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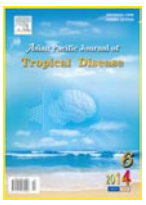

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Anti-intestinal protozoan activities of 1-hydroxy-2-hydroxymethylanthraquinone from *Coptosapelta flavescens*

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PEER REVIEW

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Comments

This is a good study in which the authors evaluated anti-amoeba and anti-giardial activities of several medicinal plants. Among them, 1-hydroxy-2-hydroxymethylanthraquinone isolated from *C. flavescens* seems to be a good candidate for use against amoeba and giardial infections. In general, writing is fine, results are interesting, the topic of MS is covered by APJTD.
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ABSTRACT

Objective: To investigate the antiprotozoal activity of medicinal plant extracts and isolated active compounds from the most active plant.
Methods: Twenty one medicinal plants with ethnobotanical use in Thailand, which were claimed to have anti-diarrhoeal or anti-parasitic activity, were screened for their anti-intestinal protozoan activity against *Entamoeba histolytica* (*E. histolytica*) and *Giardia intestinalis* (*G. intestinalis*). The most active compound was isolated and tested against *E. histolytica* and *G. intestinalis*.
Results: An acetone extract of *Coptosapelta flavescens* was the most active against both *E. histolytica* and *G. intestinalis* (minimal inhibitory concentration=125 and 15.63 μg/mL, respectively). Two anthraquinones and one naphthoquinone were isolated. The compound 1-hydroxy-2-hydroxymethylanthraquinone was the most active chemical against *E. histolytica* and *G. intestinalis* with minimal inhibitory concentration values of 20 and 2.5 μg/mL, respectively. In time killing assay, the percentage of viable *G. intestinalis*, when compare to control, after expose to compound 1-hydroxy-2-hydroxymethylanthraquinone showed significantly ($P<0.05$) lower than when exposed to a standard drug, metronidazole, at 6 and 12 h of incubation time. While for *E. histolytica*, its a=ctivity was comparable to metronidazole.
Conclusions: These observations provide preliminary evidence that 1-hydroxy-2-hydroxymethylanthraquinone from *Coptosapelta flavescens* can be considered to be a potential anti-parasitic agent against *E. histolytica* and *G. intestinalis* infections.

KEYWORDS

Antiprotozoal activity, *Coptosapelta flavescent*, Diarrhea, *Entamoeba histolytica*, *Giardia intestinalis*, Medicinal plant

1. Introduction

Intestinal infections caused by *Entamoeba histolytica* (*E. histolytica*) and *Giardia intestinalis* (*G. intestinalis*) are still major public health problems, particularly for children in most developing as well as in some of the more developed countries[1-3]. The major clinical symptoms caused by *E. histolytica* are colitis, dysentery and amoebic liver abscess whereas *G. intestinalis* may cause diarrhoea, malabsorption syndrome, cramp and weight loss[4,5]. It has been estimated that 50 million people around the world were infected by *E. histolytica* and resulted in 40-110 thousand deaths annually[6].

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Giardiasis is the most common intestinal protozoa to oCCur in humans and has been estimated at 280 million cases per year[7]. Metronidazole is the most commonly used drug to treat intestinal infections caused by *E. histolytica* or *G. intestinalis*[8]. However, unpleasant effects such as a metallic taste, headache, nausea, urticaria, pruritus and dark colored urine have been reported[9,10]. Furthermore, metronidazole is a mutagen when test in breast cancer cell lines and drug resistance varieties of the parasite have been detected[11,12]. The continuous search for new anti-protozoan compounds with high activity that are safe and produce little or no side effects is still a necessary and ongoing goal in medicine.

Medicinal plants are popular among people in developing countries because they are widely available at low cost and their products are mostly safe. Moreover, medicinal plants have a rich history of use for the treatment of diarrhoea and dysentery[13]. Several compounds from herbs have been proved to be effective against various organisms, *i.e.* curcumin from *Curcuma longa (C. longa)* is reported to have anti-bacterial, anti-oxidant, anti-HIV and anti-protozoan activities[14-16]; essential oils from *Thymbra capitata, Origanum virens, Thymus zygis* subsp. *sylvestris, Lippia graveolens* and *Zyzygium aromaticum* are active against *Giardia* as well as providing some anti-bacterial activity[17-19]. We therefore plan to evaluate the *in vitro* activity of natural products obtained from selected Thai medicinal plants claimed to have ethnobotanical use as anti-diarrhoeal or anti-parasitic agents in order to detect alternative agents that are suitable for use in preventing and treating *E. histolytica* and *G. intestinalis* infections.

2. Materials and methods

2.1. Plant materials

Twenty one medicinal plants claimed to act as agents that cured diarrhoea or parasitic infection were evaluated. The plants were dried and then extracted with acetone or ethanol. The solvent was evaporated under reduced pressure. Each dried extract was then dissolved in dimethyl sulphoxide (DMSO) at a concentration of 100 mg/mL and stored at 4 °C. The maximum concentration of DMSO in the test did not exceed 1%, and this concentration had no effect on the growth of *E. histolytica* or *G. intestinalis*.

2.2. Isolation of pure compounds from Coptosapelta flavescens

The most active plant extract against both *E. histolytica* and *G. intestinalis in vitro* was from *Coptosapelta flavescens* (*C. flavescens*) and this was further investigated. The dried and chopped whole plant (3.5 kg) was extracted three times with acetone (3 L) each for 5 d at room temperature. The combined acetone extract was evaporated to dryness under reduced pressure to afford a dark brown gum (23.7 g). The crude acetone extract was fractionated by column chromatography (CC) over silica gel with a gradient system of methanol (MeOH)-dichloromethane (CH_2Cl_2) to afford four fractions (A-D). Fraction A (0.8 g) was further separated by CC over silica gel to give six fractions (A1-A6). Fraction A2 (75.4 mg) upon CC over silica gel using gradient systems of CH_2Cl_2-Hexane and MeOH-CH_2Cl_2 afforded five subfractions (A2.1-A2.5). Subfraction A2.3 (12.7 mg) was subjected to CC over silica gel using a gradient systems of CH_2Cl_2-Hexane and MeOH-CH_2Cl_2 to yield compound 2 (3.5 mg). Fraction A4 (92.3 mg) was purified using the same procedure as the crude acetone extract to give compound 3 (3.8 mg). Fraction B (2.2 g) was further separated using the same procedure as for fraction A to give six fractions (B1-B6). Fraction B2 (300.3 mg) was purified by CC over silica gel using a gradient elution system of CH_2Cl_2-Hexane and MeOH-CH_2Cl_2 as eluents to obtain compounds 2 (35.6 mg) and 3 (4.6 mg). Subfraction B3 (331.2 mg) was separated by CC over silica gel with the same procedure as for fraction B2 to afford compound 1 (16.8 mg). Their structures were assigned by spectroscopic methods and comparison of the nuclear magnetic resonance data with those reported in literatures.

2.3. Parasite cultures

E. histolytica strain HM1:IMSS purchased from the American Type Culture Collection and a Thai strain of *G. intestinalis*, originally as described previously were used in all experiments[20]. They were cultured axenically in screw capped tubes on YI medium supplemented with 10% heat inactivated bovine serum under anaerobic conditions at 37 °C[21]. Subculture was performed every 48 h. For the assays, cells were harvested by chilling the tube on ice for 20 min to detach the monolayer and then centrifuged at 3000 r/min for 5 min. The supernatant was decanted, and cells were resuspended in fresh medium. The numbers of viable cells were calculated using a haemocytometer and 0.4% (w/v) trypan blue. The criteria for viability were motility and dye exclusion.

2.4. Screening of plant extracts and pure compounds for anti-protozoan activity

The anti-protozoan activity was performed according to standard methods as described elsewhere[22,23]. Briefly, *E. histolytica* and *G. intestinalis* trophozoites (2×10^5 cells/mL) were incubated in 96-well tissue culture plates (200 μL/well) in the presence of serial dilutions of each compound that ranged from 7.81 to 1000.00 μg/mL for crude extract and ranged from 1.25 to 80.00 μg/mL for pure compound. Each test also included metronidazole as the standard drug, at final concentrations that ranged from 1.25 to 10.00 μg/mL and an untreated control (with and without 1% DMSO). Trophozoites were incubated for 24 h at 37 °C under anaerobic conditions. After incubation, the appearance and numbers of trophozoites were scored from 1 to 4 using an inverted microscope. Their minimal inhibitory concentrations (MIC) were determined as the lowest concentration that >90% of the trophozoites rounded up and dead when compared with the control well that showed inhibition with a score of 1 according to Upcroft and Upcroft[24].

2.5.Time killing assay

The effect of the most active compounds on the viability of *E. histolytica* and *G. intestinalis* at different time intervals was compared with a standard drug, metronidazole. *E. histolytica* and *G. intestinalis* trophozoites, at a density of 2×10^5 cells/mL, were incubated in 96-well tissue culture plates (200 μL/well), in the presence of the pure compound or metronidazole at 1/2 MIC, MIC and 2 MIC concentrations for 6, 12, 24 and 48 h. At each time interval, the plate was then chilled for 20 min in an ice bath to detach trophozoites and their viable number was counted in a hemocytometer using a trypan blue exclusion assay. The percentage of viable trophozoite was calculated

against control well at the same time interval. IC_{50} value was determined by probit analysis at 24 h of incubation. Each concentration was tested in duplicate, and at least two experiments were performed on separate occasions.

2.6. Statistic analysis

The percentage of viable trophozoite exposed to the most active pure compound at each time interval was compare with percentage of viable trophozoite after exposed to metronidazole using the student *t*-test. A *P*-value of <0.05 was considered as statistically significant.

3. Results

3.1. Anti-protozoan activity of plant extracts

The *in vitro* effect of each plant extract against *E. histolytica* and *G. intestinalis* has been summarized in Table 1. The extract from *C. flavescens* showed the best activity against both *E. histolytica* and *G. intestinalis* with MICs of 125 and 15.63 μg/mL, respectively. The ethanol extract of *C. longa* inhibited both *E. histolytica* and *G. intestinalis* at concentration of 250 μg/mL whereas *Euphorbia thymifolia* (*E. thymifolia*), *Garcinia mangostana* (*G. mangostana*), *Punica granatum* (*P. granatum*), *Sandoricum koetjape* (*S. koetjape*) and *Terminalia bellirica* (*T. bellirica*) inhibited both protozoa at 250-500 μg/mL. *Derris scandens* inhibited only *E. histolytica* (MIC 500 μg/mL). *Piper betle*, *Psidium guajava*, *Rhizophora mucronata* and *Terminalia chebula* (*T. chebula*) inhibited only *G. intestinalis* at 250-500 μg/mL. The remaining extracts showed no activity (MIC≥1 000 μg/mL). The MIC of metronidazole against both *E. histolytica* and *G. intestinalis* was 2.5 μg/mL.

Table 1

Plant names, parts used and minimal inhibitory concentrations (MIC) against *E. histolytica* and *G. intestinalis in vitro*.

Plants	Part used	MIC (μg/mL)	
		E. histolytica	*G. intestinalis*
Aegle marmelos (L.) Corr.	fruit	>1 000	>1 000
Ardisia colorata Roxb.	wood	>1 000	>1 000
Centella asiatica (L.) Urb.	whole	>1 000	>1 000
C. flavescens	whole	125	15.63
C. longa L.	rhizome	250	250
Derris scandens (Roxb.) Benth.	stem	500	1 000
E. thymifolia L.	whole	500	500
G. mangostana L.	skin	500	500
Holarrhena pubescens Wall. ex G. Don	bark	1 000	1 000
Manilkara achras (Mill.) Fosberg	fruit	1 000	>1 000
Morinda citrifolia L.	fruit	>1 000	>1 000
Peltophorum pterocarpum (DC.) Backer. ex K. Heyne.	bark	1 000	1 000
Piper aurantuacum	leaf	1 000	1 000
Piper betle L.	leaf	1 000	500
Piper chaba Vahl.	fruit	1 000	1 000
Psidium guajava L.	leaf	1 000	500
P. granatum L.	skin	500	250
Rhizophora mucronata Poir	bark	>1 000	500
S. koetjape (Burm. f.) Merr.	root	500	500
T. bellirica (Gaertn.) Roxb.	fruit	500	500
Terminalia chebula Retz.	fruit	1 000	250

3.2. Purification of C. flavescens

Purification using chromatographic techniques afforded two anthraquinones and one naphthoquinone. Compound 2 was identified as 1-hydroxy-2-hydroxymethylanthraquinone which was previously isolated from *Galium verum* by Banthorpe and White[25]. 1-Hydroxy-2-methoxycarbonylanthraquinone (compound 3) was previously obtained from *Rubia wallichiana* whereas 2-amino-3-methoxycarbonyl-1,4-naphthoquinone (compound 1), as synthesized previously was isolated for the first time as a natural product[26,27].

Figure 1. *E. histolytica* trophozoites after treatment with MIC of (A) metronidazole (2.5 μg/mL), (B) compound 2 (20.0 μg/mL) and (C) untreated control at 37 °C for 24 h. Bars: 10 μm.

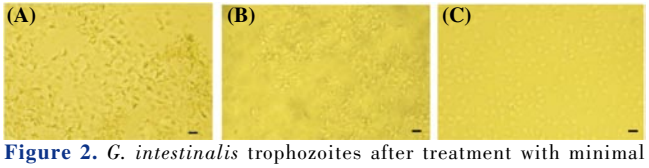

Figure 2. *G. intestinalis* trophozoites after treatment with minimal inhibitory concentration of (A) metronidazole (2.5 μg/mL), (B) compound 2 (2.5 μg/mL) and (C) untreated control at 37 °C for 24 h. Bars: 10 μm.

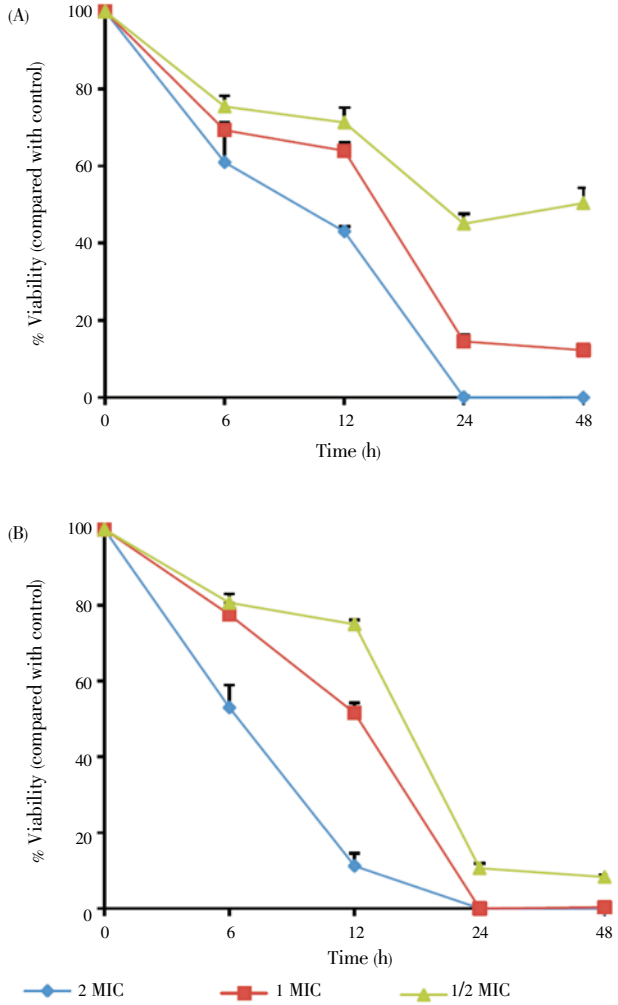

Figure 3. Percentage of viable *E. histolytica*, compared to control, after incubation with different concentrations of (A) metronidazole and (B) compound 2 for 6, 12, 24 and 48 h. Values are expressed as means and standard error of the mean.

3.3. In vitro anti-amoebic and anti-giardial activity of pure compounds isolated from C. flavescens

The MICs of compound 2 against *E. histolytica* and *G. intestinalis* were 20.0 and 2.5 μg/mL respectively. The morphology and approximate number of *E. histolytica* and *G. intestinalis* treated with compound 2 and metronidazole at the MIC concentration for 24 h under inverted microscope were shown in Figures 1A, 1B and 2A, 2B, respectively while the trophozoites from the control well were more than 90% confluent (Figures 1C and 2C). Compound 1 and compound 3 showed MIC values of >80 μg/mL which was considered to be not suitable for further study.

3.4. Time killing assay

The percentages of viable *E. histolytica* and *G. intestinalis* after exposed to metronidazole and compound 2 at 1/2 MIC, MIC and 2 MIC concentrations for 6, 12, 24 and 48 h, compare to the control at the same time interval, are shown in Figures 3 and 4, respectively. Metronidazole and compound 2 at MIC concentration decrease the number of viable *E. histolytica* and *G. intestinalis* trophozoites to <20% within 24 h. However, it is interesting to note that at 6 and 12 h the percentage of viable *G. intestinalis* expose to compound 2 significantly (P<0.05) lower than when exposed to metronidazole.

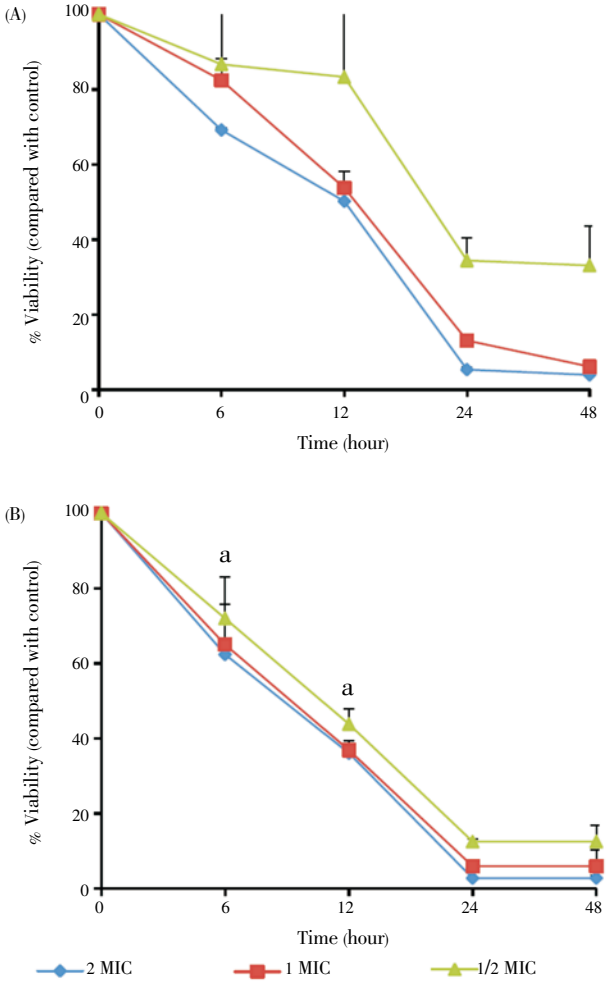

Figure 4. Percentage of viable *G. intestinalis*, compared to control, after incubation with different concentrations of (A) metronidazole and (B) compound 2 for 6, 12, 24 and 48 h. Values are expressed as means and standard error of the mean, a: P<0.05.

4. Discussion

In the present work, we have investigated the anti-protozoan activities of 21 medicinal plants (21 extracts) that have been used in Thai traditional medicine for the treatment of diarrhoea or parasitic infection. We found seven plant extracts, *C. flavescens*, *C. longa*, *E. thymifolia*, *G. mangostana*, *P. granatum*, *S. koetjape* and *T. bellirica*, exhibited both anti-amoebic and anti-giardial activities *in vitro*. These findings were in agreement with other reports such as compounds from *C. longa* and *P. granatum* were found to inhibit both *E. histolytica* and *G. intestinalis*[9,14-16].

E. thymifolia, is commonly used in folk medicine in Bangladesh for treatment of helminthiasis[28], and was found to also possess various activities such as: an anti-viral, anti-oxidant, *etc.* activities[29]. This is the first report of an extract from *E. thymifolia* that inhibited *E. histolytica* and *G. intestinalis in vitro*.

G. mangostana is a tropical plant that grows well in tropical areas including Thailand, Indonesia and Malaysia. The pericarps of *G. mangostana* have been widely used as a traditional medicine for the treatment of diarrhoea, skin infections and chronic wounds in South East Asia for many years. Medicinal properties of *G. mangostana* extract such as: an anti-oxidant, anti-tumor, anti-inflammatory, anti-allergy, anti-malarial, and anti-bacterial/viral, anti-helminthic properties have been reported[30,31]. We further added more value to a *G. mangostana* extract as it also had a low anti-*E. histolytica* and anti-giardial activities.

An aqueous extract of *S. koetjape* bark is used traditionally in Malaysia as a tonic after giving birth[32]. The biological and phamarcological properties of pure compounds isolated from different parts of *S. koetjape* have been reported to have an anti-viral[33], anti-inflammatory[32] and anti-cancer activities[34]. The roots of *S. koetjape* are used to treat intestinal disorders throughout Southeast Asia and we are the first to report its anti-*E. histolytica* and anti-*G. intestinalis* activities *in vitro*[33].

T. chebula is used as a remedy against a sore throat and cough, against diarrhoea connected to a prolapsed rectum and against ulcers and dysentery in China and Tibet[35]. The powder of *T. chebula* fruits has been used to treat chronic diarrhoea[36]. An anti-bacterial[37], anti-ciliate protozoa in ruminent[38] and anti-amoebic activities of *T. chebula* extracts have been reported[39]. We reported here its anti-giardial activity.

Results from the present study have demonstrated that an acetone extract of *C. flavescens* is the most effective against both *E. histolytica* and *G. intestinalis* growth. We therefore further isolated pure compounds from this plant and evaluated them for their anti-protozoan activities. *C. flavescens* is used in folk Thai medicine for treatment of helminthic infections. Three pure compounds were isolated, but only compound 2 exhibited both anti-amoeba and anti-giardial activities. For *E. histolytica*, at its MIC concentration (2.5 μg/mL for metronidazole and 20.0 μg/mL for compound 2), the percentage of viability trophozoite were comparable. On the other hand, at a similar MIC concentration (2.5 μg/mL for both metronidazole and compound 2), the percentage of viable *G. intestinalis* trophozoite exposed to compound 2 significantly (P<0.05) lower than when exposed to

metronidazole within 12 h of incubation. These results have indicated that compound 2 from *C. flavescens* can inhibit *G. intestinalis* quicker than metronidazole. This would be one advantage of its future therapeutic use. Compound 2 is an anthraquinone derivative and has not been studied for its bioactive activity. Anthraquinones from *Cassia* sp. are known to possess anti-fungal, anti-plasmodial, anti-inflammatory and anti-diarrhoeal activities[40].

In conclusion, several medicinal plants that have been commonly prescribed as an anti-diarhoeal or anti-pasitic remedy in Thailand showed anti-intestinal protozoan activity. Among them, 1-hydroxy-2-hydroxymethylanthraquinone isolated from *C. flavescens* seems to be a good candidate for use against amoeba and giardial infections. Further studies on the mechanism of this compound and some *in vivo* studies with regard to its effects on animals and human should be investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Intestinal infections caused by *E. histolytica* and *G. intestinalis* are major public health problems, particularly for children in most developing countries. So it is necessary to find low-cost and safe medicine. Medicinal plants are popular among people in developing countries because they are widely available at low cost and their products are mostly safe as expected. Moreover, medicinal plants have a rich history of use for the treatment of diarrhoea and dysentery.

Research frontiers

The manuscript evaluated the *in vitro* activity of natural products obtained from selected Thai medicinal plants already claimed to have ethnobotanical use as potential anti-diarrhoeal or anti-parasitic agents and detected alternative agents that are suitable for use in preventing and treating *E. histolytica* and *G. intestinalis* infections.

Related reports

Several compounds from herbs have been proved to be effective against various organisms, *i.e.* curcumin from *C. longa* is reported to have anti-bacterial, anti-oxidant, anti-HIV and anti-protozoan activities; essential oils from *Thymbra capitata*, *Origanum virens*, *Thymus zygis* subsp. *sylvestris*, *Lippia graveolens* and *Zyzygium aromaticum* are active against *Giardia* as well as providing some anti-bacterial activity.

Innovations & breakthroughs

Data regarding anti-intestinal protozoan activities of compounds from *C. flavescens* are scarce. This study has showed that 1-hydroxy-2-hydroxymethylanthraquinone from *C. flavescens* can be considered to be a potential anti-parasitic agent against *E. histolytica* and *G. intestinalis* infections.

Applications

1-hydroxy-2-hydroxymethylanthraquinone isolated from *C. flavescens* may be a hopeful candidate for use against amoeba and giardial infections. After further studies on this compound *in vitro* and *in vivo*, it is possible for the compound to be used in clinical medicine.

Peer review

This is a good study in which the authors evaluated anti-amoeba and anti-giardial activities of several medicinal plants. Among them, 1-hydroxy-2-hydroxymethylanthraquinone isolated from *C. flavescens* seems to be a good candidate for use against amoeba and giardial infections. In general, writing is fine, results are interesting, the topic of MS is covered by APJTD.

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