

# Methanolic extract of *Peganum harmala* exhibit potent activity against *Acanthamoeba castellanii* cysts and its encystment *in vitro*

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**Abstract:** *Acanthamoeba castellanii* is member of free living amoeba that may cause painful sight-threatening keratitis and life threatening encephalitis which involves central nervous system. Treatments for both infections are problematic because of the amoebic cysts resistance to therapeutic agents. Here we evaluated *in vitro* strength of methanolic seed extract of *Peganum harmala* on *Acanthamoeba* cysts and its encystment mechanism. Our results revealed seed extracts (1 to 30mg/ml) exhibited amoebicidal effects against *Acanthamoeba* cysts. Furthermore *Acanthamoeba* encystment was also inhibited in concentration dependent manner with maximum inhibition at 2µg/ml after 48h incubation. In conclusion, we demonstrated for the first time that methanolic extracts exhibit remarkable inhibition of *Acanthamoeba* cysts and encystment *in vitro* which could serve a potential new natural agent against *Acanthamoeba*.

**Keywords:** *Acanthamoeba*; amoebicidal effects; encystment; plant extracts.

## INTRODUCTION

Genus *Acanthamoeba* is member of free-living amoebae that is an opportunistic protozoan pathogen and ubiquitous in nature. *Acanthamoeba* is known to cause serious human diseases like (i) chronic granulomatous infection involving central nervous system always leading to death, (ii) disseminated infections including skin, sinuses, lungs, prostate and uterus and (iii) painful keratitis (an infection of the eye resulting in blindness) mostly related to contact lens wearers (Trabelsi *et al.* 2012). Clinical symptoms of *Acanthamoeba* granulomatous encephalitis (AGE) includes fever, headaches, neurological disorders, personality changes and coma. *Acanthamoeba* keratitis (AK) is distinguished by ophthalmalgia, photophobia, blue-red vision and blood extravasations. In some cases like the lungs, *Acanthamoeba* may cause various inflammatory foci (AP), which are escorted by the exudation of serous fluid which carry trophozoites and cysts. In case of skin which changes into various ulcerations. All above mentioned infections are usually chronic.

*Acanthamoeba* lives two stage life cycle i) active trophozoite (infectious stage) and ii) dormant cyst (non-pathogenic or inactive) stage. *Acanthamoeba* infections are always very difficult to treat and mostly ineffective. Although a number of antimicrobials have been used in the past to treat *Acanthamoeba* infections but no drug has been found to be useful. There have been few successful treatments reports which were achieved due to the use of combination of drugs against *Acanthamoeba* trophozoites

(Gautom *et al.*, 1998). In spite of this *Acanthamoeba* eradication from the infection site is very difficult because under unfavorable conditions (i.e. drug treatment), the amoebas trophozoite switch over to cyst stage and medical treatment (drugs) is mostly less effective against amoeba cysts than trophozoites due to the stiff double-wall of the cysts which enables it extremely resistant to anti-amoebic drugs. This is also problematic as cysts have the capability to resist and survive after initial successful chemotherapeutic administration and cause deterioration of the disease. Encystment is the strategic mechanism amoeba adopts to combat the external threat i.e. harsh environmental conditions and/or drug treatment. The capability of cysts to resist chemotherapeutic agents is perhaps a key factor which contributes the increased cases of protozoan infections.

There is an essential need to have a better antimicrobial chemotherapy and alternate strategies to build up therapeutic interventions. The search for new compounds originating from natural resources is an important research area. Many new natural product groups have been identified for antiparasitic properties of with their astonishing efficacy and selectivity such as plant-derived terpenes, alkaloids and phenolics (Kayser *et al.* 2003). We have previously investigated various plant extracts and reported *Peganum harmala* have shown the promising effects against *Acanthamoeba* trophozoites (Shohaib *et al.*, 2013). Therefore use of extracts could have promising outcome in the treatment of *Acanthamoeba* infections. Here, we investigated efficacy of *P. harmala* against *Acanthamoeba* cysts and its encystment mechanism to evaluate a potential therapeutic option of the extract against amoebic encystment using an *in vitro* model.

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There is no information available for such activities of any plant species against *Acanthamoeba* encystment in literature so far. Hence, we assume the present study probably is the first on this topic.

## MATERIALS AND METHODS

### *Plant collection, processing and extract preparation*

The seed of *Peganum harmala* was collected and extraction was prepared as described previously (Shohaib *et al.* 2013). Briefly 10gm of air-dried plant material in shade was finely ground and soaked in 100ml of methanol (100%). Extraction was further performed in water bath for 1 week at 25°C with constant shaking at 100 rpm. Next, extract was filtered and concentrated at 40°C under reduced pressure. It was further dried in a freeze drier and stored at 4°C refrigerator until further use in the subsequent assays.

### *Cultures of Acanthamoeba and cysts preparation*

Two pathogenic *Acanthamoeba castellanii* isolates belonging to T1 and T4 genotypes were used in this study. T1 genotype was isolated from a granulomatous amoebic encephalitis case (ATCC 50494) while T4 from a keratitis patient (ATCC 50492). *Acanthamoeba* was grown in a non-shaking incubator in 15ml of growth medium containing yeast extract 0.75% (w/v), proteose peptone 0.75% (w/v) and glucose 1.5% (w/v) in T-75 culture flasks at 30°C as described previously (Matin and Jung 2011). Growth media containing amoeba was refreshed 17-20h before subsequent experiments. This careful procedure resulted into more than 95% of the *Acanthamoeba* in the trophozoite or pathogenic form.

*Acanthamoeba* trophozoites were cultured in T-75 flasks for three weeks as described previously (Degerli *et al.* 2012). *Acanthamoeba* cysts were collected and centrifuged at 2000 rpm for 5min to collect cysts. *Acanthamoeba* cysts were tested for their viability by using trypan blue assay and 100% viable cysts were further used in subsequent experiment. The cysts were washed in PBS and harvested after adjusted a final concentration of  $1 \times 10^6$  cysts/ml.

### *Amoebicidal assay*

To evaluate the effect of extract on *Acanthamoeba* cysts amoebicidal assays were adopted as previously described (Matin *et al.*, 2007; Raza *et al.*, 2012). In brief, *Acanthamoeba* cysts were washed once with PBS. Next, cysts were harvested in 24-well tissue culture plates ( $1 \times 10^6$  amoebae per 0.5 ml per well) with various concentrations of extract (ranging 1, 5, 10, 20 and 30 mg/ml final concentration) in PBS. The numbers of *Acanthamoeba* cysts at various time intervals were determined using hemocytometer counting. For controls, *Acanthamoeba* was incubated in PBS without extracts. Viability of cysts was assessed using trypan blue exclusion test.

### *Encystment assay*

To access the capability of *Acanthamoeba* to switch over cysts form an encystment assays were performed as described previously (Dudley *et al.* 2005). In brief, *Acanthamoeba* (>95% trophozoites, at a cell density of  $5 \times 10^5$  amoeba per ml in each well) were harvested in 24-well tissue culture plates in amoeba growth medium and maintained at 30°C for 24h as described above. The cell free supernatants were collected and all the wells were washed once with HBSS to eliminate the detached amoeba, followed by the addition of various concentration of extract (0.1, 0.5, 1 and 2mg/ml) to induce encystment at 30°C for 48h. For controls, growth media was replaced by 8% glucose dissolved in RPMI1640 and RPMI1640 alone was added. Next, preliminary amoeba number was enumerated microscopically under a haemocytometer before adding together with extract. The 24-well plates were left for 48h at 30°C incubator. This was followed by the addition of 0.5% SDS to lyse the remaining amoeba trophozoites. Amoeba cysts were enumerated microscopically using a haemocytometer, both pre and post SDS treatment. Scientifically trophozoites are very sensitive to SDS are should be lysed immediately upon SDS exposure, while the cysts are resistant to SDS, ultimately will remain intact and will be encumbered. For encystment measurement, the %age of *Acanthamoeba* trophozoites transforming into cysts form was evaluated as by the following formula:

$$\frac{\text{No. of amoebae (post - SDS treatment)}}{\text{No. of amoebae (pre - SDS treatment)}} \times 100 = \% \text{Encystment}$$

## STATISTICAL ANALYSIS

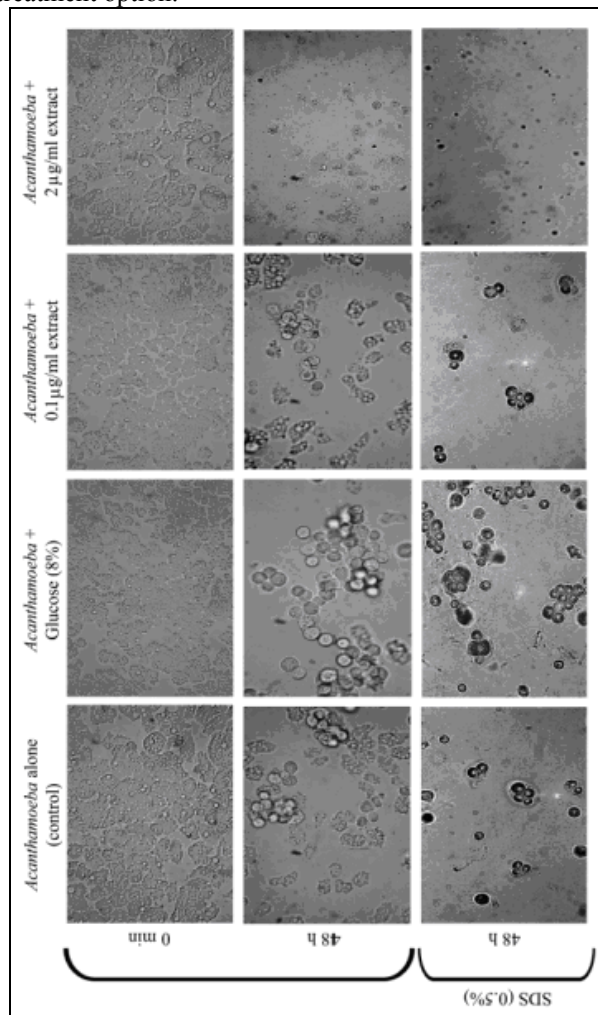
Each experiment was carried out in duplicate and three experiments were performed independently. Data are presented as mean $\pm$ SEM. The difference between tested groups and control were analyzed by Student's t test. Values of P<0.05 were considered statistically significant.

## RESULTS

Most of the currently available clinical use drugs have variable efficacy with toxic side effects and involve the long way of administration (Kayser *et al.* 2003). Recently natural sources are screened which may provide the opportunity to identify the new molecules with distinctive structure with high efficacy and more specific that could be further characterized by full or semi synthetic procedures (Holzgrave and Bechthold, 1999). For antiparasitic activity, majority of the plant species in the world flora have not been explored yet. Globally, there is a raising trend to shift resources from allopathic to traditional healthcare systems.

In most of the cases of human infection caused by *Acanthamoeba* sp., a combination of drugs (therapy) along with standard antibiotic treatment would be

preferred as it is categorically more effective than therapy done using a single drug. This is because most of the drugs have the potential of amoebistatic effects, but with no amoebicidal properties. Some drugs are very toxic to *Acanthamoeba* trophozoites, but unsuccessful against *Acanthamoeba* cysts. Up to now, there is not a single drug available which is effective against both stages of *Acanthamoeba* cysts and trophozoites. A number of reported data suggests that *Acanthamoeba* can be killed effectively as long as they are in the trophozoite forms. Therefore, selection of compounds that can reduce the amoeba encystment, together with currently available treatment options, would improve management of these dreadful infections and this may also lead to a less swear treatment option.



**Fig. 1:** *Peganum harmala* extract exhibits inhibitory effects against *Acanthamoeba* encystment. To determine the effect of extracts on *Acanthamoeba* encystment amoeba was incubated with various concentrations of extracts as described in Materials and methods. Our results revealed at maximum concentration (2 µg/ml) amoeba could not switch into cysts which clearly demonstrates the clearance of *Acanthamoeba* after 48h incubation when treated with SDS. For controls,

*Acanthamoeba* was incubated alone and with 8% glucose. The reproducible qualitative data have been presented here.

## DISCUSSION

Up to this end, it has also been shown in biochemical studies that the cyst wall is somewhat composed of cellulose (Neff and Neff 1969). Therefore, cellulose biosynthesis could be a possible target for inhibition of amoebic encystment. In the present investigation methanolic extract obtained from *Peganum harmala* seed was evaluated against *Acanthamoeba* cysts and its encystment mechanism by visual observation and haemocytometer counting using inverted microscope. Our results revealed the seed extract (1 to 30mg/ml) showed a strong inhibition against *Acanthamoeba* cysts (both T1 and T4 genotypes) dose and time dependent manner (table 1). *Acanthamoeba* cysts viability was further confirmed by trypan blue exclusion test as described in method section.

Furthermore interestingly methanolic extract also prevent *Acanthamoeba* (both T1 and T4 genotypes) towards cyst formation at maximum dose 2mg/ml (table 2), suggesting extract may contribute in disruption of amoebic encystment process which is quite interesting and novel too (fig. 1). We assume our extract may involve in cellulose biosynthesis inhibition in *Acanthamoeba* but this need to be further investigated. The property of encystment is the key bearing in mind the ability of trophozoites to switch over to cyst form in the therapy is the vital problem for the possibility of reinfection. It has been shown that this may be due to genotoxic effects of harmaline and harmine (Boeira *et al.*, 2002) and in antibiotic resistant bacteria and protozoa from poultry (Arshad *et al.* 2008), whereas a strong antileishmanial activity of harmaline towards intracellular amastigote forms of the parasite were also reported (Di-Giorgio *et al.*, 2004). The possible role of alkaloids in plant extracts of *Peganum harmala* has been evaluated previously and four known  $\beta$ -carbolin alkaloids were quantified i.e., harmaline was reported to be in the highest concentration followed by harmine and harmalol (Arshed *et al.* 2008). It is believed alternatively, it could be a synergistic effect of all alkaloids present in the extract. Our future study will be more focused on the effects of each component on biological properties of *Acanthamoeba*.

## CONCLUSIONS

In conclusion this will be our second study conducted for *Peganum harmala* for its activity against *Acanthamoeba*. Here, we have reported for the first time that seed extract of *Peganum harmala* demonstrates remarkable amoebicidal effect and inhibition of *Acanthamoeba* encystment *in vitro* which is quite novel. Future work will include identification of the active compounds, their

**Table 1:** *Peganum harmala* extract exhibits amoebicidal effects against *Acanthamoeba* cysts

		Amoebicidal effects					
		T1 genotype			T4 genotype		
		1h	24h	48h	1h	24h	48h
<i>Peganum harmala</i> (seed methanolic extract)	Control	81.38±6.74	74.86±4.25	71.52±5.13	80.07±5.32	75.19±3.99	72.25±4.19
	Amoeba+1mg/ml	78.34±3.93	53.05±2.21	41.88±3.03	77.82±2.54	51.32±0.95	40.19±1.87
	Amoeba+5mg/ml	74.65±2.88	34.82±3.5	21.09±1.98	73.19±3.11	30.53±1.46	19.4±2.08
	Amoeba+10mg/ml	69.02±4.59	20.72±4.1	10.66±3.33	66.47±1.91	19.71±2.67	8.55±2.95
	Amoeba+20mg/ml	56.89±2.91	13.54±3.23	0	53.05±3.07	9.91±4.48	0
	Amoeba+30mg/ml	21.5±6.14	0	0	17.84±5.91	0	0

**Table 2:** *Peganum harmala* extract exhibits activity against *Acanthamoeba* encystment

		SDS treatment (0.5 %) after 48h			
		T1 genotype		T4 genotype	
		Encystment (%)	Trophozoites killed (%)	Encystment (%)	Trophozoites killed (%)
<i>Peganum harmala</i> (seed methanolic extract)	<i>Acanthamoeba</i> alone	59.98±3.46	40.02±3.24	57.35±5.26	42.65±4.64
	Amoeba+8% glucose	77.63±4.74	22.37±4.25	79.4±6.48	20.25±4.91
	Amoeba+0.1mg/ml	65.04±3.99	34.96±6.03	62.24±5.6	37.76±5.86
	Amoeba+0.5mg/ml	36.27±4.57	63.73±4.25	33.47±3.38	66.53±3.17
	Amoeba+1mg/ml	18.05±2.58	81.95±2.5	16.19±1.41	83.81±1.99
	Amoeba+2mg/ml	0	100	0	100

Values are the mean +SEM of three independent experiments performed in duplicates

further characterization and mode of action against life threatening pathogen like *Acanthamoeba*.

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