

Effects of some natural products from fungal and herbal sources on *Giardia lamblia* in vivo

Aroona Chabra^{1,3}, Bahman Rahimi-Esboei², Emran Habibi¹, Taha Monadi³, Mohammad Azadbakht¹, Taher Elmi⁴, Hossein Keshavarz valian⁵, Javad Akhtari⁶, Mahdi Fakhar⁷ and Farshad Naghshvar⁸

Research Article

Cite this article: Chabra A *et al* (2019). Effects of some natural products from fungal and herbal sources on *Giardia lamblia* in vivo. *Parasitology* **146**, 1188–1198. <https://doi.org/10.1017/S0031182019000325>

Received: 30 November 2018
 Revised: 31 January 2019
 Accepted: 3 March 2019
 First published online: 22 April 2019

Key words:

Antigiardial activity; *Balb/c*; chitosan; emodin; nano-chitosan; *Rhamnus cathartica*

Author for correspondence:

Mahdi Fakhar, E-mail: mahdif53@yahoo.com;
 Mohammad Azadbakht, E-mail: azadbakhtm@hotmail.com

¹Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran; ²Department of Parasitology and Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran; ³Student Research Committee, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran; ⁴Department of Parasitology and Mycology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran; ⁵Department of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; ⁶Department of Medical Nanotechnology, Immunogenetics Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran; ⁷Department of Parasitology, Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran and ⁸Department of Pathology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

Abstract

Giardia lamblia (*G. lamblia*) is the most widely known protozoan parasite that causes human gastrointestinal infection worldwide. Some natural compounds exhibited pivotal effects against different infectious diseases. In this research, the anti-giardial activity and cytotoxicity of fungal chitosan, nano-chitosan, *Rhamnus cathartica* (*R. cathartica*) and emodin were evaluated in Balb/c mice. Genotyping of *G. lamblia* was assessed by PCR-RFLP technique. Different concentrations of mentioned compounds were used to check their anti-giardial and cytotoxicity effects on human intestinal epithelial cells (HT-29) after 24, 48 and 72 h. The *G. lamblia* strain used in the current work was genotyped and revealed as an AII assemblage. All the concentration showed acceptable activity against *G. lamblia* cysts and trophozoites in comparison to the negative and positive controls (furazolidone and metronidazole) *in vitro* ($P < 0.05$). *Giardia lamblia* cysts were susceptible after treatment in all experiments *in vivo* in comparison to negative control ($P < 0.05$). Approximately, in most of the concentration, nano-chitosan and emodin were more effective than chitosan and *R. cathartica*, respectively ($P < 0.05$). The effects of exposure times in anti-giardial and cytotoxicity effects were not statistically significant ($P > 0.05$). The maximum mortality rate (100%) was achieved at 100 and 50 $\mu\text{g kg}^{-1}$ concentrations after 48 and 72 h of exposure time, respectively. Our results provide significant information about the new anti-giardial agent and proposed the nano-chitosan and emodin for the development of new drugs against *G. lamblia* in the future.

Introduction

Giardia lamblia (*G. lamblia*), also called *G. duodenalis* or *G. intestinalis*, is a microaerophilic, flagellated, parasitic protozoan of man and animals with a worldwide distribution that causes intestinal disorders (Lee *et al.*, 2018). *Giardia lamblia* has two life stages; the trophozoite and cyst. Giardiasis is transmitted by cyst form, while the trophozoite form causes acute or chronic diarrhoea, abdominal pain, malabsorption and weight (Halliez and Buret, 2013; Zhao *et al.*, 2014). Children and infants are high-risk population, also in immune-compromised patients; the disease can result in morbidity and conducting mortality (Tůmová *et al.*, 2018). It is estimated that about 300 million people are affected by the giardiasis all over the world every year and the prevalence of giardiasis is further in poor hygiene regions (Feng and Xiao, 2011). Metronidazole, tinidazole, quinacrine, albendazole, furazolidone and paromomycin are recommended medications for giardiasis (Ordóñez-Mena *et al.*, 2017). However, the mentioned chemotherapy drugs exhibit adverse side effects, including gastrointestinal disturbances, nausea, vomiting, metallic taste, headache, leukopenia, mutagenic and carcinogenic in human (Harris *et al.*, 2001). To reduce the risks of using chemical drugs, the World Health Organization (WHO) has proposed the use of medicinal plants and other natural products and reports that approximately 80% of the world population use traditional remedies for health care (Anquez-Traxler, 2011). Up to now, ethnobotanical and ethnopharmacological investigations disclose a variety of natural products used for the treatment of *Giardiasis* which has had a variety of effects (Rahimi-Esboei *et al.*, 2013a, 2013b; Derda and Hadaš, 2014; Méabed *et al.*, 2018).

Chitosan is a partially or fully deacetylated chitin; which exists in crustacean shells, insects' cuticles and fungi cell walls (Yarahmadi *et al.*, 2016). Chitosan is biodegradable, biocompatible and non-toxic in mammalian cells (Kean and Thanou, 2010) and its antifungal, antibacterial and also antiparasitic activity has been proved in many studies (Tayel *et al.*, 2010; Rahimi-Esboei *et al.*, 2013a, 2013b; Guo *et al.*, 2018). Many pioneer investigations indicated that considering fungi as a source of chitosan over the other traditional sources such as

shrimps and non-organic source has advantageous benefits including easy access, low cost, independence of seasonal changes and safer for biomedical and healthcare applications (Ebrahimzadeh *et al.*, 2013; Naghdi *et al.*, 2014). Today a growing interest has been raised in the application of nanoparticles into anti-parasite researches.

Nanoparticles as a new form of material with outstanding biological properties and low toxicity seem that there is a high potential in crossing the physiological barrier of the body access to specific target tissues (Jahangiri and Barghi, 2018). Nanoparticles have high access to the nanoscale materials, and their toxicity is much less than the average toxic dose and acute liver damage (Jahangiri and Barghi, 2018). These results suggest the ability of nanoparticles to target different cells for drug delivery, genetic material and diagnostic factors (Wim *et al.*, 2008).

Rhamnus cathartica L. belongs to Rhamnaceae family and native in Europe, western Asia, north of America and Iran (Hamed *et al.*, 2015). The *Rhamnus* genus consists of anthraquinone (Emodin, Rhein, Aloe-emodin, frangulin and glucofrangulin, chrysophanol, and physcion), flavonols (catharticin, kaempferol and quercetin), anthrone rhamnoside, naphthalenic derivatives, flavanol glycosides (kaempferol, rhamnazin, rhamnnetin, rhamnoci-trin), steroids (stigmasterol, p-sitosterol). Emodin as one of the internal combinations of *R. cathartica* has been shown that can inhibit the activity of both monoamine oxidase and tyrosine kinase, which was reported to have antimicrobial and antineoplastic activity (Kosalec *et al.*, 2013; Hamed *et al.*, 2015). Emodin revealed remarkable anti-bacterial effects against *S. aureus*; and even better against the standard neomycin, which is a reference antibacterial agent (Chukwujekwu *et al.*, 2006). For the purposes of finding new giardiacid agents with minimal complications, it is important to design the most promising new drugs. Current study was aimed to evaluate anti-giardial activities of various concentrations of fungal chitosan isolated from *Penicillium chrysogenum* (*P. chrysogenum*), *Penicillium pinophilum* (*P. pinophilum*) and *Penicillium rubefaciens* (*P. rubefaciens*) in scale of nano, emodin delivered from *R. cathartica* and methanolic extraction of *R. cathartica* in comparison to metronidazole and furazolidone against *G. lamblia* trophozoites and cysts in the murine model (*Balb/c* mice).

Materials and methods

Chemicals supply

Emodin, quercetin, gallic acid standards, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). TYI-S-33 medium and fetal calf serum (FCS) from Gibco (India), other reagents were prepared from Merck (Germany) and Iran companies. The *Penicillium* species were obtained from Pasteur Institute of Iran.

Purification of *G. lamblia* cysts

Giardia lamblia cysts were isolated from stool samples of patients who are suffering from giardiasis and all samples were processed ordinarily within 48 h after excretion. A highly purified cyst suspension was achieved by the sucrose 0.85 M flotation method with a simplified sucrose gradient method that is previously described (Elmi T *et al.*, 2017). Purified cysts were resuspended in normal saline and stored at 4 °C for a maximum of 3 days prior to use.

DNA extraction

Purified cysts were frozen (−80 °C) and thawed (+80 °C) in 10 cycles. The genomic DNA of *G. lamblia* was extracted using a

QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. DNA samples were preserved at −20 °C until used.

PCR amplification and RFLP assay

The amplification of the *gdh* gene was performed with a forward primer (5'-TCA ACG TCA ACC GCG GCT TCC GT-3') and reverse primer (5'-GTT GTC CTT GCA CAT CTC C-3') as described by Babaei Z *et al.* (2008). The PCR mixture comprised 1 µl genomic DNA, 15 µl of PCR master mix (Amplicone, UK), 1.5 µl of each primer and 11 µl distilled water. The reactions were performed in 30 µl volumes. The DNA was amplified using BioRad Thermal Cycler under the following condition: one cycle of 94 °C for 3 min, 56 °C for 1 min and 72 °C for 2 min, followed by 35 cycles, 94 °C for 1 min, 56 °C for 20 s and 72 °C for 45 s. A final extension of 72 °C for 7 min and a 20 °C hold was used. Distilled water was used as a negative control. The PCR products were electrophoresed on Safe stain 1% (W/V) agarose gel. The positive control was gathered from by the Tehran University of Medical Sciences that was isolated from human sources, and distilled water was used as a negative control. For RFLP assay, 0.5 unit of *Bsp*LI (Fermentase, Canada) and 15 µl of PCR product were used for digestion and the restriction fragments were separated in 2% agarose gels with ethidium bromide staining (Babaei *et al.*, 2008).

Preparation of chitosan

Penicillium chrysogenum (PFCC NO: 231-3), *P. pinophilum* (PFCC NO:51681) and *P. rubefaciens* (PFCC NO:236-32) were used as a source of fungal chitosan. The fungal chitin was deacetylated by a modified method as previously described (18). After cultivation of fungal mycelia, the dry biomass was suspended with NaOH solution (1 M) and autoclaved at 121 °C for 20 min. The alkaline undissolved fractions were collected by centrifugation at 12 000 rpm for 20 min, washed with distilled water and recentrifuged to obtain pH = 7. The insoluble particles suspension was left in 2% acetic acid at 95 °C for 8 h, centrifuged, then the supernatant solution was neutralized with NaOH (2 M), the solution centrifuged and the precipitated chitosan was washed with distilled water. The degree of deacetylation (DD) of the prepared chitosan samples was determined by an acid–base titration technique. In this technique, 400 mg of prepared chitosan samples were melted in 40 mL of 0.1 M HCl at 20 °C with stirring, and then methyl orange indicator was added drop to drop (2–3 drops) to the solution and the final solution was titrated by 0.1 M NaOH. At the last stage of the titration, the colour of the solution changed from pink to orange-yellow, which was confirmed by pH meter. Five hundred grams of prepared chitosan samples were heated at 105 °C until a constant weight was reached to show the water content, and the number of free NH₂ groups in the prepared chitosan samples was calculated as follows:

$$\text{NH}_2 (\%) = \frac{(C1V1 - C2V2) \times 0.016}{G (100 - W)} \times 100\%$$

$$\text{Free NH}_2 (\%) = \frac{\text{NH}_2 (\%)}{9.94} \times 100\%$$

Molecular weight determination

The average MW of the fungal chitosan samples were determined by viscometric method. Three concentrations (10, 50 and

100 $\mu\text{g mL}^{-1}$) of fungal chitosan solutions were prepared using the solvent system of 0.1 M acetic acid and 0.2 M NaCl (1:1, v/v). Viscosity was examined using an automatic system ubbelohde capillary type viscometer, which allows the reading of flow times of the sample taken automatically without using stopwatch in triplicate at 25 °C. The intrinsic viscosity $[\eta]$ was calculated using the following formula:

$$[\eta] = KMva$$

(Mv) and $[\eta]$ were determined the viscosity and intrinsic viscosity, respectively. (K) and (a) are constants of the values that depend on the nature of polymers and solvents.

Preparation and characterization of nanoparticles

Chitosan nanoparticle is prepared using ionic gelation method (Pareesh *et al.*, 2011). Different molecular weights of chitosan were melted in acetic acid (1%) and stirred overnight at 25 °C. Tripolyphosphate (TPP) and double-distilled water were mixed together to reach a concentration of 0.05% w/v and the solution was added to chitosan nanoparticle (1:5 ratio) using an insulin syringe and was stirred at 25 °C. Ultrasonication was used for 5 min to diminish the size nanoparticles.

Photon correlation spectroscopy of the chitosan nanoparticles was performed by a Dynamic Light Scattering (DLS) instrument (Nano-ZS; Malvern, UK) to determine the size profile of the preparation including zeta average size, zeta potential and polydispersity index (PDI). Size of the nanoparticles further confirmed by SEM (Seron Technology, AIS2100, Babol Noshirvani University of Technology, Mazandaran, Iran).

Plant collection and extraction

The bark of *R. cathartica* was collected from the forest area of Mazandaran Province, northern Iran, 1500 m above sea level during November 2017. The plant was botanically authenticated by Prof Mohammad Azadbakht (Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran) and deposited in the herbarium (herbarium NO: E₁-212-111) of the Faculty of Pharmacy, Mazandaran University of Medical Sciences (Sari, Iran).

The crushed and air-dried bark were extracted by maceration in methanol (5 × 3 and 3 × 1 L, respectively) for 72 h at room temperature and away from light, then filtered with the filter paper and dried in a rotary evaporator with bath at 40 °C to obtain a crude extract.

Determination of total flavonoids content

The total flavonoid contents of the crude extract was determined by the aluminium chloride colorimetric method with a little modification (Sembiring *et al.*, 2018). Quercetin (Sigma-Aldrich) was used to make the calibration curve. Two milligrams of the quercetin sample was added to a 25 mL volumetric flask with the solvent of 80% ethanol solution to obtain a 200 $\mu\text{g mL}^{-1}$ stock solution and diluted to 100, 50, 25 and 12.5 $\mu\text{g mL}^{-1}$. A total of 0.5 mL of the prepared solutions were mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water, respectively. Then the samples were incubated in room temperature for 30 min and the absorbance was measured at 415 nm. The blank sample was prepared by (the addition of the same mixture without the sample) the substitution of aluminium chloride with distilled water. Ten milligrams of the crude extracts were made with 95% ethanol solution to the volume of 10 mL in a volumetric flask and followed by the same

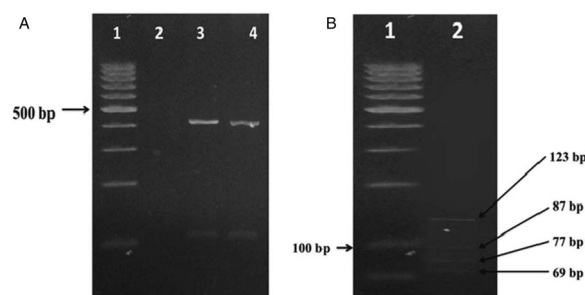


Fig. 1. (A) Electrophoretic separation of PCR product from DNA amplified at the *gdh* locus of *G. lamblia*, lane 1; 100 bp marker, lane 2; negative control (distilled water), line 3; positive control and line 4; PCR products from clinical samples. (B) Lane 1; 50 bp marker, lane 2; digestion of PCR product of *G. lamblia*.

solutions described above. The total contents of flavonoid were indicated as microgram of quercetin equivalents/g DW ($\mu\text{g QE/g DW}$). The samples analysed were conducted in triplicates.

Determination of total phenols content

Total phenolic content was determined using Folin–Ciocalteu reagent as the method adopted from Sembiring *et al.* (2018) with slight modifications. Gallic acid (Sigma-Aldrich) was used as the standard to plot the standard curve. The extracts were prepared at the concentration of 1000 $\mu\text{g mL}^{-1}$, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 1:10 with deionized water) and incubated at room temperature for 10 min. Then, the sample was added by 2 mL of NaHCO_3 (7.5%) and incubated at room temperature for 60 min. The absorbance of all samples was measured at 765 nm vs blank. The results are presented as gallic acid equivalents per μg of the dry weight of plant ($\mu\text{g GA}/\mu\text{g extract}$) and expressed as means of triplicate analysis.

DPPH free radical scavenging activity

The methodology of scavenging 1, 1-diphenyl-2-picrylhydrazyl stable free radical (DPPH) was adopted from Sembiring (2018). Briefly, 8 mg solution of DPPH was dissolved in a 100 mL volumetric flask with the solvent of methanol to obtain a concentration of 80 $\mu\text{g mL}^{-1}$. Two millilitres of different concentrations of the extracts in methanol was added to 2 mL of prepared DPPH solution. Then the mixture was shaken and incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm vs the blank, prepared by replacing the extract with methanol. The control was made with ascorbic acid instead of extracts at the same concentrations. The DPPH scavenging capability was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance of the test compound. The IC_{50} value, which is the extract concentration providing 50% inhibition, was calculated as $\mu\text{g mL}^{-1}$ from the dose–response curve. All spectrometric analyses were conducted in triplicate and averaged.

High-performance liquid chromatography analysis

The assay of extract base of the emodin was performed by the high-performance liquid chromatography (HPLC) apparatus [Knauer-YF 50-C18, 100A column (250 mm × 4.6 mm with C18 pre-column) plus quaternary gradient pump (pu-2089) plus

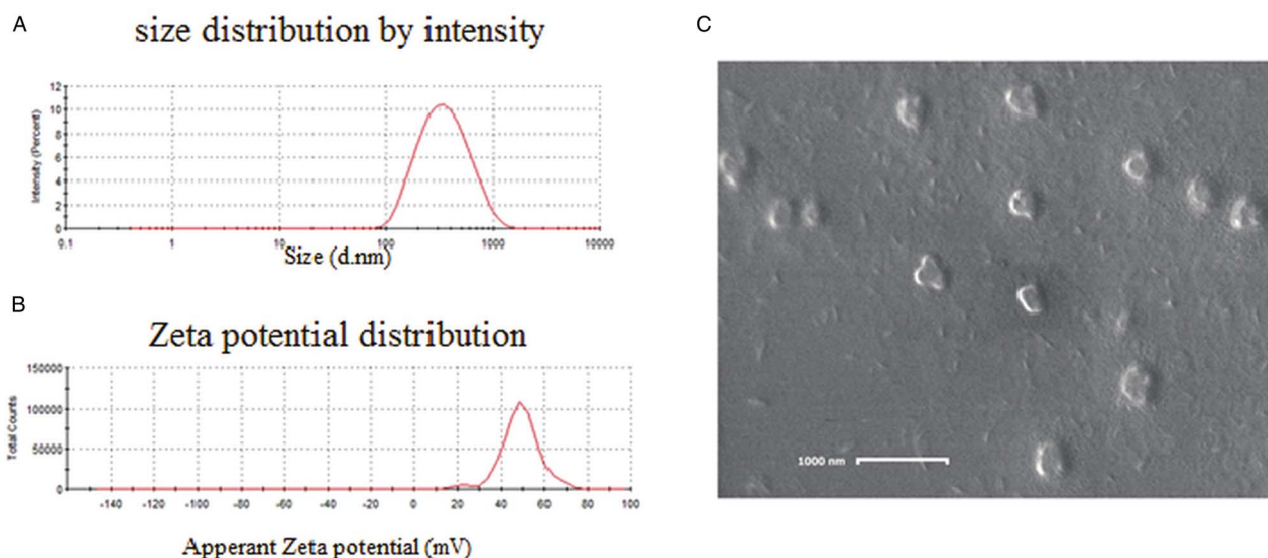


Fig. 2. (A) DLS showing the size distribution of chitosan nanoparticles; (B) zeta potential of chitosan nanoparticles showed good positive charge; (C) SEM image of chitosan nanoparticles showed proper morphology.

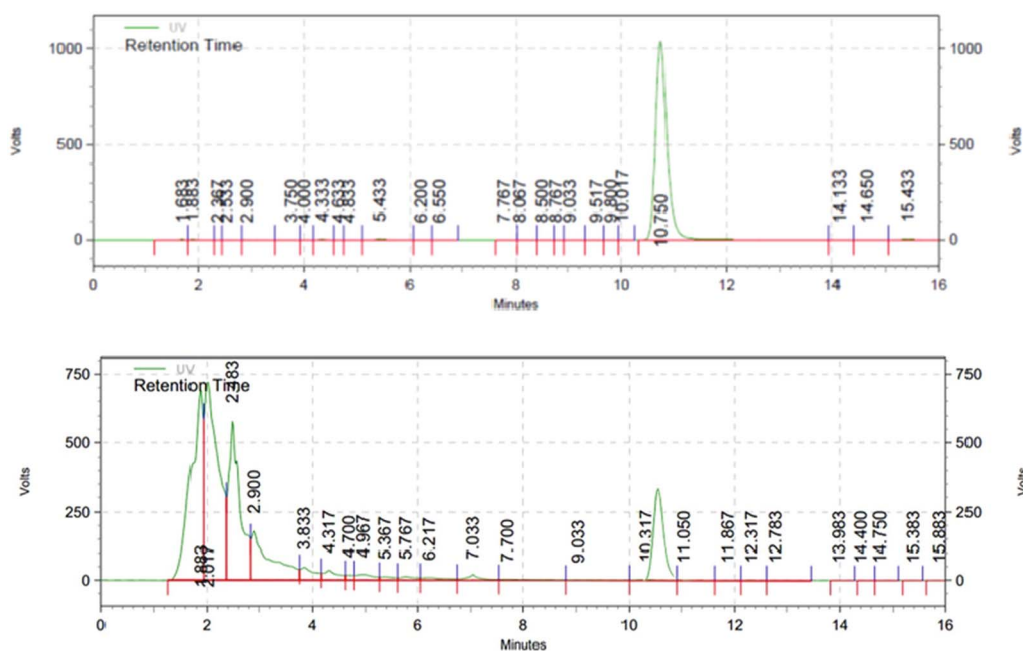


Fig. 3. HPLC chromatogram of standard emodin (up) and methanolic extract of *R. cathartica* (down); wavelength 254 nm; flow rate, 1 mL min⁻¹; injection volume, 20.0 mL; concentration, 200 mM each.

UV-E4310 intelligent UV/vis detector, 254 nm]. The sample was eluted with isocratic mobile phase consisted of acetonitrile and water (65: 35) HPLC grade at the flow rate of 1 mL min⁻¹ with 20 min retention time (RT).

In vitro anti-giardial assay

The total extract and natural products were used with the following concentration: chitosan: 100, 200, 400 µg mL⁻¹, nano-chitosan: 100, 200, 400 µg mL⁻¹, emodin; 200 µg mL⁻¹, *R. cathartica*: 50, 100, 200 µg mL⁻¹, metronidazole: 400 mg mL⁻¹, furazolidone: 250 mg mL⁻¹, at 30, 60, 180 min. Dimethyl sulfoxide

(DMSO) and phosphate-buffered saline (PBS) were considered as negative controls, otherwise metronidazole and furazolidone as positive controls.

The excystation was done according to the method described by Golami *et al.* (2016). Trophozoite forms were cultured in TYI-S-33 medium enriched with bovine bile, 20% heat-inactivated FCS, penicillin (200 mg mL⁻¹) and streptomycin (200 mg mL⁻¹) at 37 °C in borosilicate glass tubes (PYREX®). Two millilitres of each solution and 1 × 10⁴ washed trophozoites were added to the test tubes. The contents of the tubes were gently mixed and incubated at 37 °C for 30, 60 and 180 min. At the end of each incubation time, the upper phase was carefully removed, 1 mL of 0.1%

Table 1. The mortality rate of *G. lamblia* trophozoite in different groups, *in vitro*

| Time concentration | | | 30 min | 60 min | 180 min | P-value* |
|---------------------------------|---------------------------|-----|---------------|---------------|---------------|----------|
| Chitosan | 100 $\mu\text{g mL}^{-1}$ | I | 72 \pm 2.04 | 82 \pm 2.67 | 89 \pm 3.9 | 0.406 |
| | | II | 74 \pm 1.09 | 82 \pm 3.24 | 83 \pm 3.87 | |
| | | III | 71 \pm 3.1 | 78 \pm 1.67 | 82 \pm 2.89 | |
| | 200 $\mu\text{g mL}^{-1}$ | I | 77 \pm 2.3 | 86 \pm 3.23 | 95 \pm 4.5 | 0.390 |
| | | II | 76 \pm 4.5 | 82 \pm 4.32 | 93 \pm 2.9 | |
| | | III | 76 \pm 3.6 | 87 \pm 3.21 | 94 \pm 3.8 | |
| | 400 $\mu\text{g mL}^{-1}$ | I | 87 \pm 3.2 | 95 \pm 5.76 | 99 \pm 6.3 | 0.671 |
| | | II | 85 \pm 5.25 | 94 \pm 5.3 | 100 \pm 1.2 | |
| | | III | 83 \pm 4.56 | 92 \pm 4.2 | 99 \pm 0.9 | |
| Nano-chitosan | 100 $\mu\text{g mL}^{-1}$ | I | 75 \pm 3.15 | 81 \pm 4.67 | 89 \pm 1.3 | 0.547 |
| | | II | 71 \pm 2.78 | 79 \pm 3.2 | 90 \pm 2.9 | |
| | | III | 76 \pm 2.34 | 78 \pm 2.89 | 88 \pm 2.1 | |
| | 200 $\mu\text{g mL}^{-1}$ | I | 79 \pm 1.45 | 89 \pm 4.56 | 97 \pm 3.2 | 0.398 |
| | | II | 77 \pm 3.76 | 87 \pm 3.21 | 95 \pm 2.2 | |
| | | III | 79 \pm 2.45 | 87 \pm 5.4 | 96 \pm 1.9 | |
| | 400 $\mu\text{g mL}^{-1}$ | I | 85 \pm 4.35 | 93 \pm 6.5 | 99 \pm 2.7 | 0.586 |
| | | II | 87 \pm 3.24 | 92 \pm 5.4 | 99 \pm 3.8 | |
| | | III | 88 \pm 5.34 | 93 \pm 3.9 | 100 \pm 0.1 | |
| Emodin | 200 $\mu\text{g mL}^{-1}$ | | 90 \pm 5.23 | 98 \pm 2.89 | 100 \pm 1.1 | 0.747 |
| <i>R. cathartica</i> | 50 $\mu\text{g mL}^{-1}$ | | 81 \pm 4.32 | 91 \pm 4.3 | 97 \pm 2.1 | 0.483 |
| | 100 $\mu\text{g mL}^{-1}$ | | 81 \pm 3.23 | 89 \pm 3.7 | 98 \pm 1.9 | 0.487 |
| | 200 $\mu\text{g mL}^{-1}$ | | 79 \pm 2.76 | 91 \pm 5.6 | 98 \pm 2.1 | 0.399 |
| Metronidazole | | | 83 \pm 34 | 94 \pm 4.31 | 100 \pm 1.5 | 0.312 |
| Furazolidone | | | 81 \pm 5.67 | 89 \pm 1.98 | 97 \pm 3.4 | 0.348 |
| Negative control (DMSO and PBS) | | | 1 \pm 0.1 | 1 \pm 0.2 | 2 \pm 0.2 | |
| P-value** | | | <0.001 | <0.001 | <0.001 | |

*Effect of time.

**Effect of concentration.

(I: *P. chrysogenum*, II: *P. pinophilum*, III: *P. rubefaciens*).

eosin stain (MW: 691.86) were added and incubated for 15 min. The viability of were microscopically determined by counting 500 cysts.

In vivo anti-giardial assay

Thirty male Balb/c mice (20 ± 2 g) at the age of at least 2 weeks were used in six groups (five mice in every group) and all animals were kept under standard environments. Stool examination ensured that mice were free from all possible protozoan infections. Mice were infected intragastrically with 1×10^3 cysts. One week after treatment, three consecutive fecal examinations were performed to prove the experimental infections. After infection, mice were treated intragastrically with fungal chitosan (10, 50, 100 $\mu\text{g kg}^{-1}$), nano-chitosan (50 $\mu\text{g kg}^{-1}$), emodin (100 mg kg^{-1}), *R. cathartica* (50, 100, 200 mg kg^{-1}) in comparison to metronidazole (10 mg kg^{-1}) and furazolidone (10 mg kg^{-1}) daily, separately. The mentioned natural products that dissolved in DMSO and 1 mL PBS were in contrast to metronidazole and furazolidone as a positive control. The negative control group received the PBS alone. After 24, 48 and 72 h of treatment, stool examinations were done to evaluate the viability of cysts. One gram of feces was homogenized in 10 cc of formal saline and the viability of cysts was done

by 0.1% eosin vital staining and a haemocytometer (Dyab *et al.*, 2016).

Cytotoxicity assay

Human intestinal epithelial cells (HT-29) were seeded into 96-well plates (Nunc, Roskilde, Denmark) in DMEM (100 μl) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin except for the last row which contained only 100 μl of DMEM which was considered as the blank. Cells then were incubated at 37 $^{\circ}\text{C}$ in a humidity of 95 and 5% CO_2 for 24 h. Plant extracts in concentrations of 50, 100, 200 mg mL^{-1} were added to each well in triplicate. After 24, 48 and 72 h of incubation time, 10 μL of MTT solution (Sigma, UK) (5 mg mL^{-1} in PBS) was added to each well, including controls. After 3 h of incubation at 37 $^{\circ}\text{C}$, the supernatant was removed and 100 μL of DMSO that was purchased from Merck (Darmstadt, HE, Germany) was added. Finally, the absorbance at 570 nm was measured by a microtiter plate reader (BioTek ELX800, Winooski, VT, USA) (Lakshmi and Bai, 2016) and the viability was calculated by below formula:

$$\text{Viability (\%)} = \frac{\text{OD Test}}{\text{OD Control}} \times 100$$

Table 2. The mortality rate of *G. lamblia* cyst in in different groups, *in vitro*

| Time concentration | | | 30 min | 60 min | 180 min | P-value* |
|-------------------------------|---------------------------|-----|---------------|---------------|---------------|----------|
| Chitosan | 100 $\mu\text{g mL}^{-1}$ | I | 66 \pm 2.9 | 66 \pm 1.2 | 82 \pm 4.2 | 0.302 |
| | | II | 68 \pm 5.3 | 67 \pm 2.8 | 78 \pm 3.7 | |
| | | III | 65 \pm 3.2 | 71 \pm 3.1 | 78 \pm 2.9 | |
| | 200 $\mu\text{g mL}^{-1}$ | I | 70 \pm 1.8 | 79 \pm 3.9 | 87 \pm 3.2 | 0.399 |
| | | II | 69 \pm 2.6 | 76 \pm 2.04 | 89 \pm 4.8 | |
| | | III | 70 \pm 3.2 | 81 \pm 4.02 | 90 \pm 5.2 | |
| | 400 $\mu\text{g mL}^{-1}$ | I | 79 \pm 4.9 | 86 \pm 5.9 | 96 \pm 5.8 | 0.432 |
| | | II | 78 \pm 4.2 | 88 \pm 4.3 | 97 \pm 6.3 | |
| | | III | 76 \pm 4.2 | 88 \pm 3.89 | 93 \pm 4.5 | |
| Nano-chitosan | 100 $\mu\text{g mL}^{-1}$ | I | 71 \pm 3.4 | 79 \pm 2.87 | 84 \pm 3.21 | 0.576 |
| | | II | 69 \pm 4.8 | 74 \pm 3.87 | 87 \pm 2.98 | |
| | | III | 70 \pm 3.7 | 75 \pm 2.54 | 82 \pm 3.8 | |
| | 200 $\mu\text{g mL}^{-1}$ | I | 73 \pm 3.2 | 86 \pm 3.2 | 93 \pm 2.56 | 0.239 |
| | | II | 71 \pm 2.9 | 83 \pm 4.6 | 90 \pm 8.54 | |
| | | III | 74 \pm 2.4 | 82 \pm 3.5 | 93 \pm 6.43 | |
| | 400 $\mu\text{g mL}^{-1}$ | I | 79 \pm 3.5 | 89 \pm 5.4 | 95 \pm 3.82 | 0.475 |
| | | II | 83 \pm 2.5 | 88 \pm 4.3 | 94 \pm 4.32 | |
| | | III | 82 \pm 3.9 | 88 \pm 3.9 | 98 \pm 6.54 | |
| Emodin | 200 $\mu\text{g mL}^{-1}$ | | 86 \pm 3.6 | 92 \pm 4.3 | 95 \pm 3.22 | 0.794 |
| <i>R. cathartica</i> | 50 $\mu\text{g mL}^{-1}$ | | 75 \pm 2.56 | 87 \pm 3.4 | 93 \pm 4.32 | 0.372 |
| | 100 $\mu\text{g mL}^{-1}$ | | 77 \pm 4.32 | 84 \pm 4.8 | 95 \pm 5.1 | 0.424 |
| | 200 $\mu\text{g mL}^{-1}$ | | 71 \pm 3.09 | 86 \pm 2.7 | 96 \pm 4.9 | 0.153 |
| Metronidazole | | | 79 \pm 3.56 | 89 \pm 3.9 | 96 \pm 4.76 | 0.214 |
| Furazolidone | | | 77 \pm 2.89 | 95 \pm 2.8 | 92 \pm 3.29 | 0.189 |
| Negative control (DMSO & PBS) | | | 1 \pm 0.2 | 1 \pm 0.15 | 2 \pm 0.3 | |
| P-value** | | | <0.001 | <0.001 | <0.001 | |

*Effect of time.

**Effect of concentration.

(I: *P. chrysogenum*, II: *P. pinophilum*, III: *P. rubefaciens*).

Histopathological investigation

At the end of the treatment, the kidneys and livers of each animal were removed and fixed in 10% formalin. The organs sliced transversely and paraffin embedded to prepare 5 μm thick sections. The sections were stained with haematoxylin and eosin on a slide and evaluated under a light microscope and photographed by a camera microscope (Labomed, LX400) at 400x magnification. The level of liver damage was diagnosed by hepatocellular necrosis, level of inflammatory in portal area, and lymphocytic inflammatory infiltrations were scored in which grade 0 = no damage, 1 = very low level of damage, 2 = mild damage, 3 = moderate damage and 4 = severe damage. The level of kidney damage was confirmed by the survey of the glomerular inflammation, tubular necrosis and intracellular inflammation. All procedures were performed in triplicate.

Data analysis

Statistical analysis was carried out using SPSS (version 16) Software. Data were given as the mean \pm standard deviation (s.d.). Paired sample *t*-test, one-way ANOVA and K^2 square have been done to determine the differences between groups and after treatment. *P* value <0.05 considers defining significant variation.

Results

Giardia lamblia genotyping

Gdh gene was amplified and digested by restriction *Bsp*LI enzyme. PCR products in the first step had a molecular weight band of 458 bp that fragment by restriction enzymes and the results revealed that the isolate is characteristic four-band pattern of assemblage AII (Fig. 1).

Molecular weight of chitosan and DD and viscosity

The DD was achieved 75.1, 64, 57.9, viscosity 10.1, 9, 9.3 and MW 1.3×10^5 , 9.3×10^4 , 5.3×10^4 for *P. chrysogenum*, *P. pinophilum*, *P. rubefaciens*, respectively.

Morphology analysing by SEM

The particle size data of chitosan NPs by DLS showed (Fig. 2A) a size of 387 nm with a PDI of about 0.23. SEM (Fig. 2C) images show that the average size is around 300 nm with spherical morphology. Surface charge and thereby the stability of the prepared nanoparticle systems was determined by zeta potential measurements. Zeta potential value was found to be 48.7 mV

Table 3. Mean percentage of excreted cysts before and after treatment in comparison to control groups *in vivo* ($n=5$, mice per a group)

| Time treatment | | Before treatment (~%) | After 24 h (~%) | After 48 h (~%) | After 72 h (~%) | P-value* |
|---|-------------------------|-----------------------|-------------------|------------------|------------------|----------|
| Chitosan-10 $\mu\text{g kg}^{-1}$ | I | 57.63 \pm 0.55 | 50.83 \pm 0.85 | 45.4 \pm 1.28 | 38.63 \pm 0.81 | 0.001 |
| | II | 56.3 \pm 1.13 | 47.4 \pm 1.34 | 53.8 \pm 1.47 | 41.4 \pm 5.99 | |
| | III | 55.67 \pm 1.53 | 33.97 \pm 4.63 | 63.2 \pm 1.93 | 31.83 \pm 1.40 | |
| Chitosan-50 $\mu\text{g kg}^{-1}$ | I | 57.3 \pm 0.61 | 34.33 \pm 1.46 | 25.03 \pm 1.05 | 21.7 \pm 3.84 | 0.017 |
| | II | 57.3 \pm 1.54 | 31.7 \pm 5.12 | 21.1 \pm 1.85 | 19.23 \pm 2.99 | |
| | III | 57.33 \pm 3.51 | 27.64 \pm 15.96 | 23.77 \pm 2.82 | 17.67 \pm 2.55 | |
| Chitosan-100 $\mu\text{g kg}^{-1}$ | I | 60.0 \pm 2.0 | 33.43 \pm 1.40 | 17.60 \pm 7.71 | 1.0 \pm 1.0 | 0.006 |
| | II | 51.2 \pm 18.13 | 29.53 \pm 4.83 | 15.87 \pm 3.07 | 1.0 \pm 1.0 | |
| | III | 55.03 \pm 3.36 | 17.1 \pm 3.21 | 11.87 \pm 3.45 | 3.0 \pm 1.0 | |
| Nano-chitosan 10 $\mu\text{g kg}^{-1}$ | I | 54.3 \pm 2.54 | 37.33 \pm 2.46 | 28.03 \pm 2.05 | 19.7 \pm 2.84 | 0.001 |
| | II | 56.3 \pm 1.54 | 29.7 \pm 4.12 | 22.1 \pm 2.85 | 18.23 \pm 1.8 | |
| | III | 55.3 \pm 1.64 | 28.64 \pm 11.96 | 21.77 \pm 1.82 | 16.07 \pm 1.05 | |
| Nano-chitosan-50 $\mu\text{g kg}^{-1}$ | I | 57.5 \pm 1.34 | 30.3 \pm 2.5 | 19.60 \pm 0.6 | 2.0 \pm 0.7 | <0.001 |
| | II | 57.7 \pm 2.54 | 19.03 \pm 4.83 | 15.3 \pm 2 | 3.0 \pm 1.0 | |
| | III | 53.3 \pm 1.34 | 15.1 \pm 2.01 | 10.64 \pm 2.75 | 3.0 \pm 2.1 | |
| Nano-chitosan 100 $\mu\text{g kg}^{-1}$ | I | 54.4 \pm 2.35 | 22.27 \pm 2.25 | 11.6 \pm 1.51 | 1.0 \pm 1.0 | 0.004 |
| | II | 54.83 \pm 2.56 | 21.3 \pm 1.13 | 11.13 \pm 3.38 | 2.0 \pm 1.0 | |
| | III | 55.3 \pm 2.46 | 11.57 \pm 6.44 | 9.27 \pm 4.19 | 1.0 \pm 1.0 | |
| Emodin | 100 mg kg^{-1} | 59.63 \pm 5.5 | 24.27 \pm 5.02 | 16.8 \pm 3.5 | 1.0 \pm 1.0 | <0.001 |
| <i>R. cathartica</i> | 50 mg kg^{-1} | 56.57 \pm 1.07 | 52.87 \pm 2.9 | 30.17 \pm 8.66 | 23.7 \pm 2.47 | 0.002 |
| | 100 mg kg^{-1} | 54.67 \pm 2.65 | 43.57 \pm 1.29 | 30.87 \pm 3.49 | 18.17 \pm 4.11 | |
| | 200 mg kg^{-1} | 53.0 \pm 1.54 | 42.53 \pm 4.08 | 21.17 \pm 3.38 | 5.3 \pm 4.01 | |
| Metronidazole | | 51.1 \pm 0.75 | 27.57 \pm 1.16 | 13.8 \pm 0.75 | 3.97 \pm 0.057 | 0.001 |
| Furazolidone | | 54.3 \pm 0.61 | 30.93 \pm 1.01 | 18.17 \pm 0.67 | 6.2 \pm 0.82 | 0.001 |
| Negative control (DMSO and PBS) | | 54.93 \pm 0.95 | 24.47 \pm 1.17 | 48.1 \pm 0.66 | 72.07 \pm 0.21 | |

*The differences between groups before and after treatment.
(I: *P. chrysogenum*, II: *P. pinophilum*, III: *P. rubefaciens*).

(Fig. 3B). This value lies in the stable range, indicating that this nanoparticle system was stable.

Phytochemical screening

Total flavonoid and phenolic contents

The yield of extract was 17%. The total flavonoid content was 23.15 ± 1.355 mg CE/g DW in reference to the standard curve ($y = 0.0063x - 0.0055$, $r^2 = 0.999$). The total phenolic content was 351.66 ± 4.85 mg GAE/g DW in reference to the standard curve ($Y = 0.0028x - 0.0362$, $r^2 = 0.999$).

DPPH free radical scavenging activity

The calculated IC_{50} value of methanolic extract was 74.46 ± 1.89 $\mu\text{g mL}^{-1}$ while for positive control ascorbic acid it was 57.92 ± 2.21 $\mu\text{g mL}^{-1}$.

HPLC separation

The isocratic HPLC chromatogram of emodin exhibited the narrower and sharp peak in 10.75 min (Fig. 2). The methanolic extract of *R. cathartica* reported the shorter peak in approximately

the same RT in 10.31 min (Fig. 3). The similar chromatograms proved the existence of emodin in *R. cathartica* methanolic extract.

In vitro anti-giardial activity

The mortality rate of trophozoites and cysts at the highest concentration and exposure time were (99 ± 6.3 and 96 ± 5.8) and (99 ± 2.7 and 95 ± 3.82) for common and nano form of *P. chrysogenum*, (100 ± 1.2 and 97 ± 6.3) and (99 ± 3.8 and 94 ± 4.32) for common and nano form of *P. pinophilum*, (99 ± 0.9 and 93 ± 4.5) and (100 ± 0.1 and 98 ± 6.54) for common and nano form of *P. rubefaciens*, (100 ± 1.1 and 95 ± 3.22) for emodin, (98 ± 2.1 and 96 ± 4.9) for *R. cathartica*, (100 ± 1.5 and 96 ± 4.76) for metronidazole, (97 ± 3.4 and 92 ± 3.29) for furazolidone, (2 ± 0.8 and 2 ± 0.3) for negative control. The toxicity of these treatments on trophozoites is independent to exposure time without any significant difference ($P > 0.05$). The mortality rate of trophozoites and cysts in treatment groups was similar to positive control ($P > 0.05$), otherwise significantly higher than negative control ($P < 0.05$). Concentration as a main variable has a significant effect on mortality rate (Tables 1 and 2). Nano-chitosan has a better effect than chitosan; however, the difference was not significant ($P > 0.05$).

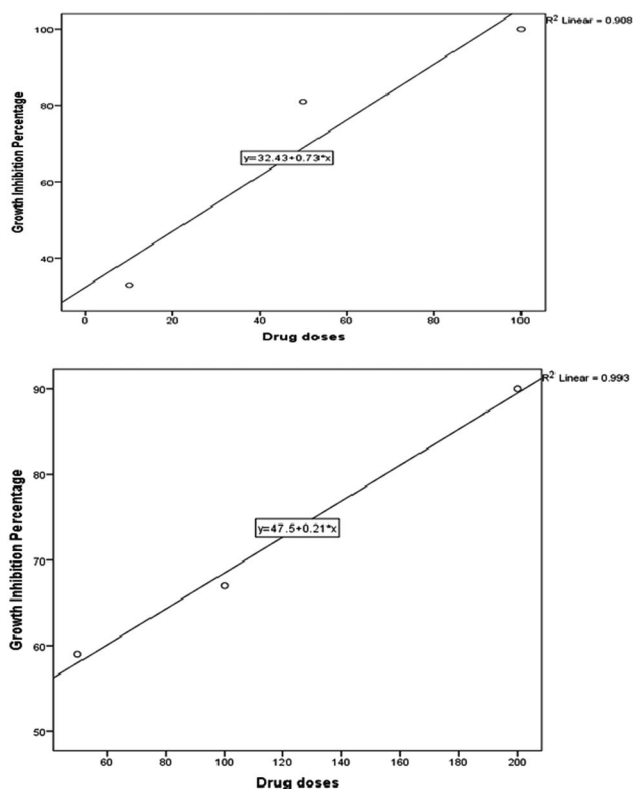


Fig. 4. (A) Determination of EC₅₀ values of fungal chitosan on *Giardia lamblia* *in vivo*, (B) Determination of EC₅₀ values of *R. cathartica* extract on *Giardia lamblia* *in vivo*.

In vivo anti-giardial activity

As shown in Table 3, the excretion of cysts was decreased after treatment in comparison to negative control ($P < 0.05$). Concentration was another important factor in this study, but in all cases there was no significant difference. Emodin as the most important contents of *R. cathartica* was more effective against *G. lamblia* than the total extract ($P < 0.05$).

Approximately, in most of the concentration, all fungal chitosan, nano-chitosan and emodin were more effective than metronidazole and furazolidone against *G. lamblia* in mice model.

The excretion of the cysts was increased by the increase of the chitosan concentration and there are significant differences among cyst excretion in the concentration of 10 and 100 $\mu\text{g kg}^{-1}$ ($P < 0.05$). Of course, the time as an effective factor influenced the mortality rate of *G. lamblia* cysts and trophozoites; by increasing the time of exposure, the mortality rate is being increased.

Since the results of the study in different doses of chitosan show a linear relationship with the doses of the parasite, according to the linearity obtained from the regression equation ($y = 32.43 + 0.73X$), EC₅₀ of chitosan at a dose of 24 mg mL^{-1} was calculated. On the other hand, the linear regression equation for the extract of *R. cathartica* showed ($y = 47.5 + 0.21X$), the EC₅₀ value is based on the line of 12 mg (Fig. 4). However, the regression equation for the nano-chitosan was not linear and the EC₅₀ was predicted as 12 $\mu\text{g mL}^{-1}$.

Cytotoxicity activity

The tetrazolium-based colorimetric MTT assay indicated that fungal chitosan, nano-chitosan and *R. cathartica* methanolic extract in different concentrations and emodin had no toxicity effects on the HT-29 cells viability after 24, 48 and 72 h ($P > 0.05$) (Table 4 and Fig. 5). By prolonging exposure time, no significant toxicity has been observed in all groups ($P > 0.05$).

Histopathology examination

The histopathological examinations of the histological sections of the livers and kidneys in different groups (chitosan, nano-chitosan, emodin, *R. cathartica*, metronidazole and furazolidone) after 72 h of treatment were represented, respectively. No evidence of abnormality, tissue degeneration, inflammation and necrosis was existed in liver and kidney sections in all groups. Hepatocytes were normal with polygonal shape and normal sinusoidal and portal space. Kidney tissue and glomerular morphology and tubular epithelia were normal in all groups.

Discussion

In the present study, the anti-giardial activity of fungal chitosan, nano-chitosan, emodin, nano-emodin and methanolic extract of *R. cathartica* evaluated *in vivo*. Natural products are valuable sources for developing new drugs over centuries (Koehn and Carter, 2005). Owing to the growing rate of resistance to giardiasis drugs, introduction of new agents are essential for effective treatment (Croft *et al.*, 2006). Up to now, the genus *Rhmannus* has not been studied for anti-giardial effects, however there are reports of the antimicrobial effect of the species (Kosalec *et al.*, 2013). *Rhmannus cathartica* showed equal anti-giardial activity against trophozoites and cyst of *G. lamblia* in comparison to metronidazole and furazolidone as positive controls without any significant differences ($P = 0.09$). Flavonoids, phenolics and anthraquinones compounds in this genus are effective in antimicrobial activities and this is due to the ability to penetrate into the microorganisms cells (Sultana *et al.*, 2007; Qadir *et al.*, 2017). These compounds could bind to the nucleotide-binding site of multiple drugs proteins with an associated heighten the accumulation of intracellular drug as a modulator. Several investigations have also indicated an anti-giardial activity of a variety of total herbal extracts such as *Artemisia annua* (IC₅₀ = 51 mg mL^{-1}), *Sambucus ebulus* (IC₅₀ = 43 mg mL^{-1}) (Rahimi-Esboei *et al.*, 2013a, 2013b).

The IC₅₀ values described in these studies variety from 0.8 to 300 $\mu\text{g mL}^{-1}$, but the majority of plant extracts presented IC₅₀ values more than 100 $\mu\text{g mL}^{-1}$ (Calzada *et al.*, 2006; Barbosa *et al.*, 2007; Machado *et al.*, 2010). Emodin with anthraquinone structure is one of the main compositions of *R. cathartica* extract and has anti-parasitic effects; better than positive controls and common treatments in an *in vivo* situation (Srinivas *et al.*, 2007; Li *et al.*, 2016). The reports on anthraquinones parasitocidal activities are limited and the mechanism of action is not cleared yet. Anthraquinones, especially emodin, can selectively inhibit the casein kinase II and tyrosine kinases activity (Li *et al.*, 2016). Protein tyrosine kinases play significant roles in the regulation of cell growth by catalysing the transfer of phosphate from ATP to the hydroxyl of tyrosine. The conjugated double-bond system in aromatic rings with the resonant properties causes the tremendous antimicrobial effects. The resonance in anthraquinone structure creates the electron donors (HD) and acceptors leading to form hydrogen bonds with the cell membrane of microbial and leakage of the intracellular contents (Srinivas *et al.*, 2007; Li *et al.*, 2016; Venkatachalam *et al.*, 2016). Chukwujekwu *et al.* (2009) reported that emodin had antibacterial effects against *Bacillus subtilis* and *Staphylococcus aureus* with Minimum Inhibitory Concentration (MIC) values of 7.8×10^{-3} and 3.9×10^{-3} mg mL^{-1} , respectively. Emodin and three anthraquinone derivatives isolated from *Rheum emodi* had antimalarial activity on Pfk₁ strain (*Palladium falciparum* chloroquine resistant strain) with the IC₅₀ of 2.28, 2.49 and 2.48 μM , respectively, and without any cytotoxicity on monkey kidney cell line (Pandeti *et al.*, 2014).

Table 4. Cytotoxicity results of the different groups on the HT-29 cells

| Treatment | Concentration | 24 h | 48 h | 72 h | P-value* |
|-------------------------------|-------------------------|--------------|--------------|--------------|----------|
| Chitosan | I | 1.23 ± 0.015 | 0.93 ± 0.02 | 0.65 ± 0.01 | 0.832 |
| | II | 1.14 ± 0.01 | 0.90 ± 0.01 | 0.64 ± 0.012 | |
| | III | 1.10 ± 0.02 | 0.78 ± 0.015 | 0.63 ± 0.015 | |
| Nano-chitosan | I | 1.25 ± 0.015 | 1.06 ± 0.015 | 0.68 ± 0.015 | 0.171 |
| | II | 1.21 ± 0.01 | 1.06 ± 0.026 | 0.68 ± 0.01 | |
| | III | 1.20 ± 0.01 | 0.98 ± 0.067 | 0.66 ± 0.01 | |
| Emodin | 100 mg mL ⁻¹ | 1.19 ± 0.01 | 0.90 ± 0.10 | 0.76 ± 0.01 | 0.123 |
| <i>R. cathartica</i> | 50 mg mL ⁻¹ | 1.26 ± 0.05 | 1.07 ± 0.02 | 0.88 ± 0.016 | 0.251 |
| | 100 mg mL ⁻¹ | 1.24 ± 0.02 | 1.03 ± 0.01 | 0.88 ± 0.01 | 0.225 |
| | 200 mg mL ⁻¹ | 1.19 ± 0.01 | 0.88 ± 0.015 | 0.73 ± 0.01 | 0.162 |
| | 400 mg mL ⁻¹ | 0.93 ± 0.047 | 0.59 ± 0.068 | 0.34 ± 0.01 | 0.078 |
| Metronidazole | | 1.22 ± 0.015 | 0.98 ± 0.03 | 0.43 ± 0.01 | 0.051 |
| Furazolidone | | 1.08 ± 0.05 | 0.79 ± 0.035 | 0.46 ± 0.006 | 0.067 |
| Negative control (DMSO & PBS) | | 1.32 ± 0.04 | 1.13 ± 0.06 | 0.94 ± 0.01 | 0.072 |
| P-value** | | 0.602 | 0.567 | 0.074 | |

*Effect of exposure time on toxicity of drugs on normal cell line.

**The toxicity of drugs on normal cell line.

(I: *P. chrysogenum*, II: *P. pinophilum*, III: *P. rubefaciens*).

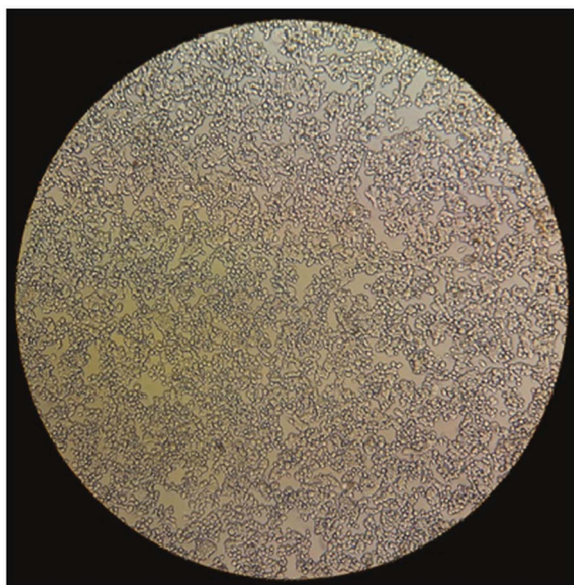


Fig. 5. Fresh HT-29 cells after treatment by Chitosan, *R. cathartica* and emodin.

Chitosan in high acetylation was more effective in comparison to the low acetylation form. Similar findings revealed the significant difference in the efficacy of fungal chitosan in comparison to untreated group in the concentration of 10 µg mL⁻¹ against *G. lamblia* cysts (Yarahmadi et al., 2016). The modification of chitosan nanoparticles using sodium TPP by ionotropic gelation method revealed more advantages over the common form.

This modification can promote the bioavailability, specificity and sensitivity and decreasing toxicity in human body (Ebrahimzadeh et al., 2013). The SEM showed that the pure chitosan has a plain, smooth, compact and homogeneous surface without pores, whereas, chitosan nanoparticles have a rough, porous and larger exposed surface which increases adsorption. Several researchers reported that the chitosan nanoparticles have

the spherical shape, solid dense cubical or rectangular structure and rough surface morphology (Wim et al., 2008; Jahangiri et al., 2018; Wardani et al., 2018).

Thus, the smaller the particle, the higher will be its mobility and surface interaction, resulting in enhancing the antimicrobial activity and the bioavailability of poorly water-soluble molecules and could improve the efficacy of the particle-based oral drug delivery systems (Sui et al., 2010; Pilon et al., 2015; Jahangiri et al., 2018). *Rhamnus cathartica* methanolic extract, emodin and chitosan had no cytotoxicity effects on the HT-29 cells viability after 24, 48 and 72 h. However, after many times (72 h) high deacetylated chitosan, high deacetylate nano-chitosan and *R. cathartica* methanolic extract in the concentration of 400 mg mL⁻¹ reduced the cell viability to <50%.


Several *in vivo* and *in vitro* studies have confirmed the non-toxicity effect of chitosan on human cell lines, and the FDA has approved chitosan for clinical applications (Baldrick, 2010). Also, it is approved for dietary applications in Japan, Italy and Finland (Kim, 2013). Biodegradability and biocompatibility as some of their particular properties make them appropriate for medical application (Kucharska et al., 2010; Kim, 2013). Although the particular mechanism of antiprotozoal activity of chitosan is not fully explained, but the effect on the cell wall of parasites is the suggested mechanism (Tayel et al., 2010; Rahimi-Esboei et al., 2013a, 2013b).

Chitosan is more effective than the majority of other plant extracts. Additionally, the effect observed on viability revealed advantageous for the development of potential giardicidal treatments from chitosan.

Conclusion

The effects of different therapies on trophozoite have been better than cysts. But contrary to the previous mindset that we thought that trophozoite was more sensitive than cyst; the difference between cysts and trophozoites was not significant in this study. Due to differences in cellular structure and cell wall in cyst and trophozoite, this study has shown that there is no significant

difference in drug effects. The chitosan derived from fungi, nano-chitosan, *R. cathartica* extract and emodin may be used as important compounds for the development of novel therapeutic drugs for the treatment of giardiasis. Mentioned remedies showed to be safe for mammalian cells by *in vitro* and *in vivo* tests. Also, the nano-chitosan synthesis improved the therapeutic response in lower dosage. In addition, the chitosan has been commonly available, easily prepared and low-priced. Therefore, nano-chitosan was accepted as a good candidate among natural giardiacidal agents.

Author ORCIDs.  Aroona Chabra, 0000-0002-2432-0362

Acknowledgements. Special thanks to Mr. Danial Chabra (Faculty of Food Science & Industry, Gorgan Agricultural Sciences University, Gorgan, Iran) for language editing.

Financial support. This work was supported by a grant from Student Research Committee (No: 72 & 106 & 107) and Pharmaceutical Science Research Center, Faculty of Pharmacy (No: 2843), Mazandaran University of Medical Sciences, Sari, Iran.

Conflict of interest. None.

Ethical standards. The experimental protocols were approved by the ethical committee of Mazandaran University of Medical Sciences, in agreement with the guidelines for the use and care of laboratory animals and the treatment started after the initial infection.

References

- Anquez-Traxler C (2011) The legal and regulatory framework of herbal medicinal products in the European Union: a focus on the traditional herbal medicines category. *Drug Information Journal* **45**, 15–23.
- Babaei Z, Oormazdi H, Akhlaghi L, Rezaie S, Razmjou E, Soltani-Arabshahi SK, Meamar AR and Hadighi R (2008) Molecular characterization of the Iranian isolates of *Giardia lamblia*: application of the glutamate dehydrogenase gene. *Iranian Journal of Public Health* **37**, 75–72.
- Baldrick P (2010) The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology and Pharmacology* **56**, 290–299.
- Barbosa E, Calzada F and Campos R (2007) *In vivo* anti-giardial activity of three flavonoids isolated of some medicinal plants used in Mexican traditional medicine for the treatment of diarrhea. *Journal of Ethnopharmacology* **109**, 552–554.
- Calzada F, Y´opez-Mulia L and Aguilar A (2006) *In vitro* susceptibility of *Entamoeba histolytica* and *Giardia lamblia* to plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *Journal of Ethnopharmacology* **108**, 367–370.
- Chukwujekwu JC, Coombes PH, Mulholland DA and van Staden J (2006) Emodin, an antibacterial anthraquinone from the roots of *Cassia occidentalis*. *South African Journal of Botany* **72**, 295–297.
- Croft SL, Sundar S and Fairlamb AH (2006) Drug resistance in leishmaniasis. *Clinical microbiology reviews* **19**, 111–126.
- Derda M and Hadaš E (2014) The use of phytotherapy in diseases caused by parasitic protozoa. *Acta Parasitologica* **60**, 1–8.
- Dyab AK, Yones DA, Ibrahim ZZ and Hassan TM (2016) Anti-giardial therapeutic potential of dichloromethane extracts of *Zingiber officinale* and *Curcuma longa* *in vitro* and *in vivo*. *Parasitology Research* **115**, 2637–2645.
- Ebrahimzadeh MA, Chabra A, Gharaei-Fathabad E and Pourmorad F (2013) Preparation of chitosan from *Penicillium* spp. and determination of their degree of deacetylation. *Indian Journal of Biotechnology* **12**, 231–235.
- Elmi F, Gholami S, Rahimi-Esboei B, Garaili Z, Najm M and Tabatabaie F (2017) Comparison of sensitivity of sucrose gradient, Wet mount and formalin – ether in detecting protozoan *Giardia lamblia* in stool specimens of BALB/c mice. *Journal of Pure and Applied Microbiology* **11**, 105–109.
- Feng Y and Xiao L (2011) Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. *Clinical Microbiology Reviews* **24**, 110–140.
- Golami Sh, Rahimi-Esboei B, Mousavi P, Marhaba Z, Yousefi MR and Rahimi MT (2016) Survey on efficacy of chloroformic extract of *Artemisia annua* against *Giardia lamblia* trophozoite and cyst *in vitro*. *Journal of Parasitic Diseases* **40**, 88–92.
- Guo J, Sun X, Yin H, Wang T, Li Y, Zhou C, Zhou H, He S and Cong H (2018) Chitosan microsphere used as an effective system to deliver a linked antigenic peptides vaccine protect mice against acute and chronic *Toxoplasmosis*. *Frontiers in Cellular and Infection Microbiology* **23**, 163.
- Halliez MC and Buret AG (2013) Extra-intestinal and long term consequences of *Giardia duodenalis* infections. *World Journal Gastroenterol* **19**, 8974–8985.
- Hamed M, Refahy L and Abdel-aziz M (2015) Evaluation of antimicrobial activity of some compounds isolated from *Rhamnus cathartica* L. *Oriental Journal of Chemistry* **31**, 1133–1140.
- Harris JC, Plummer S and Lloyd D (2001) Anti-giardial drugs. *Applied Microbiology and Biotechnology* **57**, 614–619.
- Hashemi SA, Abediankenari S, Ghasemi M, Azadbakht M, Yousefzadeh Y and Dehpour AA (2011) The Effect of Fig Tree Latex (*Ficus carica*) on Stomach Cancer Line. *Iran Red Crescent Med J* **13**, 272–275.
- Jahangiri A and Barghi L (2018) Polymeric nanoparticles: review of synthesis methods and applications in drug delivery. **1**, 38–47.
- Kean T and Thanou M (2010) Biodegradation, biodistribution and toxicity of chitosan. *Advanced Drug Delivery Reviews* **62**, 3–11.
- Kim S.-K. *Marine Nutraceuticals: Prospects and Perspectives*, 1st Edn. by Se-Kwon Kim (Editor). US: CRC Press, 2013 – Medical – 464.
- Koehn FE and Carter GT (2005) The evolving role of natural products in drug discovery. *Nat Rev Drug Discov* **4**, 206–220.
- Kosalec I, Kremer D, Locatelli M, Epifano F, Genovese S, Carlucci G, Randi M and ZovkoKoncic M (2013) Anthraquinone profile, antioxidant and antimicrobial activity of bark extracts of *Rhamnus alaternus*, *R. fallax*, *R.intermedia* and *R. pumila*. *Food Chemistry* **136**, 335–341.
- Kucharska M, Ciechańska D and Niekraszewicz A (2010) Potential use of chitosan – based material in medicine. *Progress on Chemistry and Application of Chitin and its Derivatives* **15**, 58–90.
- Lee YJ, Han DG, Ryu JH, Chae JB, Chae JS, Yu DH, Park J, Park BK, Kim HC and Choi KS (2018) Identification of zoonotic *Giardia duodenalis* in Korean native calves with normal feces. *Parasitology Research* **117**, 1969–1973.
- Li L, Song X, Yin Z, Jia R, Li Z, Zhou X, Zou Y, Li L, Yin L, Yue G, Ye G, Lv C, Shi W and Fu Y (2016) The antibacterial activity and action mechanism of emodin from *Polygonum cuspidatum* against *Haemophilus parvus* *in vitro*. *Microbiological Research* **186–187**, 139–145.
- Machado M, Augusto M, Salgueiro LD, Cavaleiro C, José BA and Do Céu Sousa CM (2010) Anti-Giardia activity of phenolic-rich essential oils: effects of *Thymbra capitata*, *Origanum virens*, *Thymus zygis* subsp. *sylvestris*, and *Lippia graveolens* on trophozoites growth, viability, adherence, and ultrastructure. *Parasitology Research* **106**, 1205–1215.
- Mébed EMH, Abou-Sreya AIB and Roby MHH (2018) Chemical analysis and giardiacidal effectiveness of the aqueous extract of *Cymbopogon citratus* stapf. *Parasitology Research* **117**, 1745–1755.
- Naghdi M, Zamani A and Karimi K (2014) A sulfuric-lactic acid process for efficient purification of fungal chitosan with intact molecular weight. *International Journal of Biological Macromolecules* **63**, 158–162.
- Ordóñez-Mena JM, McCarthy ND and Fanshawe TR (2017) Comparative efficacy of drugs for treating giardiasis: a systematic update of the literature and network meta-analysis of randomized clinical trials. *Journal of Antimicrobial Chemotherapy* **27**, 596–606.
- Pandeti S, Gunjan S, Paidipelli S, Tripathi R and Tadigoppula N (2014) Anti-malarial activity of New emodin derivatives against *Plasmodium falciparum* chloroquine resistant strain. *Natural Products Chemistry and Research* **2**, 150.
- Pareash N, Patel L, Patel J and Patel JK (2011) Development and testing of novel temoxifen citrate loaded chitosan nanoparticles using ionic gelation method. *Der Pharmacia Sinica* **2**, 17–25.
- Pilon L, Spricigo PC, Miranda M, Moura MR, Assis OBG, Mattoso LHC and Ferreira MD (2015) Chitosan nanoparticle coatings reduce microbial growth on fresh-cut apples while not affecting quality attributes. *International Journal of Food Science and Technology* **50**, 440–448.
- Qadir MA, Shahzadi SK, Bashir A, Munir A and Shahzad S (2017) Evaluation of phenolic compounds and antioxidant and antimicrobial activities of some common herbs. *International Journal of Analytical Chemistry*, 1–6.
- Rahimi Esboei B, Ebrahimzadeh MA, Gholami SH and Fallah Omrani V (2013a) Anti-giardial activity of *Sambucus ebulus*. *European Review for Medical and Pharmacological Sciences* **17**, 2047–2050.
- Rahimi-Esboei B, Fakhar M, Chabra A and Hosseini M (2013b) *In vitro* treatments of *Echinococcus granulosus* with fungal chitosan, as a novel biomolecule. *Asian Pacific Journal of Tropical Biomedicine* **3**, 930–935.

- Sembiring EN, Elya B and Sauriasari RJPJ** (2018) Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of *Caesalpinia bonduc* (L.) roxb. *Pharmacognosy Journal* **10**, 123–127.
- Srinivas G, Babykutty S, Sathiadevan PP and Srinivas P** (2007) Molecular mechanism of emodin action: transition from laxative ingredient to an anti-tumor agent. *Medicinal Research Reviews* **27**, 591–608.
- Sui B, Fang L, Chi Y and Zhang C** (2010) Nano-Reconfigurable cells with hybrid circuits of single-electron transistors and MOSFETs. *IEEE Transactions on Electron Devices* **57**, 2251–2257.
- Sultana B, Anwar F and Przybylski R** (2007) Antioxidant activity of phenolic components present in barks of *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam. *Trees Food Chemistry* **104**, 1106–1114.
- Tayel AA, Moussa S, el-Tras WF, Knittel D and Opwis K** (2010) Anticandidal action of fungal chitosan against *Candida albicans*. *International Journal of Biological Macromolecules* **247**, 454–457.
- Tůmová P, Mazánek L, Lecová L, Dluhošová J, Typovská H, Kotrašová V, Tichácková V and Nohýnková E** (2018) A natural zoonotic giardiasis: infection of a child *via* giardia cysts in pet chinchilla droppings. *Parasitology International* **67**, 759–762.
- Venkatachalam M, Girard-Valenciennes E, Caro Y, Dufossé L and Fouillaud M** (2016) Anthraquinones and derivatives from marine-derived fungi: structural diversity and selected biological activities. *Marine Drugs* **14**, 64.
- Wardani G, Mahmiah M and Sudjarwo SA** (2018) In vitro antibacterial activity of chitosan nanoparticles against *Mycobacterium tuberculosis*. *Pharmacognosy Journal* **10**, 162–166.
- Wim H De Jong and Borm PJA** (2008) Drug delivery and nanoparticles: applications and hazards. *International Journal of Nanomedicine* **3**, 133–149.
- Yarahmadi M, Fakhar M, Ebrahimzadeh MA, Chabra A and Rahimi-Esboei B** (2016) The anti-giardial effectiveness of fungal and commercial chitosan against *Giardia intestinalis* cysts in vitro. *Journal of Parasitic Diseases* **40**, 75–80.
- Zhao Z, Haiju d, Wang R, Zhao W and Chen G** (2014) Genotyping and subtyping *Cryptosporidium parvum* and *Giardia duodenalis* carried by flies on dairy farms in Henan, China. *Parasites & Vectors* **7**, 190.