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Brief Communication

Antiamoebic activities of flavonoids against pathogenic free-living amoebae, *Naegleria fowleri* and *Acanthamoeba* species

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Abstract

Free-living amoebae (FLA) rarely cause human infections but can invoke fatal infections in the central nervous system (CNS). No consensus treatment has been established for FLA infections of the CNS, emphasizing the urgent need to discover or develop safe and effective drugs. Flavonoids, natural compounds from plants and plant-derived products, are known to have antiprotozoan activities against several pathogenic protozoa parasites. The anti-FLA activity of flavonoids has also been proposed, while their antiamoebic activity for FLA needs to be emperically determined. We herein evaluated the antiamoebic activities of 18 flavonoids against *Naegleria fowleri* and *Acanthamoeba* species which included *A. castellanii* and *A. polyphaga*. These flavonoids showed different profiles of antiamoebic activity against *N. fowleri* and *Acanthamoeba* species. Demethoxycurcumin, kaempferol, resveratrol, and silybin (A+B) showed in vitro antiamoebic activity against both *N. fowleri* and *Acanthamoeba* species. Apigenin, costunolide, (–)-epicatechin, (–)-epigallocatechin, rosmarinic acid, and (–)-trans-caryophyllene showed selective antiamoebic activity for *Acanthamoeba* species. Luteolin was more effective for *N. fowleri.* However, afzelin, berberine, (±)-catechin, chelerythrine, genistein, (+)-pinostrobin, and quercetin did not exhibit antiamoebic activity against the amoeba species. They neither showed selective antiamoebic activity with significant cytotoxicity to C6 glial cells. Our results provide a basis for the anti-FLA activity of flavonoids, which can be applied to develope alternative or supplemental therapeutic agents for FLA infections of the CNS.

Keywords: *Naegleria fowleri*, *Acanthamoeba*, flavonoids, antiamoebic activity

Free-living amoebae (FLA) are protozoa that live autonomously in diverse environments, such as soil and fresh water, and feed on bacteria, fungi, and algae. However, *Naegleria fowleri, Balamuthia mandrillaris*, and *Acanthamoeba* species are pathogenic and can infect humans and cause life-threatening diseases [1]. Although human infections by these FLA are rare, they can cause rapidly progressive and severe central nervous system (CNS) infections that are almost fatal [1].

Primary amebic meningoencephalitis (PAM) is a brain infection caused by *Neglaria fowleri*. The amoeba enters the brain via the olfactory neuroepithelium and induces an acute hemorrhagic-necrotizing meningoencephalitis accomplished with fulminating inflammation [2]. The rapid progression of PAM can cause death (mortality rate >97%) within 1 or 2 weeks after the initial exposure [2]. *Acanthamoeba* species cause diseases such as acanthamoeba keratitis and granulomatous amebic encephalitis (GAE) in humans [3]; GAE also has

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Conflict of interest
The authors declare no conflict of interest.

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a high lethality of up to 90%, particularly in immunocompromised humans [3]. Such high fatalities of PAM and GAE are mainly attributed to the lack of a reliable and rapid diagnosis method and effective therapeutic drugs. The currently recommended drug treatment regimens either alone or in combination for PAM and GAE have typically been used for treating bacterial and fungal infections [2] but provide no guarantee for successful treatment, and undesirable side effects of these drugs are also a concern. Therefore, the development of novel effective drugs for PAM and GAE is urgently required.

Natural compounds from plants have been used to manage or treat diverse diseases throughout human history [4]. Recently, large screening approaches to find natural compounds with antiamoebic or amoebicidal activity against *N. fowleri* and *Acanthamoeba* species from diverse natural resources have been performed [5-9]. These studies demonstrated the amoebicidal or antiamoebic effects of natural compounds or plant extracts against *N. fowleri* and *Acanthamoeba* species, suggesting these compounds have potential applications as therapeutic candidates or supplemental compounds for PAM and GAE.

Flavonoids, natural compounds isolated from plants and plant-derived products, have gained attention as attractive alternative drugs or leads for protozoan parasites such as *Entamoeba histolytica*, *Giardia intestinalis*, *Cryptosporidium parvum*, and *Trypanosoma cruzi* [10-13]. Several flavonoids have recently been proposed as potential candidates for anti-FLA drug development based on these studies [14]. However, their antiamoebic activity for FLA has not yet been experimentally determined. Herein, we evaluated the antiamoebic activity of selected flavonoids against trophozoites of *N. fowleri* and *Acanthamoeba* species.

Based on a literature review [10,14], a selection of 18 flavonoids was made according to their potential antiprotozoan activities on other protozoan parasites and included (−)-epicatechin, (−)-epigallocatechin, (±)-catechin hydrate, demethoxycurcumin, luteolin, resveratrol, silybin (A+B) mixture, rosmarinic acid, (−)-trans-caryophyllene, costunolide, kaempferol, apigenin, afzelin, quercetin, genistein, berberine, chelerythrine chloride, and (+)-pinostrobin. Azithromycin dihydrate and miltefosine were included as control drugs. All flavonoids and chemicals were purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma) or distilled water at 100 mM. The purity of all flavonoids was >95%.

Neglaria fowleri (Carter NF69 strain, ATCC 30215) trophozoites were cultured and maintained in Nelson's medium containing 5% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco) at 37°C. *A. castellanii* (ATCC-30868) and *A. polyphaga* (ATCC-30461) were axenically cultured and maintained in peptone-yeast-glucose medium supplemented with 1% penicillin/streptomycin at 25°C.

C6 rat glial cells (ATCC CCL-107) were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO_2 incubator.

Neglaria fowleri trophozoites (5×10^4 cells/well) were seeded on 96-well microplates (Thermo Fisher Scientific, Waltham, MA, USA) in Nelson's medium and incubated at 37°C overnight. *A. castellanii* and *A. polyphaga* trophozoites (5×10^4 cells/well) were seeded on 96-well microplates (Thermo Fisher Scientific) in peptone-yeast-glucose medium and incubated at 25°C overnight. Different concentrations (0–400 μM) of each flavonoid were used to treat *N. fowleri* and *Acanthamoeba* trophozoites followed by incubation at 37°C or 25°C

for 48 h, respectively. Azithromycin and miltefosine were included as control drugs with the same procedures. Morphological changes of the amoebae were observed microscopically every 12 h. The viability of the amoebae was accessed with a colorimetric method using the CellTiter-Blue Cell viability assay (Promega, Madison, WI, USA). All experiments were performed in triplicate with 3 replicates. Data are presented as mean ± standard deviation (SD) of 3 independent experiments. The inhibitory concentration 50 (IC_{50}) of each compound was calculated using GraphPad Prism 9.1.0 software (GraphPad Software, San Diego, CA, USA). Amoebae treated with 0.1% DMSO were used as negative controls, representing 100% cell viability.

The potential cytotoxicity of each flavonoid against C6 glial cells was analyzed. Cells were seeded in 96-well microplates (Thermo Fisher Scientific; 2×10^4 cells/well) and incubated overnight until 80% confluence. Serially diluted flavonoids (0–400 μM) or control drugs were used to treat cells as described above. Morphological alterations of the cells were observed via microscopic examination. Cell viability was determined using the CellTiter-Blue Cell viability assay (Promega). All experiments were performed in triplicate with 3 replicates. Data are given as mean ± SD of 3 independent assays. The cytotoxicity concentration 50 (CC_{50}) of each flavonoid was calculated using GraphPad Prism 9.1.0 software (GraphPad Software). The sensitivity index (SI) was determined by the ratio between CC_{50} and IC_{50}. Cells treated with 0.1% DMSO, which was confirmed not to induce the morphological change of the cells under microscopic examination, were used as controls with 100% cell viability.

In vitro anti-*N. fowleri* activities of 18 flavonoids were evaluated by incubating the amoeba with various concentrations of each flavonoid. Six of the 18 flavonoids showed anti-*N. fowleri* activity with IC_{50} values < 50 μM (Table 1). Demethoxycurcumin, luteolin, and kaempferol had low IC_{50} values < 30 μM and high SI values > 7 and was more effective than miltefosine, a reference drug. Resveratrol and silybin (A+B) also showed low IC_{50} against *N. fowleri* but displayed partial effects on C6 glial cells, producing lower SI values of 4.68 and 4.13, respectively. Chelerythrine had the most robust antiamoebic activity against *N. fowleri* (IC_{50} = 11.83 ± 1.15 μM) but showed potent cytotoxicity against C6 glial cells, producing low CC_{50} and SI values. The other 12 flavonoids showed weak anti-*N. fowleri* activity (IC_{50} > 65 μM) and low SI values (< 4).

The in vitro anti-*Acanthamoeba* activity of the flavonoids was analyzed. The IC_{50} values of the flavonoids slightly differed by *Acanthamoeba* species, and *A. polyphaga* was generally more sensitive than *A. castellanii*. Among the 18 flavonoids, 10 showed antiamoebic activity against *A. castellanii* and *A. polyphaga* (Table 1). (−)-Epicatechin, (−)-epigallocatechin, demethoxycurcumin, apigenin, resveratrol, rosmarinic acid, silybin (A+B), costunolide, and kaempferol had low IC_{50} < 50 μM for both *Acanthamoeba* species. In particular, (−)-epicatechin, (−)-epigallocatechin, demethoxycurcumin, rosmarinic acid, and kaempferol displayed high SI values > 8. Apigenin, resveratrol, silybin (A+B), costunolide, and (−)-trans-caryophyllene also showed potential antiamoebic activity against *Acanthamoeba* but also partially affected C6 glial cells, producing relatively low CC_{50} and SI values. Luteolin was effective only for *A. polyphaga*. The other seven flavonoids, (±)-catechin, afzelin, quercetin, genistein, berberine, chelerythrine, and (+)-pinostrobin, did not exhibit potential or selective antiamoebic activity against either *Acanthamoeba* species.

Table 1. Anti-amoebic activities of flavonoids against *N. fowleri* and *Acanthamoeba* species

Classification	Compounds	*N. fowleri* (IC_{50} ± SD, μM)	*A. castellanii* (IC_{50} ± SD, μM)	*A. polyphaga* (IC_{50} ± SD, μM)	C6 glial cells (CC_{50} ± SD, μM)	SI[b]	SI[c]	SI[d]
Flavan-3-ols (Flavanols)	(–)-Epicatechin	102.62 ± 0.97	33.38 ± 0.68	25.02 ± 0.14	318.62 ± 0.82	3.11	9.55	12.73
	(–)-Epigallocatechin	114.59 ± 0.48	32.14 ± 1.03	24.16 ± 0.16	278.98 ± 0.30	2.44	8.68	11.55
	(±)-Catechin	> 344.51	> 344.51	> 344.51	> 344.51	-	-	-
Beta-diketone	Demethoxycurcumin[a]	27.07 ± 0.35	36.09 ± 0.82	21.93 ± 0.18	> 295.55	> 11.10	> 8.19	> 13.48
Flavones	Luteolin	27.45 ± 0.61	> 350.21	40.23 ± 0.47	> 350.21	> 12.72	-	> 8.71
	Apigenin	94.65 ± 0.58	43.39 ± 0.80	28.33 ± 0.13	243.09 ± 0.98	2.57	5.60	8.58
Polyphenols	Resveratrol[a]	47.19 ± 0.36	40.03 ± 0.39	31.35 ± 0.08	220.78 ± 0.09	4.68	5.52	7.04
	Rosmarinic acid	77.69 ± 0.97	31.66 ± 0.31	22.41 ± 0.78	> 277.54	> 3.57	> 8.77	> 12.38
Flavonolignans	Silybin (A+B)[a]	24.20 ± 0.27	24.46 ± 0.56	15.82 ± 0.06	99.89 ± 0.78	4.13	4.08	6.31
Sesquiterpene	Costunolide	65.56 ± 0.58	32.02 ± 1.14	35.14 ± 0.74	191.19 ± 1.18	2.92	5.97	5.44
	(–)-trans-Caryophyllene	84.78 ± 0.65	65.06 ± 1.42	44.77 ± 1.41	220.36 ± 2.42	2.60	3.39	4.92
Flavonols	Kaempferol[a]	27.82 ± 0.33	21.63 ± 1.28	28.61 ± 1.17	333.82 ± 0.99	12.00	15.43	11.67
	Afzelin	> 230	> 230	> 230	> 230	-	-	-
	Quercetin	> 330	> 330	320.81 ± 0.33	> 330	-	-	> 1.03
Isoflavonoids	Genistein	157.38 ± 0.57	> 370	> 370	103.93 ± 1.08	0.66	-	-
Alkaloid	Berberine	> 260	> 260	> 260	159.65 ± 1.23	-	-	-
	Chelerythrine	11.83 ± 1.15	148.10 ± 1.38	101.89 ± 1.40	16.43 ± 0.45	1.39	-	-
Flavonoid	(+)-Pinostrobin	> 370	> 370	> 370	> 370	-	-	-
Antibiotics	Azithromycin	14.67 ± 1.65	12.11 ± 0.20	4.12 ± 0.11	86.24 ± 0.93	5.88	7.12	20.93
Chemical	Miltefosine	153.34 ± 1.30	213.23 ± 1.22	227.45 ± 0.36	> 245	> 1.60	> 1.15	> 1.08

[a]Flavonoids showed effective anti-amoebic activities against both *N. fowleri* and *Acanthamoeba* species.
[b]Sensitivity index (CC_{50} C6 glial cells / IC_{50} *N. fowleri*).
[c]Sensitivity index (CC_{50} C6 glial cells / IC_{50} *A. castellanii*).
[d]Sensitivity index (CC_{50} C6 glial cells / IC_{50} *A. polyphaga*).

The 18 flavonoids showed different profiles of antiamoebic activity against *N. fowleri* and *Acanthamoeba* species. However, demethoxycurcumin and kaempferol showed promising in vitro antiamoebic activity against both *N. fowleri* and *Acanthamoeba* species with low IC_{50} values (< 40 μM) and high SI values (> 8). The overlapping antiamoebic activities of demethoxycurcumin and kaempferol against both *N. fowleri* and *Acanthamoeba* species suggest these flavonoids could be candidates for drug development for both PAM and GAE. Resveratrol and silybin (A+B) also showed reasonable IC_{50} and SI values against both *N. fowleri* and *Acanthamoeba* species. The antiamoebic activities of kaempferol, demethoxycurcumin, and resveratrol have been reported previously. Kaempferol was shown to block the proliferation and invasion of *E. histolytica* trophozoites in vitro through the degradation of the cytoskeleton [15] and to induce programmed cell death in *N. fowleri* [16]. Demethoxycurcumin and resveratrol were toxic to *A. castellanii* trophozoites and inhibited the binding of the trophozoites to host cells [17]. These studies support the hypothesis that these flavonoids are attractive candidates for anti-FLA drug development. However, further investigations are necessary to understand the underlying antiamoebic mechanisms of these flavonoids at the molecular level and evaluate their biosafety.

The anti-*Acanthamoeba* activities of (−)-epicatechin, (−)-epigallocatechin, apigenin, and rosmarinic acid have been partially reported [7,10,18]. Consistent with these studies, we confirmed the anti-*Acanthamoeba* activities of these 4 flavonoids herein, although they did not show significative anti-*N. fowleri* activities. Interestingly, these flavonoids had different IC_{50} values against *A. castellanii* and *A. polyphaga*, suggesting further investigation is required

on these inconsistent antiamoebic activities against different *Acanthamoeba* species and underlying molecular mechanisms of these flavonoids for anti-*Acanthamoeba* activities.

Apigenin and luteolin reportedly show potential cytotoxicity against murine macrophages J774A.1 but did induce programmed cell death in *A. castellanii* [7]. However, the 2 flavones did not show significative cytotoxicity to C6 glial cells in our study, suggesting further evaluation is needed of these flavones as potential anti-*Acanthamoeba* drug candidates. The antiamoebic activity of luteolin is also a novel finding obtained in this study. Luteolin showed promising IC_{50} values (< 30 μM) and high SI values (> 11) for *N. fowleri* and *A. polyphaga*, implying substantial and selective antiamoebic activity without toxicity to animal cells but was not effective against *A. castellanii*. Further studies are therefore required to investigate the molecular mechanism of the antiamoebic activity of luteolin and evaluate its effectiveness as a drug candidate.

A recent in silico analysis demonstrated that chelerythrine and berberine are attractive drug candidates for PAM [19], and the strong antiamoebic activity of chelerythrine against *N. fowleri* was confirmed in this study to support the in silico results. However, chelerythrine also showed strong toxicity to C6 glial cells, producing very low CC_{50} and SI values. Considering the high antiamoebic activity of the alkaloid for the *N. fowleri*, studies using derivatives of chelerythrine should be performed to reduce the toxicity to human cells. Berberine did not affect either *N. fowleri* or *Acanthamoeba* species.

In addition to the 18 flavonoids evaluated in this study, flavonoids such as anemonin, conessine, curcumin, isotrilobine, mangostin, and quassin have been proposed as potential candidates with anti-FLA activities because of their known antiamoebic activities for *E. histolytica* [14]. Further analyses of the anti-FLA activities of these flavonoids would be interesting in drug repurposing.

In conclusion, we experimentally confirmed that 11 flavonoids possess antiamoebic activities against *N. fowleri* and *Acanthamoeba* species. These flavonoids exhibited different antiamoebic profiles against the amoeba species and can be applied as alternative or supplement therapeutic agents to treat PAM and GAE. Further studies to determine the molecular mechanisms of the antiamoebic activities of these flavonoids and in vivo studies to evaluate their clinical significances are necessary. Significant variances in drug susceptibility among *N. fowleri* strains and isolates have also been reported [20], suggesting that additional studies to evaluate the antiamoebic activity of flavonoids for different genotypes or clinical isolates of the amoeba are also required. Moreover, studies to evaluate the antiamoebic activity of flavonoids, alone or in combination with other drugs, are recommended to support the design or formulation of effective treatment regimens.

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