Parasitology

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

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Research Article

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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 antigiardial 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 antigiardial 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 antigiardial and cytotoxicity effects were not statistically significant ($P > 0.05$). The maximum mortality rate (100%) was achieved at 100 and 50 $\mu g\ kg^{-1}$ concentrations after 48 and 72 h of exposure time, respectively. Our results provide significant information about the new antigiardial 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*., 2013*a*, 2013*b*; Derda and Hadaś, 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*., 2013*a*, 2013*b*; Guo *et al*., 2018). Many pioneer investigations indicated that considering fungi as a source of chitosan over the other traditional sources such as

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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, napthalenic derivatives, flavanol glycosides (kaempferol, rhamnazin, rhamnetin, rhamnocitrin), 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 giardiacidal agents with minimal complications, it is important to design the most promising new drugs. Current study was aimed to evaluate antigiardial 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 freezed (−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 anegative control. For RFLP assay, 0.5 unit of *BspLI* (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 NH_2 groups in the prepared chitosan samples was calculated as follows:

$$NH_2\ (\%) = \frac{(C1V1 - C2V2) \times 0.016}{G\ (100 - W)} \times 100\%$$

$$\text{Free}\ \ NH_2\ (\%) \frac{NH_2\ (\%)}{9.94\ \ \%} \times 100\%$$

Molecular weight determination

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

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100 μg 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 [η] was calculated using the following formula:

$$[\eta] = \mathrm{KM}v\mathrm{a}$$

(Mv) and [η] 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 (Paresh *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: E$_1$-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 μg mL^{-1} stoke solution and diluted to 100, 50, 25 and 12.5 μg 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 solutions described above. The total contents of flavonoid were indicated as microgram of quercetin equivalents/g DW (μg QE/g DW). The samples analysed were conducted in triplicates.

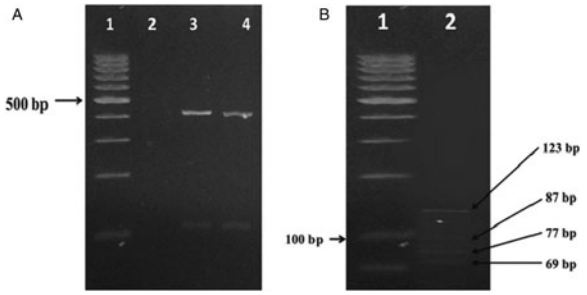

Fig. 1. (A) Electrophoretic separation of PCR product from DNA amplified at the *gdh* locus of *G. lamblia*, lane 1; 100 bp marker, lane 2; negative control (distilled water), line 3; positive control and line 4; PCR products from clinical samples. (B) Lane 1; 50 bp marker, lane 2; digestion of PCR product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

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 μg 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 (μg GA/μg 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 μg 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:

$$\%\ \text{Inhibition} = \frac{Ac - As}{Ac} \times 100$$

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 μg 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

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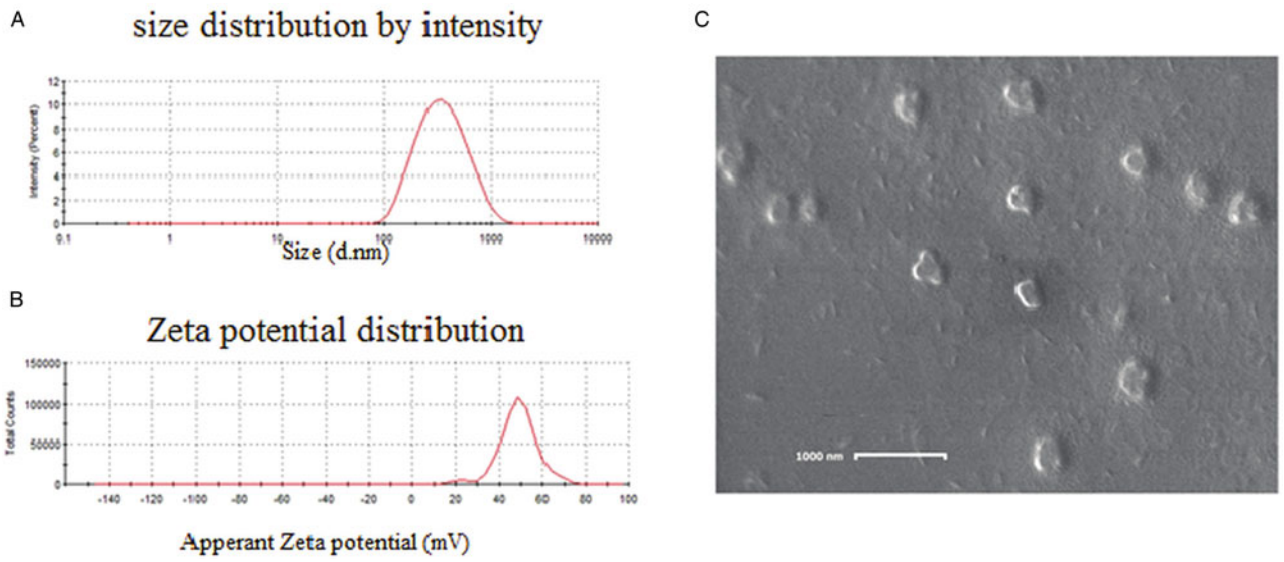

Fig. 2. (A) DLS showing the size distribution of chitosan nanoparticles; (B) zeta potential of chitosan nanoparticles showed good positive charge; (C) SEM image of chitosan nanoparticles showed proper morphology.

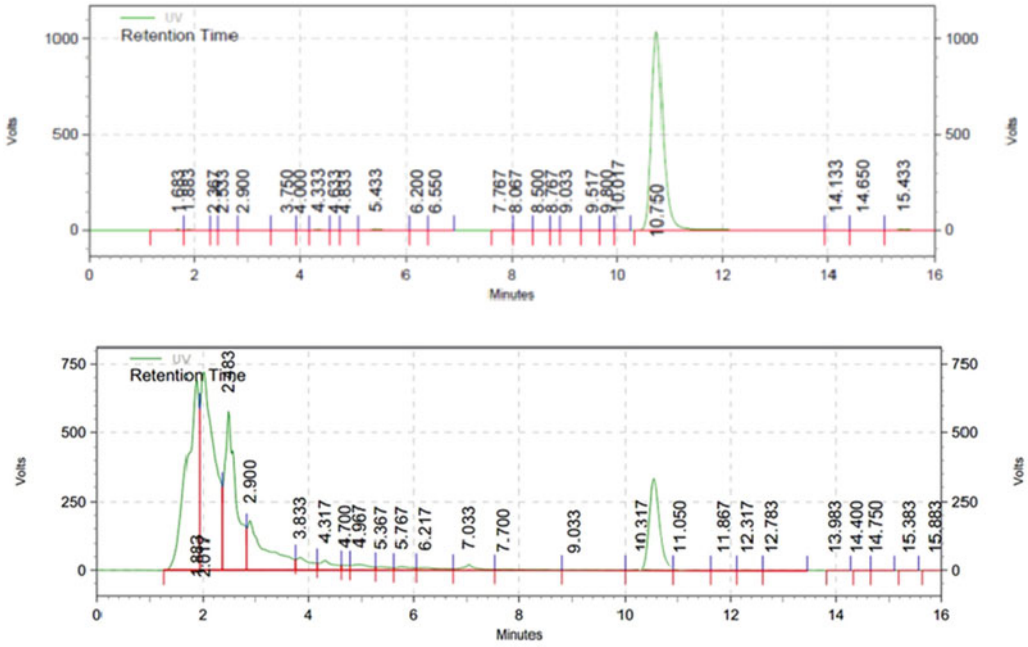

Fig. 3. HPLC chromatogram of standard emodin (up) and methanolic extract of *R. cathartica* (down); wavelength 254 nm; flow rate, 1 mL min^{-1}; injection volume, 20.0 mL; concentration, 200 mM each.

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 *antigiardial 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 excystations were 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 1×10^4 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%

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

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

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

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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 $\mu g\ kg^{-1}$	I	57.63 ± 0.55	50.83 ± 0.85	45.4 ± 1.28	38.63 ± 0.81	
	II	56.3 ± 1.13	47.4 ± 1.34	53.8 ± 1.47	41.4 ± 5.99	0.001
	III	55.67 ± 1.53	33.97 ± 4.63	63.2 ± 1.93	31.83 ± 1.40	
Chitosan-50 $\mu g\ kg^{-1}$	I	57.3 ± 0.61	34.33 ± 1.46	25.03 ± 1.05	21.7 ± 3.84	
	II	57.3 ± 1.54	31.7 ± 5.12	21.1 ± 1.85	19.23 ± 2.99	0.017
	III	57.33 ± 3.51	27.64 ± 15.96	23.77 ± 2.82	17.67 ± 2.55	
Chitosan-100 $\mu g\ kg^{-1}$	I	60.0 ± 2.0	33.43 ± 1.40	17.60 ± 7.71	1.0 ± 1.0	
	II	51.2 ± 18.13	29.53 ± 4.83	15.87 ± 3.07	1.0 ± 1.0	0.006
	III	55.03 ± 3.36	17.1 ± 3.21	11.87 ± 3.45	3.0 ± 1.0	
Nano-chitosan 10 $\mu g\ kg^{-1}$	I	54.3 ± 2.54	37.33 ± 2.46	28.03 ± 2.05	19.7 ± 2.84	
	II	56.3 ± 1.54	29.7 ± 4.12	22.1 ± 2.85	18.23 ± 1.8	0.001
	III	55.3 ± 1.64	28.64 ± 11.96	21.77 ± 1.82	16.07 ± 1.05	
Nano-chitosan-50 $\mu g\ kg^{-1}$	I	57.5 ± 1.34	30.3 ± 2.5	19.60 ± 0.6	2.0 ± 0.7	
	II	57.7 ± 2.54	19.03 ± 4.83	15.3 ± 2	3.0 ± 1.0	<0.001
	III	53.3 ± 1.34	15.1 ± 2.01	10.64 ± 2.75	3.0 ± 2.1	
Nano-chitosan 100 $\mu g\ kg^{-1}$	I	54.4 ± 2.35	22.27 ± 2.25	11.6 ± 1.51	1.0 ± 1.0	
	II	54.83 ± 2.56	21.3 ± 1.13	11.13 ± 3.38	2.0 ± 1.0	0.004
	III	55.3 ± 2.46	11.57 ± 6.44	9.27 ± 4.19	1.0 ± 1.0	
Emodin	100 mg kg^{-1}	59.63 ± 5.5	24.27 ± 5.02	16.8 ± 3.5	1.0 ± 1.0	<0.001
R. cathartica	50 mg kg^{-1}	56.57 ± 1.07	52.87 ± 2.9	30.17 ± 8.66	23.7 ± 2.47	
	100 mg kg^{-1}	54.67 ± 2.65	43.57 ± 1.29	30.87 ± 3.49	18.17 ± 4.11	0.002
	200 mg kg^{-1}	53.0 ± 1.54	42.53 ± 4.08	21.17 ± 3.38	5.3 ± 4.01	
Metronidazole		51.1 ± 0.75	27.57 ± 1.16	13.8 ± 0.75	3.97 ± 0.057	0.001
Furazolidone		54.3 ± 0.61	30.93 ± 1.01	18.17 ± 0.67	6.2 ± 0.82	0.001
Negative control (DMSO and PBS)		54.93 ± 0.95	24.47 ± 1.17	48.1 ± 0.66	72.07 ± 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 $\mu g\ mL^{-1}$ while for positive control ascorbic acid it was 57.92 ± 2.21 $\mu g\ mL^{-1}$.

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 *antigiardial 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 ($P > 0.05$).

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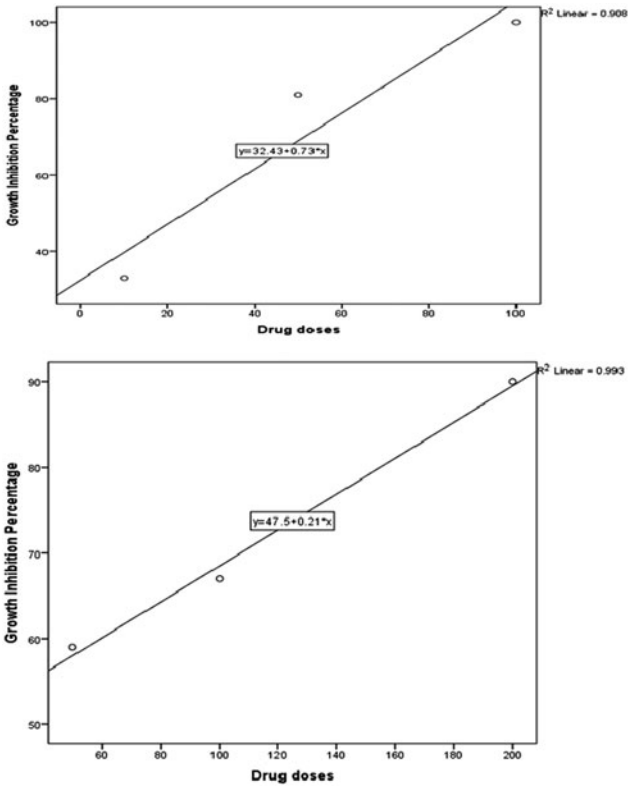

Fig. 4. (A) Determination of EC50 values of fungal chitosan on *Giardia lamblia in vivo*, (B) Determination of EC50 values of *R. cathartica* extract on *Giardia lamblia in vivo*.

In vivo *antigiardial 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 $\mu g\ kg^{-1}$ ($P < 0.05$). 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 ($y = 32.43 + 0.73X$), EC 50 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 $\mu g\ mL^{-1}$.

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, emodine, *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 antigiardial 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 *Rhamnus* has not been studied for antigiardial effects, however there are reports of the antimicrobial effect of the species (Kosalec *et al.*, 2013). *Rhamnus 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 antigiardial 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.*, 2013*a*, 2013*b*).

The IC_{50} values described in these studies variety from 0.8 to 300 $\mu g\ mL^{-1}$, but the majority of plant extracts presented IC_{50} values more than 100 $\mu g\ mL^{-1}$ (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 parasiticidal 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 PfK_1 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).

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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	
	II	1.14 ± 0.01	0.90 ± 0.01	0.64 ± 0.012	0.832
	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	
	II	1.21 ± 0.01	1.06 ± 0.026	0.68 ± 0.01	0.171
	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
R. cathartica	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*).

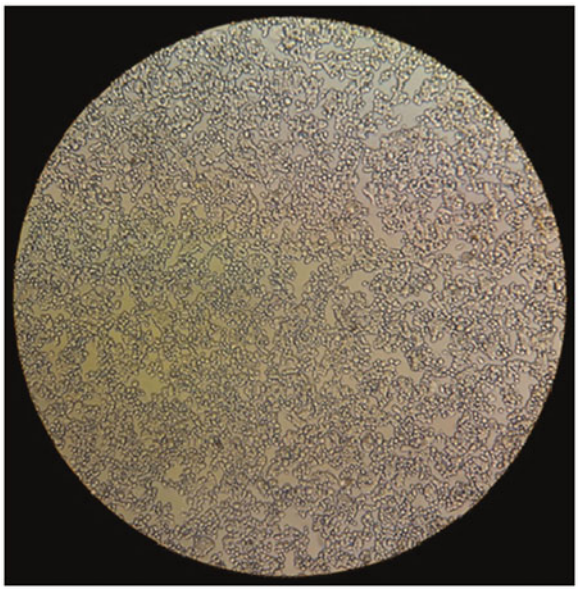

Fig. 5. Fresh HT-29 cells after treatment by Chitosan, *R. cathartica* and emodin.

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 $\mu g\ mL^{-1}$ 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.*, 2013*a*, 2013*b*).

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

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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.

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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.

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