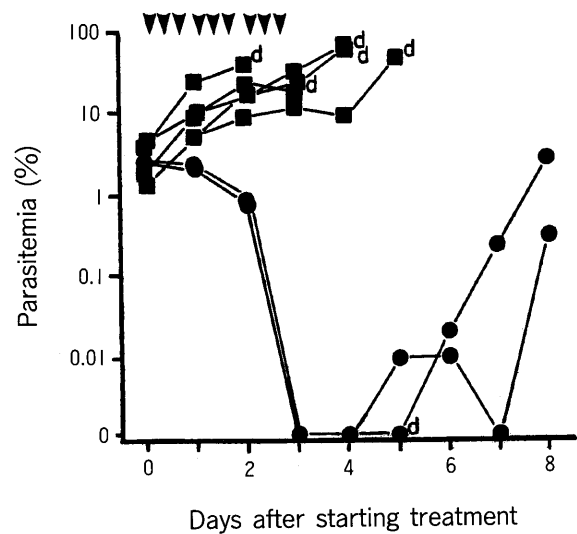


Fig. 3 *In vivo* experiment 3. *P. berghei*-infected mice were given the *H. macrophylla* leaf extract orally (●—●, 3 mice) or physiological saline (■—■, 5 mice) for 3 days. A mark (▼) indicates the leaf-extract treatment, and (d) indicates died.

group died on day 5, 2 days after the parasites had disappeared and after the leaf-extract treatment. We do not have a conclusive explanation for the death of this mouse, but it is possible that it died from acute peritonitis due to an accidental perforation of the stomach or esophagus by the stomach tube inserted during the leaf-extract treatment.

Discussion

H. macrophylla is a native Japanese plant which grows in Japanese domestic gardens. This species was exported several hundred years ago to China as a Japanese flower and then it spread to Southeast and Central Asia. Sir J. Banks introduced this plant, harvested in China, to the Royal Botanic Garden in England in 1789. Dr. P.F. von Siebold, who lived in Japan from 1823 to 1829, also introduced this plant as *Hydrangea otaksa* (This name is not used now) when he returned to his native Holland. *H. macrophylla* is currently known for its beautiful flower and is commonly planted in Asian, European and American countries. *D. febrifuga*, which belongs to the plant family *Hydrangeaceae*, as *H. macrophylla*, has been used as an anti-malarial medicine in China. Febrifugine and iso-febrifugine were isolated from the root of *D. febrifuga* and their anti-malarial activity has been confirmed (9, 10). We expected that *H. macrophylla* leaves contain an anti-malarial substance similar to that of *D. febrifuga* leaves.

We extracted the leaves of 13 common Japanese plants, including *H. macrophylla*, and tested their anti-malarial activity *in vitro* and *in vivo*. The leaf-extract of *H. macrophylla* was effective against the human malaria parasite, *P. falciparum*, *in vitro*. We determined the IC_{50} of *H. macrophylla* against *P. falciparum* as being $0.18 \mu\text{g/ml}$ and the IC_{50} for a normal mammalian cell line (NIH 3T3-3) was $7.2 \mu\text{g/ml}$. According to Professor Y. Oshima, Tohoku University, the IC_{50} of *D. febrifuga* extract against *P. falciparum* and in the mammalian cell (FM3A cells, a mouse cell line) are $0.025 \mu\text{g/ml}$ and $8 \mu\text{g/ml}$, respectively (personal communication). Thus, the efficacy of the *H. macrophylla* leaf extract is promising.

For the *in vivo* test, *H. macrophylla* completely suppressed the growth of *P. berghei*, a rodent malaria, when it was used 3 times a day for 3 days (3.6 mg for each dose). Administration of treatment twice daily also was effective in 4 of 5 mice at suppressing parasite

growth. We tested the remaining 12 plants' leaf-extracts on mice infected with *P. berghei* and found none of them to be effective against rodent malaria (Data not shown). Thus, we conclude that the *H. macrophylla* leaf extract can suppress parasite growth if oral treatment with the extract starts on the same day as the administration of inoculation with the parasite.

Oral treatment with *H. macrophylla* 3 times a day for 3 days of mice with a level of parasitemia at 2.7% suppressed malaria parasites to non-detectable levels. Parasitemia increased to detectable levels 2 to 3 days after finishing the treatment. The efficacy of the *H. macrophylla* leaf extract against malaria parasites *in vivo* was confirmed. Under experimental conditions, *i.e.*, at a high level of parasitemia of the rodent malaria parasite, *P. berghei*, it was nearly impossible to eliminate the parasite completely from peripheral blood. When sulfamonomethoxine, an anti-malarial medicine, is used against *P. berghei*-infected mice with a parasitemia of 1 to 3%, it suppresses parasitemia to non-detectable levels. However, the parasites do appear later in mouse blood (11). The existence of dormant parasites in the erythrocyte stage is a possible explanation for this observation (12).

Two among 8 mice in experiments 1 and 2 died during treatment with the *H. macrophylla* extract when it was administered 3 times per day to total more than 10 mg of crude leaf extract per day, per mouse. We assume that the total amount of extract was too much for the mice, because the IC_{50} for mammalian cells (NIH 3T3-3) was $7.2 \mu\text{g/ml}$. On the other hand, like apricot-toxin or tapioca-toxin, *H. macrophylla* contains cyanogenic glycosides (13). These substances cause nausea, vomiting, and sometimes sudden death. We were unable to conclusively determine the cause of death. However, purification of the effective substance from the crude leaf-extract will most likely resolve glycoside-related toxicity. The effective dose and the toxic dose of the purified substance also remain to be determined.

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