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Antifilarial activity *in vitro* and *in vivo* of some flavonoids tested against *Brugia malayi*

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ABSTRACT

We evaluated the antifilarial activity of 6 flavonoids against the human lymphatic filarial parasite *Brugia malayi* using an *in vitro* motility assay with adult worms and microfilariae, a biochemical test for viability (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)-reduction assay), and two animal models, *Meriones unguiculatus* (implanted adult worms) and *Mastomys coucha* (natural infections). *In vitro,* naringenin and hesperetin killed the adult worms and inhibited (>60%) MTT-reduction at 7.8 and 31.2 μg/ml concentration, respectively. Microfilariae (mf) were killed at 250–500 μg/ml. The half maximal inhibitory concentration (IC_{50}) of naringenin for motility of adult females was 2.5 μg/ml. Flavone immobilized female adult worms at 31.2 μg/ml (MTT > 80%) and microfilariae at 62.5 μg/ml. Rutin killed microfilariae at 125 μg/ml and inhibited MTT-reduction in female worms for >65% at 500 μg/ml. Naringin had adulticidal effects at 125 μg/ml while chrysin killed microfilariae at 250 μg/ml. *In vivo*, 50 mg/kg of naringenin elimiated 73% of transplanted adult worms in the *Meriones* model, but had no effect on the microfilariae in their peritoneal cavity. In *Mastomys*, the same drug was less effective, killing only 31% of the naturally acquired adult worms, but 51%, when the dose was doubled. Still, effects on the microfilariae in the blood were hardly detectable, even at the highest dose. In summary, all 6 flavonoids showed antifilarial activity *in vitro*, which can be classed, in a decreasing order: naringenin > flavone = hesperetin > rutin > naringin > chrysin. In jirds, naringenin and flavone killed or sterilized adult worms at 50 mg/kg dose, but in *Mastomys*, where the parasite produces a patent infection, only naringenin was filaricidal. Thus naringenin and flavone may provide a lead for design and development of new antifilarial agent(s). This is the first report on antifilarial efficacy of flavonoids.

1. Introduction

Human lymphatic filariasis (LF), caused by the nematode parasites *Brugia malayi*, *Brugia timori* and *Wuchereria bancrofti*, affects 120 million people worldwide, of which 40 million people show the chronic disease manifestations: elephantiasis and hydrocele (http://www.globalnetwork.org; WHO, 2006). A further one billion people (18% of the world's population) are at risk of infection (http://www.globalnetwork.org). The disease is the second leading cause of permanent and long-term disability worldwide (Molyneux et al., 2003). The Global Programme to Eliminate Lymphatic Filariasis (GPELF) started using yearly doses of multi-drug regimen in a mass drug administration (MDA) program for at least five years (http://www.filariasis.org) (Molyneux and Zagaria, 2002) to interrupt transmission and reduce morbidity. However, it appears unlikely that the MDA regimen will be sufficient to eliminate LF in all endemic areas (Molyneux et al., 2003). Numerous technical challenges threaten the success of such eradication programmes (Dadzie et al., 2003; Molyneux et al., 2003), including limited efficacy of available drugs diethylcarbamazine (DEC) and ivermectin given alone or in combination, against adult filarial worms.

Killing the adult worms or sterilizing the female worms is considered to be one of the best strategies. The need for an adulticidal (macrofilaricidal) and or sterilizing agent was specially emphasized by WHO because: (a) a single female adult worm can produce thousands of microfilariae (mf), (b) in an infected host, adult worms are numerically fewer than the millions of mf; and (c) adult worms are considered responsible for some of the severe and debilitating manifestations of the disease. There are however several other advantages associated with killing the adult worms like the slow release of host-protective antigens; similarly sterilizing the worms

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Table 1
Flavonoids, their main source(s) in nature and their reported biological activities.

Class	Compound	Main source(s)	Biological activity
Flavones	Flavone	Grains, leafy vegetables and herbs	Anti-inflammatory (Hamalainen et al., 2007)
	Chrysin (5,7-dihydroxyflavone)	Passion flower (*Passiflora incarnate*), propolis	Anti-inflammatory (Woo et al., 2005), anxiolytic (Brown et al., 2007; Wolfman et al., 1994), prevents cell death (Izuta et al., 2008), promotes cell death (Jaganathan and Mandal, 2009)
Flavonols	Rutin (quercetin-3-rhamno-glucoside)	Buckwheat, Fava D'Anta tree, citrus fruits, Onions, black tea	Venotonic (Benavente-Garcia and Castillo, 2008), VEGF release inhibition (Schindler and Mentlein, 2006)
Flavanones	Naringenin (4′,5,7-trihydroxyflavanone), Naringin (naringenin-7- rhamnoglucoside)	Grapefruit, orange, tomato skin	Antioxidant (Cavia-Saiz et al., 2010; Hsu and Yen, 2006), lipid lowering, anticancer (So et al., 1996), CYP3A4 and CYP1A2 inhibition (Doostdar et al., 2000; Ghosal et al., 1996), VEGF release inhibition (Schindler and Mentlein, 2006)
	Hesperetin (4′-methoxy 3′,5,7-trihydroxyflavanone)	Citrus fruits	Anti-inflammatory (Emim et al., 1994; Hirata et al., 2005), hypolipidemic (Monforte et al., 1995; Ohtsuki et al., 2003), sedative (Guzman-Gutierrez and Navarrete, 2009; Loscalzo et al., 2008)

will help to maintain premunition of host in endemic areas that will interfere with development of in-coming L_3s in to adult worms. Recently, several investigators including our group, reported many new potential antifilarial natural products isolated from plants or synthesized in laboratory (Khunkitti et al., 2000; Lakshmi et al., 2004; Misra et al., 2007; Sahare et al., 2008a,b; Tripathi et al., 2003). We have also shown that some benzopyrone derivatives have macrofilaricidal activity against rodent filariids (Tripathi et al., 2000). Since benzopyrones are related to flavonoids we are currently exploring plant flavonoids to get new leads for developing antifilarials.

Flavonoids are one of the largest (>6000 identified) and most widely distributed groups of secondary plant metabolites (Harborne and Williams, 2000; Kuhnau, 1976) and are found in practically all photosynthesizing plants. Flavonoids are polyphenolic compounds showing a common basic C6–C3–C6 skeleton structure consisting of a heterocyclic pyran or pyrone ring flanked by a benzene ring on each side. Flavonoids are grouped into 6 main classes: (1) flavones (e.g. apigenin, flavone, chrysin), (2) flavanones (e.g. naringenin, hesperetin), (3) flavonols (e.g. quercetin and quercetin-3-rhamnoglucoside or rutin), (4) flavan-3-ols (e.g. +catechin, −epicatechin), (5) isoflavones (e.g. genistein, daidzein) and (6) anthocyanins (e.g. cyanidin, pelargonidin), on the basis of the oxidative status and the number and type of substituents on the heterocyclic ring. Extensive modifications with additional hydroxyl groups in benzene rings and methylation or glycosylation of hydroxyl groups result in more diversity. Flavonoids are mainly present in plants as glycosides; aglycones occur less frequently (Harborne and Williams, 2000). In plants, the different flavonoids have diverse functions that include providing much of the colour to flowers and fruits, symbiotic relationship with N_2-fixing rhizobia, protection from UV, pathogens (Friedman, 2007) and insects, allelopathy and inhibition of auxin transport (Buer et al., 2010). Interest in the possible health benefits of flavonoids has increased owing to their potent antioxidant and free-radical scavenging activities observed *in vitro*. Important dietary sources of flavonoids in Western societies are onions (flavonols), cocoa (proanthocyanidins), tea, apples, grape fruit and red wine (flavonols and catechins), citrus fruits (flavanones), berries and cherries (anthocyanidins), and soy (isoflavones). There is growing evidence from human feeding studies that the absorption and bioavailability of specific flavonoids is much higher than originally believed (Hollman et al., 1995). However, epidemiologic studies exploring the role of flavonoids in human health have been inconclusive. Some studies support a protective effect in cardiovascular disease and cancer, other studies demonstrate no effect, and a few studies suggest potential harm (reviewed by Ross and Kasum, 2002).

As mentioned above, one of the functions of flavonoids is to protect the plants from pathogens. Recent isolated reports show that some flavonoids and flavonoid-containing extracts possess anthelmintic and nematicidal activity: Thus, flavones induced embryonic and larval lethality in the pinewood nematode, *Bursaphelenchus xylophilus* and in the free-living nematode *Caenorhabditis elegans* (Kim et al., 2009; Lee et al., 2008) and showed anthelmintic activity (Ayers et al., 2008). Propolis extract containing flavonoids protected faba bean plants from nematode infection (Noweer and Dawood, 2009). Flavonol glycosides such as rutin also possess anthelmintic activity (Barrau et al., 2005). Among flavonones, 5,7-dihydroxyflavanone (pinocembrine) showed fasciolicide, ovicide and larvicide activities on newly excysted *Fasciola hepatica*, on infective eggs of the nematode *Ascaridia galli* and on 3rd stage larvae of the biting fly *Stomoxys calcitrans*, respectively (Del Rayo Camacho et al., 1991). Some prenylated flavanones were also found active against plant nematode *Rotylenchulus reniformis* (Shakil et al., 2008). Flavan-3-ols and their derivatives such as epigallocatechingallate were found to be potent anthelmintics (Mukai et al., 2008) and inhibit egg hatching and larval development in nematodes (Molan et al., 2003).

This information encouraged us to investigate whether flavonoids have the potential to kill or affect human lymphatic dwelling filarial nematode *B. malayi* also. For this purpose we randomly selected 6 pure compounds belonging to different classes of flavonoids and assayed them in 2 *in vitro* systems against mf and adult worms and in 2 animal models of the infection. Use of pure flavonoids is to ensure that the observed effect is due to the flavonoid alone and to avoid the difficulties in interpreting the data associated with the use of crude or semi-purified extracts which may contain many other substances. The flavonoids selected for the present study, their main source(s) in nature and their reported biological activities are presented in Table 1.

2. Materials and methods

2.1. Chemicals and reagents

Rutin (quercetin-3-rhamnoglucoside), Flavone, Naringenin (4′,5,7-trihydroxyflavanone), Hesperetin (4′-methoxy 3′,5,7-trihydroxyflavanone), Chrysin (5,7-dihydroxyflavone), Naringin (Naringenin-7-rhamnoglucoside) and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were procured from Sigma–Aldrich, USA. All other chemicals and reagents were reagent grade and procured from local suppliers.

2.2. Parasites

The human filarial parasite *B. malayi* was maintained in *Mastomys coucha* (Murthy et al., 1983) and jirds (*Meriones unguiculatus*) (Murthy et al., 1997) as described elsewhere. Microfilariae (mf) and adult worms freshly harvested from the peritoneal cavity of the jirds exposed to infective 3rd stage larvae (L_3) 5–6 months back were washed thoroughly in HBSS and used for *in vitro* assays and for transplantation into peritoneal cavity of the jirds.

2.3. In vitro assays

The efficacy of compounds to affect the viability of adult worms and mf *in vitro* was assessed using the motility and MTT reduction assays (Murthy and Chatterjee, 1999). Test and reference compounds were dissolved in DMSO and used at a concentration range of 3.13– 500 μg/ml (using two-fold serial dilutions). Diethylcarbamazine (DEC) was used as reference compound. DMSO was used in place of test agent solution for controls.

2.4. Assessment of in vitro efficacy

The viability of the treated worms was assessed by calculating percent inhibition in motility and MTT reduction over DMSO treated worms (Murthy and Chatterjee, 1999) using a total of 4 worms (in replicates of 2 worms) for motility and MTT assays and 160–200 mf (in replicates of 40–50 mf/100 μl/well) for motility assay per test concentration. Parasite motility was assessed under a microscope after 24 h exposure to test substance and scored as: 0 = dead; 1–4 = loss of motility (1 = 75%; 2 = 50%; 3 = 25% and 4 = no loss of motility). Loss of motility is defined as the inability of the worms to regain pretreatment level motility even after incubating in fresh medium *minus* the test agent at 37 °C for 25–30 min and is expressed as percentage (%) of control. 100% inhibition in motility of female adults or mf and or ≥50 inhibition in MTT reduction ability of adult parasites was considered acceptable antifilarial activity.

2.5. Determination of IC_{50}

For IC_{50} (the concentration at which the parasite motility was inhibited by 50%) determination, the adult worm (total 4; one female/1 ml/well) or mf (40–50 mf/100 μl/well) were incubated in multi-well test plates with the test agents or DEC at concentration ranging from 3.13 to 500 μg/ml (obtained by two-fold serial dilution at each step). Experiments were run in duplicate and incubations were carried out in replicates for 24 h at 37 °C. Percent inhibition in motility was determined as described above and the data were transferred into a graphic program (MS Excel) and IC_{50} was calculated by linear interpolation between the two concentrations above and below 50% (Huber and Koella, 1993).

2.6. Determination of cytotoxic concentration 50 (CC_{50})

The cytotoxicity assay of the test substances was done following the method of Page et al. (1993) with some modifications (Mosmann, 1983). Briefly, VERO Cell line C1008 (African green monkey kidney cells) was seeded in 96-well microtiter plates (Nunc, Denmark) at 0.1×10^6 cells/ml (100 μl per well) in DMEM supplemented with 10% heat inactivated FBS. A three-fold serial dilution of the test substance (starting from $>20 \times LC_{100}$ concentration of the test agent) in test medium was added. Plates with a final volume of 100 μl per well were incubated in 5% CO_2 atmosphere at 37 °C. After 72 h incubation 10 μl of 0.025% Resazurin in phosphate buffered saline (PBS; pH 7.2) was added as viability indicator (Ahmed et al., 1994). After an additional incubation for 4 h the plate was read in a fluorescence reader (Synergy HT plate reader, Biotek, USA) at excitation wavelength of 530 nm and an emission wavelength of 590 nm. Data were transferred into a graphic program (MS Excel) and CC_{50} was calculated by linear interpolation between the two concentrations above and below 50% inhibition (Huber and Koella, 1993). The assay was run in replicates in each of two independent experiments.

Selectivity Index (SI) of the agents were computed by the formula $SI = CC_{50}/IC_{50}$.

2.7. In vivo efficacy

2.7.1. Host–parasite models

All the experiments in animals were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. Throughout the study, the animals were housed in climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light–dark cycles) controlled animal quarters. They were fed standard rodent chow supplemented with dried shrimps and had free access to drinking water.

M. coucha and jirds infected with *B. malayi* were used for testing the flavonoids. At the end of observation period the treated and control animals were autopsied under deep anesthesia following the guidelines of Institutional Animal Ethics Committee.

2.7.2. Administration of the test agents and reference drug

The test agents were pulverized to fine powder and suspended in 0.1% Tween-80 in distilled water. DEC was prepared in distilled water. In jirds the test agents and DEC were administered at 25 mg/kg body weight through s.c. route for 5 consecutive days. In *M. coucha* the test agent was administered at 25, 50 and 100 mg/kg body weight i.p. × 5 consecutive days. The suspensions/solutions of the test and reference compounds were prepared fresh daily before administration.

2.7.3. B. malayi-jird model

8–10 weeks old male jirds received intraperitoneal transplantation of adult worms isolated from peritoneal cavity of infected jirds as described elsewhere (Gaur et al., 2007). Each animal received 10 female and 5 male adult worms. On day 2 or 3 post-adult worm transplantation (p.a.t.), the peritoneal fluid was aspirated and checked for the presence of mf. Test or reference compound administration was started on day 7 or 8 p.a.t. The animals were sacrificed on day 42 post initiation of treatment (p.i.t.) and parasites were collected and counted (Gaur et al., 2007).

2.7.4. B. malayi–M. coucha model

Animals harbouring 5–7 months old *B. malayi* infection and showing progressive rise in microfilraemia were selected for the study. Microfilaremia was monitored by examination of night-blood as described elsewhere (Murthy et al., 1983) before initiation of treatment (day 0), on days 7/8 and 14, and thereafter at fort-

Table 2
In vitro activity of flavonoids on adult worms and microfilariae of *Brugia malayi* (values are mean ± SD).

Compound	Effect on female adult worm			Effect on microfilariae		CC_{50}[c] (μg/ml)	SI for adult motility
	LC_{100}[a] (μg/ml) in motility assay	IC_{50} (μg/ml) in motility assay[b]	%Inhibition in MTT reduction at LC_{100}	LC_{100}[a] (μg/ml) in motility assay	IC_{50} (μg/ml) in motility assay[b]		
Rutin (quercetin-3-rhamnoglucoside)	>500	–	66 ± 5.4[d]	125	74.3 ± 7.2	269.2 ± 26.9	1.1 ± 0.1
Flavone	31.2	22.6 ± 2.8	82 ± 19.1	62.5	23.7 ± 0.5	67.2 ± 16.8	3.0 ± 0.8
Naringenin (4′,5,7-trihydroxyflavanone)	7.8	2.5 ± 0.2	62 ± 13.7	500	297.3 ± 5.2	178.8 ± 23.5	72.7 ± 9.6
Hesperetin (4′-methoxy 3′,5,7-trihydroxy flavanone)	31.2	18.2 ± 2.6	63 ± 20.0	250	88.4 ± 17.0	116.4 ± 21.8	5.9 ± 0.5
Chrysin (5,7-dihydroxyflavone)	>500	–	20 ± 27.6[d]	250	105.1 ± 21.1	223.4 ± 5.5	0.9 ± 0.0
Naringin (Naringenin-7-rhamnoglucoside)	125	78.8 ± 11.5	NI	>500	–	120.6 ± 22.0	1.5 ± 0.3
DEC-C	500	288.7 ± 15.1	64 ± 9.8	500	353.6 ± 17.9	9102.9 ± 1058.5	31.9 ± 3. 7

[a] 100% reduction in motility indicates 100% death of parasite.
[b] IC_{50} = concentration of the agent at which 50% inhibition in motility was achieved.
[c] CC_{50} = concentration at which 50% of cells are killed; SI = Selectivity Index (= CC_{50}/IC_{50}).
[d] Achieved at 500 μg/ml; DEC-C = diethylcarbamazine-citrate; NI = no inhibition; a total of 4 female worms at each concentration was used in two experiments.

nightly intervals till day 90 p.i.t. The animals were sacrificed on day 90 p.i.t.

2.7.5. Assessment of microfilaricidal efficacy

Microfilaricidal efficacy of the test agents was expressed as percent reduction in mf count over the pretreatment level (Chatterjee et al., 1992; Gaur et al., 2007; Lämmler and Wolf, 1977).

2.7.6. Assessment of macrofilaricidal and worm sterilization efficacy

Treated and untreated animals were killed on day 42 (jird) or day 90 (*M. coucha*) p.i.t. Adult worms were recovered from the peritoneal cavity washings (jird) or various tissues such as heart, lungs, and testes (*M. coucha*). Tissues were teased gently to avoid any damage to the worms. Parasites were microscopically examined for motility, cell adherence on their surface, death, or encapsulation and all the surviving females were teased individually in a drop of saline to examine the condition of intrauterine contents (Chatterjee et al., 1992; Gaur et al., 2007).

Macrofilaricidal/adulticidal efficacy of the treatments was assessed and expressed as percent reduction in adult worm recovery in treated group over untreated animals. In case of transplanted jirds, the condition of worms recovered was recorded as live, dead, encapsulated, or recovered as calcified fragments. For assessing sterilization of female worms, any death, abnormality, or distortion of embryonic stages were recorded and used to determine the percentage of female worms showing abnormal embryos among the live female worms. As 2–10% of embryos/female worm from even untreated animals show abnormalities, only those worms with at least 60% embryos abnormal were considered sterile. The %sterile worms in treated groups were compared with the %sterile worms in untreated control animals.

2.8. Statistical analysis

The Newman–Keuls multiple comparison test and the Student's *t*-test were used to analyze the data.

3. Results and discussion

3.1. In vitro efficacy

The effects of flavonoids on female adult parasites and mf *in vitro* are shown in Table 2. Naringenin and hesperetin produced 100% inhibition in motility and >60% inhibition in MTT reduction of female worms at 7.8 and 31.25 μg/ml respectively, but mf were killed only at higher concentrations (250–500 μg/ml). Flavone completely inhibited only motility of mf and female worms (LC_{100}: 31.25–62.5 μg/ml), whereas rutin produced 100% inhibition in mf motility and >65% inhibition in MTT reduction of the female parasites at 500 μg/ml. Naringin killed adults at 125 μg/ml, but showed no effect on mf even at 500 μg/ml. Chrysin killed mf at 250 μg/ml but was ineffective on adults up to 500 μg/ml. DEC killed the adults, affected motility of mf and caused 64% inhibition in MTT reduction in the female parasites at 500 μg/ml. We earlier used DEC at 400 μg/ml to get around 70% inhibition of worm motility (Singh et al., 2008). The vehicle exposed parasites showed no inhibition in motility of adult or mf stages or inhibition of MTT reduction.

In summary, all the 6 flavonoids showed antifilarial activity *in vitro*; the activity of the flavonoids can be graded as naringenin > flavone = hesperetin > rutin > naringin > chrysin. Naringenin also showed high SI (>70) for motility of adult parasites.

3.2. In vivo efficacy

All the 6 flavonoids were tested in *B. malayi*/jird model. The macrofilaricidal and worm-sterilizing efficacy of the flavonoids in this model is shown in Table 3. Naringenin at 50 mg/kg dose showed significant macrofilaricidal efficacy (~73%; $P < 0.01$). Flavone at this dose showed 38% macrofilaricidal effect which was not considered as promising. The rest of the flavonoids did not show any macrofilaricidal or sterilizing effect. Also, none of the flavonoids had any effect on the mf in the peritoneal cavity of animals. The reference drug DEC citrate (25 mg/kg) was also ineffective against peritoneal mf and adult worms. Parasites recovered from untreated control animals were healthy with around 14% female parasites sterile.

Naringenin, which has shown promising macrofilaricidal activity in *B. malayi*/jird model was further evaluated in *B. malayi*/*M. coucha* model. Table 4 shows the effect of naringenin on microfilaremia and adult worms in this model. Naringenin showed 51, 31 and 12% macrofilaricidal activity at 100, 50 and 25 mg/kg dose levels, with 75, 45 and 23% female worm sterilization, respectively. In summary, naringenin showed dose dependent macrofilaricidal and female worm sterilization efficacy though the efficacy was good and acceptable only at 100 mg/kg dose level.

As expected the reference drug DEC at 50 mg/kg (i.p.) caused more than 70% ($P < 0.001$) reduction in microfilaraemia on day 7

Table 3
In vivo antifilarial activity of flavonoids and DEC against transplanted adult worms of *Brugia malayi* in jird (values are mean ± SD).

Compound (Dosing: 50 mg/kg, s.c. × 5d)	No. of animals	Effect on microfilariae in peritoneal cavity	No. live worms			Sterile female worm count[b]
			Male	Female	Total[a]	
Rutin (quercetin-3-rhamnoglucoside)	4	No effect	4 ± 1.2	7 ± 2.0	11 ± 3.1	0 ± 0.6 (4 ± 6.4)
Flavone	4	No effect	3 ± 0.6	5 ± 3.1	8 ± 3.6 (38 ± 28.8)*	2 ± 1.0 (47 ± 36.7)
Naringenin (4′,5,7-trihydroxy flavanone)	4	No effect	1 ± 0.7	3.00 ± 2.8	4 ± 3.5 (73 ± 28.3)**	1 ± 0.7 (50 ± 70.7)
Hesperetin (4′, methoxy, 3′,5,7 trihydroxy flavanone)	4	No effect	4 ± 1.0	8 ± 2.1	12 ± 2.5	1 ± 0.6
Chrysin (5,7 dihydroxy flavone)	4	No effect	4 ± 1.5	8 ± 1.5	12 ± 2.0	1.00 ± 1.7
Naringin (Naringinin-7-rhamnoglucoside)	4	No effect	4 ± 1.0	8 ± 1.2	12 ± 0.6	1 ± 0.6
DEC-C[c]	6	No effect	2 ± 1.5	8 ± 1.5	10 ± 2. 9 (15 ± 23.8)	1 ± 1.0
Untreated Control	4	No effect	4 ± 1.3	9 ± 0.8	13 ± 1.9	1 ± 0.5 (14 ± 6.0)

[a] Values in parentheses are %reduction over control.
[b] Values in parentheses are %sterile females among live female worms.
[c] DEC-C: diethylcarbamazine-citrate (25 mg/kg, s.c. × 5d).
* $P < 0.05$.
** $P < 0.01$.

p.i.t. in this model. In these animals 20% female worms were sterile but no macrofilaricidal activity was detected.

Untreated control animals (*M. coucha*) showed progressive rise in microfilaraemia till termination of the experiment. About 20% of live female worms recovered from these animals were sterile (Table 4).

All the flavonoids tested were well tolerated in both the animal models and there were no signs of behavioral or other changes that can be related to flavonoid treatment in the animals throughout the observation period.

The dosing route and schedules used in the animal assays (25 mg/kg, s.c. × 5d in jird and 25, 50 and 100 mg/kg, i.p. × 5d in *M. coucha*) are the standard procedures in our laboratory for testing new pure compounds for antifilarial activity (Fatma et al., 1989; Gaur et al., 2008; Lämmler, 1977; Sadanaga et al., 1984; Singh et al., 2008; Suswillo and Denham, 1977). The parenteral routes of administrations are intentionally selected to bypass the uncertainties posed by differences in alimentary absorption and 1st pass hepatic handling of flavonoids after oral dosing. Once the efficacy is identified by s.c./i.p. routes, the active compounds are followed-up by studies with oral dosing, determination of plasma levels, ADME, etc. We are indeed planning these studies on the active compounds.

The mechanism by which the flavonoids affect the viability of filarial parasites is not known. In the present study, naringenin has shown promising adulticidal activity with (in *B. malayi*/*M. coucha*) or without (in *B. malayi*/jird) female sterilizing activity. Naringenin has been claimed to show antioxidant activity (Cavia-Saiz et al., 2010). But it is difficult to relate the antioxidant activity directly as the cause of antifilarial activity. On the other hand, induction of apoptosis was found to be related to antioxidant activity by Hsu and Yen (2006) and this might be responsible for at least the worm sterilizing effect of the flavonoid. As for the identity of the antifilarial pharmacophore in the flavonoid, it appears from the present study that the host's metabolism, if any, of naringenin has little effect on the activity because naringenin was effective on the parasite both *in vitro* and in the hosts. However, one may also take into consideration the observation that host metabolism might transform one class of flavonoid into another resulting in generation of new pharmacological activity or the loss of previous activity (Nikolic and van Breemen, 2004). This may be responsible for the absence of notable *in vivo* activity for the rest of flavonoids that were otherwise active *in vitro* in the present study. In conclusion, all the 6 flavonoids showed antifilarial activity *in vitro*; the activity can be graded as naringenin > flavone = hesperetin > rutin > naringin > chrysin. In the jird model, only naringenin and flavone showed, in that order, potent macrofilaricidal efficacy at 50 mg/kg dose whereas only naringenin showed both macrofilaricidal and female sterilizing activity in a dose dependent manner in the *M. coucha* model.

Table 4
In vivo antifilarial activity of Naringenin (4′,5,7-trihydroxyflavanone) and DEC against *Brugia malayi* in *Mastomys coucha* (values are mean ± SD).

Compound	Dose (mg/kg) i.p. × 5d (*N*)	Microfilariae/10 μl tail blood on different days post initiation of treatment			No. live worms			Sterile female worm count[b]
		Day 0	Day 7/8	Day 90	Male	Female	Total[a]	
Naringenin (4′,5,7-trihydroxyflavanone)	100 (6)	71 ± 23.9	92 ± 35.8	79 ± 25.1	3 ± 0.8	7 ± 1.6	10 ± 2.2 (51 ± 11.1)***	5 ± 1.7 (75 ± 17.8)***
	50 (6)	89 ± 27.0	108 ± 45.5	111 ± 21.7	5 ± 0.9	9 ± 1.9	14 ± 2.1 (31 ± 5.9)*	4 ± 1.2 (45 ± 4.7)***
	25 (6)	59 ± 26.6	75 ± 13.8	201 ± 37.0	4 ± 1.1	13 ± 1.5	17 ± 4.2 (12 ± 7.6)	3 ± 1.7 (23 ± 14.4)
DEC-C	50 (6)	108 ± 63.3	9 ± 5.4	141 ± 78.8	6 ± 1.8	14 ± 4.0	20 ± 3.5	4 ± 6.1 (21 ± 29.5)
Untreated control	Vehicle (6)	54 ± 31.7	114 ± 52.6	170 ± 64.2	7 ± 3.6	12 ± 3.0	19 ± 3.0	2 ± 1.4 (20 ± 9.6)

N = no. of animals.
[a] Values in parentheses are %reduction over control.
[b] Values in parentheses are %sterile females among live female worms; DEC-C: diethylcarbamazine-citrate.
* $P < 0.05$.
*** $P < 0.001$.

Thus, naringenin and flavone may provide a lead for design and development of new antifilarial agent(s). This is the first report on antifilarial efficacy of flavonoids.

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References

Ahmed, S.A., Gogal Jr., R.M., Walsh, J.E., 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J. Immunol. Methods 170, 211–224.

Ayers, S., Zink, D.L., Mohn, K., Powell, J.S., Brown, C.M., Murphy, T., Brand, R., Pretorius, S., Stevenson, D., Thompson, D., Singh, S.B., 2008. Flavones from *Struthiola argentea* with anthelmintic activity *in vitro*. Phytochemistry 69, 541–545.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology 131, 531–538.

Benavente-Garcia, O., Castillo, J., 2008. Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. J. Agric. Food Chem. 56, 6185–6205.

Brown, E., Hurd, N.S., McCall, S., Ceremuga, T.E., 2007. Evaluation of the anxiolytic effects of chrysin, a *Passiflora incarnata* extract, in the laboratory rat. Aana J. 75, 333–337.

Buer, C.S., Imin, N., Djordjevic, M.A., 2010. Flavonoids: new roles for old molecules. J. Integr. Plant Biol. 52, 98–111.

Cavia-Saiz, M., Busto, M.D., Pilar-Izquierdo, M.C., Ortega, N., Perez-Mateos, M., Muniz, P., 2010. Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. J. Sci. Food Agric. 90, 1238–1244.

Chatterjee, R.K., Fatma, N., Murthy, P.K., Sinha, P., Kulshreshtha, D.K., Dhawan, B.N., 1992. Macrofilaricidal activity of the stem bark of *Streblus asper* and its major active constituents. Drug Dev. Res. 26, 67–78.

Dadzie, Y., Neira, M., Hopkins, D., 2003. Final report of the Conference on the eradicability of Onchocerciasis. Filaria J. 2, 2.

Del Rayo Camacho, M., Sanchez, B., Quiroz, H., Contreras, J.L., Mata, R., 1991. Pinocembrine: a bioactive flavanone from *Teloxys graveolens*. J. Ethnopharmacol. 31, 383–389.

Doostdar, H., Burke, M.D., Mayer, R.T., 2000. Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology 144, 31–38.

Emim, J.A., Oliveira, A.B., Lapa, A.J., 1994. Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. J. Pharm. Pharmacol. 46, 118–122.

Fatma, N., Sharma, S., Chatterjee, R.K., 1989. 2,2′-Dicarbomethoxyamino-5,5′-dibenzimidazolyl ketone—a new antifilarial agent. Acta Trop. 46, 311–321.

Friedman, M., 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Mol. Nutr. Food Res. 51, 116–134.

Gaur, R.L., Dixit, S., Sahoo, M.K., Khanna, M., Singh, S., Murthy, P.K., 2007. Anti-filarial activity of novel formulations of albendazole against experimental brugian filariasis. Parasitology 134, 537–544.

Gaur, R.L., Sahoo, M.K., Dixit, S., Fatma, N., Rastogi, S., Kulshreshtha, D.K., Chatterjee, R.K., Murthy, P.K., 2008. Antifilarial activity of *Caesalpinia bonducella* against experimental filarial infections. Indian J. Med. Res. 128, 65–70.

Ghosal, A., Satoh, H., Thomas, P.E., Bush, E., Moore, D., 1996. Inhibition and kinetics of cytochrome P4503A activity in microsomes from rat, human, and cdna-expressed human cytochrome P450. Drug Metab. Dispos. 24, 940–947.

Guzman-Gutierrez, S.L., Navarrete, A., 2009. Pharmacological exploration of the sedative mechanism of hesperidin identified as the active principle of *Citrus sinensis* flowers. Planta Med. 75, 295–301.

Hamalainen, M., Nieminen, R., Vuorela, P., Heinonen, M., Moilanen, E., 2007. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm., 45673.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Hirata, A., Murakami, Y., Shoji, M., Kadoma, Y., Fujisawa, S., 2005. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. Anticancer Res. 25, 3367–3374.

Hollman, P.C., de Vries, J.H., van Leeuwen, S.D., Mengelers, M.J., Katan, M.B., 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am. J. Clin. Nutr. 62, 1276–1282.

Hsu, C.L., Yen, G.C., 2006. Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity. Mol. Nutr. Food Res. 50, 1072–1079.

Huber, W., Koella, J.C., 1993. A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. Acta Trop. 55, 257–261.

Izuta, H., Shimazawa, M., Tazawa, S., Araki, Y., Mishima, S., Hara, H., 2008. Protective effects of Chinese propolis and its component, chrysin, against neuronal cell death via inhibition of mitochondrial apoptosis pathway in SH-SY5Y cells. J. Agric. Food Chem. 56, 8944–8953.

Jaganathan, S.K., Mandal, M., 2009. Antiproliferative effects of honey and of its polyphenols: a review. J. Biomed. Biotechnol. 2009, 830616.

Khunkitti, W., Fujimaki, Y., Aoki, Y., 2000. *In vitro* antifilarial activity of extracts of the medicinal plant *Cardiospermum halicacabum* against *Brugia pahangi*. J. Helminthol. 74, 241–246.

Kim, K.S., Kawasaki, I., Chong, Y., Shim, Y.H., 2009. Inhibition of overexpressed CDC-25.1 phosphatase activity by flavone in *Caenorhabditis elegans*. Mol. Cells 27, 345–350.

Kuhnau, J., 1976. The flavonoids A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet 24, 117–191.

Lakshmi, V., Kumar, R., Gupta, P., Varshney, V., Srivastava, M.N., Dikshit, M., Murthy, P.K., Misra-Bhattacharya, S., 2004. The antifilarial activity of a marine red alga Botryocladia leptopoda, against experimental infections with animal and human filariae. Parasitol. Res. 93, 468–474.

Lämmler, G., 1977. Experimental chemotherapy and chemoprophylaxis of filariasis. Pesticide Sci. 8, 563–576.

Lämmler, G., Wolf, E., 1977. Chemoprophylactic activity of filaricidal compounds on *Litomosoides carinii* infection of *Mastomys natalensis* (author's transl). Tropenmed. Parasitol. 28, 205–225.

Lee, Y.U., Kawasaki, I., Lim, Y., Oh, W.S., Paik, Y.K., Shim, Y.H., 2008. Inhibition of developmental processes by flavone in *Caenorhabditis elegans* and its application to the pinewood nematode *Bursaphelenchus xylophilus*. Mol. Cells 26, 171–174.

Loscalzo, L.M., Wasowski, C., Paladini, A.C., Marder, M., 2008. Opioid receptors are involved in the sedative and antinociceptive effects of hesperidin as well as in its potentiation with benzodiazepines. Eur. J. Pharmacol. 580, 306–313.

Misra, N., Sharma, M., Raj, K., Dangi, A., Srivastava, S., Misra-Bhattacharya, S., 2007. Chemical constituents and antifilarial activity of *Lantana camara* against human lymphatic filariid *Brugia malayi* and rodent filariid *Acanthocheilonema viteae* maintained in rodent hosts. Parasitol. Res. 100, 439–448.

Molan, A.L., Meagher, L.P., Spencer, P.A., Sivakumaran, S., 2003. Effect of flavan-3-ols on in vitro egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. Int. J. Parasitol. 33, 1691–1698.

Molyneux, D.H., Bradley, M., Hoerauf, A., Kyelem, D., Taylor, M.J., 2003. Mass drug treatment for lymphatic filariasis and onchocerciasis. Trends Parasitol. 19, 516–522.

Molyneux, D.H., Zagaria, N., 2002. Lymphatic filariasis elimination: progress in global programme development. Ann. Trop. Med. Parasitol. 96 (Suppl. 2), S15–S40.

Monforte, M.T., Trovato, A., Kirjavainen, S., Forestieri, A.M., Galati, E.M., Lo Curto, R.B., 1995. Biological effects of hesperidin, a Citrus flavonoid (note II): hypolipidemic activity on experimental hypercholesterolemia in rat. Farmaco 50, 595–599.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mukai, D., Matsuda, N., Yoshioka, Y., Sato, M., Yamasaki, T., 2008. Potential anthelmintics: polyphenols from the tea plant *Camellia sinensis* L. are lethally toxic to *Caenorhabditis elegans*. J. Nat. Med. 62, 155–159.

Murthy, P.K., Chatterjee, R.K., 1999. Evaluation of two in vitro test systems employing *Brugia malayi* parasite for screening of potential antifilarials. Curr. Sci. 77, 1084–1089.

Murthy, P.K., Murthy, P.S.R., Tyagi, K., Chatterjee, R.K., 1997. Fate of infective larvae of *Brugia malayi* in the peritoneal cavity of Mastomys natalensis and *Meriones unguiculatus*. Folia Parasitol. (Praha) 44, 302–304.

Murthy, P.K., Tyagi, K., Roy Chowdhury, T.K., Sen, A.B., 1983. Susceptibility of *Mastomys natalensis* (GRA strain) to a subperiodic strain of human *Brugia malayi*. Indian J. Med. Res. 77, 623–630.

Nikolic, D., van Breemen, R.B., 2004. New metabolic pathways for flavanones catalyzed by rat liver microsomes. Drug Metab. Dispos. 32, 387–397.

Noweer, E.M., Dawood, M.G., 2009. Efficiency of propolis extract on faba bean plants and its role against nematode infection. Commun. Agric. Appl. Biol. Sci. 74, 593–603.

Ohtsuki, K., Abe, A., Mitsuzumi, H., Kondo, M., Uemura, K., Iwasaki, Y., Kondo, Y., 2003. Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. J. Nutr. Sci. Vitaminol. (Tokyo) 49, 447–450.

Page, C., Page, M., Noel, C., 1993. A new fluorimetric assay for cytotoxicity measurements *in vitro*. Int. J. Oncol. 3, 473–476.

Ross, J.A., Kasum, C.M., 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. 22, 19–34.

Sadanaga, A., Hayashi, Y., Tanaka, H., Nogami, S., Shirasaka, A., 1984. Evaluation of the transplantation method of adult *Brugia malayi* into the jird *Meriones unguiculatus*, for testing macrofilaricides. Jpn. J. Exp. Med. 54, 275–277.

Sahare, K.N., Anandharaman, V., Meshram, V.G., Meshram, S.U., Gajalakshmi, D., Goswami, K., Reddy, M.V., 2008a. *In vitro* effect of four herbal plants on the motility of *Brugia malayi* microfilariae. Indian J. Med. Res. 127, 467–471.

Sahare, K.N., Anandharaman, V., Meshram, V.G., Meshram, S.U., Reddy, M.V., Tumane, P.M., Goswami, K., 2008b. Anti-microfilarial activity of methanolic extract of *Vitex negundo* and *Aegle marmelos* and their phytochemical analysis. Indian J. Exp. Biol. 46, 128–131.

Schindler, R., Mentlein, R., 2006. Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. J. Nutr. 136, 1477–1482.

Shakil, N.A., Pankaj, Kumar, J., Pandey, R.K., Saxena, D.B., 2008. Nematicidal prenylated flavanones from *Phyllanthus niruri*. Phytochemistry 69, 759–764.

Singh, B.K., Mishra, M., Saxena, N., Yadav, G.P., Maulik, P.R., Sahoo, M.K., Gaur, R.L., Murthy, P.K., Tripathi, R.P., 2008. Synthesis of 2-sulfanyl-6-methyl-1,4-dihydropyrimidines as a new class of antifilarial agents. Eur. J. Med. Chem. 43, 2717–2723.

So, F.V., Guthrie, N., Chambers, A.F., Moussa, M., Carroll, K.K., 1996. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr. Cancer 26, 167–181.

Suswillo, R.R., Denham, D.A., 1977. A new system of testing for filaricidal activity using transplanted adult *Brugia* in the jird. J. Parasitol. 63, 591–592.

Tripathi, R.P., Tiwari, V.K., Misra-Bhattacharya, S., Tyagi, K., Srivastava, V.M., Murthy, P.K., 2003. 7- O-[4-methyl piperazine-1-(2-acetyl)]-2H-1-benzopyran-2-one: a novel antifilarial lead compound. Acta Trop. 87, 215–224.

Tripathi, R.P., Tripathi, R., Bhaduri, A.P., Singh, S.N., Chatterjee, R.K., Murthy, P.K., 2000. Antifilarial activity of some 2H-1-benzopyran-2-ones (coumarins). Acta Trop. 76, 101–106.

WHO, 2006. Global programme to eliminate lymphatic filariasis. Wkly Epidemiol. Rec. 81, 221–232.

Wolfman, C., Viola, H., Paladini, A., Dajas, F., Medina, J.H., 1994. Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from *Passiflora coerulea*. Pharmacol. Biochem. Behav. 47, 1–4.

Woo, K.J., Jeong, Y.J., Inoue, H., Park, J.W., Kwon, T.K., 2005. Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity. FEBS Lett. 579, 705–711.