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A Study on the Amoebicidal Effect of *Nigella Sativa* Aqueous and Alcoholic Extracts and Wheat Germ Agglutinin on Pathogenic *Acanthamoeba*

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Abstract

Background: *Acanthamoeba* keratitis is a corneal disease associated predominantly with contact lens wear. Treatment of *Acanthamoeba* keratitis has been fairly successful using chlorhexidine but may be accompanied by drug toxicity and development of resistance. Medicinal plants can be an alternative resource of novel anti-protozoal drugs with high effectiveness and low toxicity. *Nigella sativa* seeds are considered one of the potential natural sources in folk medicine. Wheat germ agglutinin (WGA) has shown therapeutic effects against protozoa.

Objective: is to study the *in vitro* effect of *Nigella sativa* aqueous and alcoholic extracts as well as wheat germ agglutinin on pathogenic *Acanthamoeba spp*. in comparison to chlorhexidine 0.02%.

Methods: Collection of corneal samples for isolation of *Acanthamoebae*, preparation of non-nutrient agar-*Escherichia coli* plates for cultivation of the obtained samples,microscopic examination of the plates for detection and identification of *Acanthamoeba* growth daily for two weeks, preparation of the study medications (*Nigella sativa* aqueous and alcoholic extracts as well as WGA). Preparation of chlorhexidine 0.02% as a drug control. Evaluation of the amoebicidal effect of different concentrations of *N. Sativa* aqueous extract, *N. sativa* alcoholic extract and WGA in comparison to chlorhexidine 0.02%.Determining the parasite inhibition percentage and the minimal lethal concentration (MLC) of each medication.

Results: The MLC of *N. sativa* aqueous extracton *Acanthamoeba* trophozoites was 5 mg/ml after 24 h incubation and 500 µg/ml after 48 hrs. The drug control (chlorhexidine 0.02%) had an inhibition percentage of 100% at incubation durations of 24, 48 and 72 hrs. The MLC of *N. sativa* aqueous extract on *Acanthamoeba* cysts was 30 mg/ml after 24 hrs and 25 mg/ml after 48 hrs with a highly significant ($p<0.001$) and a significant ($p<0.05$) differences respectively compared to the drug control. Chlorhexidine 0.02% had an inhibition percentage of 56% after 24 hrs, 64% after 48 hrs and 80% after 72 hrs incubation. The MLC of *N. sativa* alcoholic extract on *Acanthamoeba* trophozoites was 10 mg/ml after 24 hr incubation and 500 µg/ ml after 48 hrs. The MLC of *N. sativa* alcoholic extract on *Acanthamoeba* cysts was 30 mg/ml after 24 hrs incubation with a highly significant difference ($p<0.001$) compared to the drug control. The MLC of WGA on *Acanthamoeba* trophozoites was 50 µg /ml after 24, 48 and 72 hrs incubation which was similar to the drug control. Wheat germ agglutinin concentration of 1 mg/ml caused inhibition of *Acanthamoeba* cysts with percentages of 60% and 68% after 24 and 48 hrsincubation respectively which were higher than those caused by the drug control (56% after 24 hrs and 64% after 48 hrs) with statistically insignificant difference ($p>0.05$). Inhibition percentage of 80% was obtained by concentration of 2 mg/ml after 72 hrs incubation which was similar to the drug control. The same inhibition percentage (80%) was also obtained by the same concentration after 48 hrs incubation which was significantly higher ($p<0.05$) than the

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drug control (64%).

Conclusion: *N. sativa* aqueous and alcoholic extracts as well as WGA showed that these agents had considerable lethal effects on *Acanthamoeba* trophozoites and cysts. These effects were found to be dose and time dependent. They were also found comparable to or even superior than the effect of the commonly used chlorhexidine (0.02%) as a chemotherapeutic.

Keywords: *Acanthamoebae spp*; *Nigella sativa*; Wheat germ agglutinin

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Introduction

Acanthamoebae are ubiquitous potentially pathogenic free-living amoebae that occur worldwide in soil and water. They are tolerant of a wide range of osmolarity, enabling them to survive in distilled water, tissue culture media, mammalian body fluids and sea water [1].

Acanthamoeba keratitis is a corneal disease associated predominantly with contact lens wear. The occurrence of *Acanthamoeba* keratitis has been rising since 1990 in correlation to the increasing number of contact lens wearers [2].

Treatment of *Acanthamoeba* keratitis has been fairly successful using a variety of drugs. Brolene, a commercially available eye medication containing propamidine isethionate and dibromopropamidine isethionate, is found to be effective but may be accompanied by drug toxicity and development of resistance [3].

Polyhexamethylene biguanide and chlorhexidine have proved to be effective with treatment success of 78% and 86% respectively [4]. However, side effects in the form of cataract, iris atrophy and peripheral ulcerative keratitis have been attributed to the use of those drugs [5].

Medicinal plants can be an alternative resource of novel anti-protozoal drugs with high effectiveness and low toxicity [6].

Nigella sativa seeds are considered one of the potential natural sources in folk medicine [7]. Crude extracts (aqueous and alcoholic) and essential oil of *N. sativa* proved to have many therapeutic effects. *Nigella sativa* alcoholic extract is found to be as effective as Metronidazole in the cure of giardiasis [8]. Aqueous extract has demonstrated a potential therapeutic effect against *Blastocystis hominis* [9] and *Trichomonas vaginalis* [10]. *Nigella sativa* oil was found to have a significant therapeutic effect on experimental infection with *Cryptosporidium parvum* [11], *Trypanosoma brucei* [12] and *Toxoplasma gondii* [13].

Wheat germ agglutinin (WGA) has produced dose-related growth inhibition of *Giardia lamblia* trophozoites *in vitro* [14].

So the aim of the present work is to study the *in vitro* effect of *Nigella sativa* aqueous and alcoholic extracts as well as wheat germ agglutinin on pathogenic *Acanthamoeba*.

Material and Methods

The current study was conducted at The Medical Parasitology Department, Faculty of Medicine, Ain Shams University during the period from June 2010 to June 2011.

Plant materials and extraction procedure:

Preparation of *Nigella sativa* aqueous extract [10]: *Nigella sativa* seeds, obtained from the local market, were washed to remove any debris and air-dried. A weight of 125 g seeds was crushed then boiled in distilled water (500 ml) for 90 minutes and filtered through gauze. The filtrate was evaporated under reduced pressure, lyophilized and preserved in the deep freezer (–20°C) till it was used.

Preparation of *Nigella sativa* alcoholic extract [15]: *Nigella sativa* seeds were freed of dust. A weight of 125 g seeds was crushed in a domestic grinder then soaked in absolute ethanol in a beaker for 5 days at room temperature. The amount of ethanol was just enough to adequately cover the seeds. They were filtered under UV light using a filter paper. Ethanol was evaporated from the filtrate using a rotary evaporator. The prepared extract was collected and stored at –20 °C.

Wheat germ agglutinin (WGA): It was obtained as a highly purified lyophilized powder L9640 (Sigma Corporation).

Preparation of the drug control: chlorhexidine 0.02% [4]: Chlorhexidine digluconate 20% was obtained from Sigma Corporation. Chlorhexidine 0.02% was prepared by diluting one (1) ul of chlorhexidine 20% with one (1) ml of distilled water. *Nigella sativa* aqueous and alcoholic extracts were mixed in distilled water to get concentrations of 500 µg/ml, 1 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml and 30 mg/ml. Wheat germ agglutinin was dissolved in distilled water to get concentrations of 50, 100, 150, 250, 500 µg/ml, 1 mg/ml, and 2 mg/ml. Chlorhexidine was tested in a concentration of 0.02% as a drug control.

Acanthamoeba spp. Isolation

Corneal scrapings were obtained from patients attending The Corneal Outpatient Clinic, Research Institute of Ophthalmology, Giza, who were clinically suspected to have *Acanthamoeba* keratitis. The samples were kept in 5 ml screw-capped vials containing Page Amoeba Saline solution (PAS) to betransported to the laboratory for cultivation. The specimens were inoculated directly onto the surface of 1.5% non-nutrient agar (NNA) plates seeded with *Escherichia coli* bacterial suspension and incubated in a humidified chamber at 30°C [16]. The presence of

Acanthamoeba could be seen by the clear tracks on the *E. coli* lawn NNA produced by the feeding trophozoites of *Acanthamoeba.* Examination of the agar plate surface for the presence of amoebic growth was carried out daily for up to 14 days with a light and inverted microscopes using x40 objective. *Acanthamoeba* was identified by the specific morphology of cyst and trophozoite. Subcultures were done after 2 weeks from positive cultures with confirmed amoebic growth by cutting a small square of agar using a sterile scalpel and placed upside down on new NNA-*E.coli* plates. The plates were incubated in humidified chambers at 30ºC and examined after 24 h. Performing sub-culturing several times facilitated the isolation of *Acanthamoeba*. *Acanthamoeba* cysts were collected from 3-week cultures. The agar surfaces were flooded with 5 ml of phosphate buffered saline (PBS) and were gently scraped with an inoculating loop. Cysts were harvested from the suspension by centrifugation at 350 × *g* for 10 min. The supernatant was aspirated, and the sediment was washed twice in PBS in order to eliminate most of the bacteria. Cysts in the resultant suspension were counted with a hemacytometer and the suspension was standardized to be $25\times10_4$/ml [17].

Experimental design:

In order to evaluate the *in vitro* amoebicidal activity of *Nigella sativa* aqueous and alcoholic extracts as well as WGA. Chlorhexidine 0.02% as a drug control *Acanthamoeba* were incubated with different concentrations of *Nigella sativa* aqueous and alcoholic extracts (500 µg/ml and 1,5,10,15,20,25,30 mg/ml), and WGA (50,150,250,500 µg/ml and 1,2 mg/ml), for different incubation periods (24, 48 and 72 h). One hundred microliters (100 µl) of the calibrated trophozoite suspension ($1\times10_5$/ml) were inoculated into each well of a 96-well plate and then the plate was left for 30 min to avoid disturbance of the adherence of amoebae onto the wells' surface. Then, the PBS solution was removed and 100 µl of each concentration of the plant extracts were added into wells. The plate was sealed and incubated at 30°C for different incubation periods. Also in a separate well 100 µl of the calibrated cyst suspension ($25\times10_4$/ml). In addition, controls containing only the parasite in PBS as a parasite control and parasite plus 0.02% chlorhexidine gluconate (prepared from a solution 20% in H_2O CHX, C-9394; Sigma) as a reference drug control, were submitted to the same procedure. Each experiment was performed in triplicate. After each incubation period, 100 µl from each test and control wells were transferred into 100 µl of 0.3% basic methylene blue media. Unstained (viable) and stained (nonviable) parasite were enumerated in the haemocytometer, 10 min after stain addition. For cultures containing no viable cysts, an additional test was performed to confirm the results obtained. To evaluate their viability, it was inoculated onto NNA-*E.coli* plate, incubated at 30°C for additional 72 h and examined to detect any viable cysts or trophozoites [18].

Evaluation of the drug efficacy was done by:

- Counting the number of trophozoites ad cyst separetly using the haemocytometer after each period of incubation.
- Calculation of the percent of growth reduction according to the equation [19]
- Percent of growth reduction = a – b / a × 100
- Where; a = Mean number of trophozoites / cysts in parasite control cultures.
- b = Mean number of trophozoites / cysts in drug treated cultures.
- Determination of the minimal inhibitoryconcentration (MIC)as thelowest concentration ofthetestedplant extractsandchlorhexidine 0.02% in which no viable organism observed [20].

Statistical evaluations

Statistical analysis was performed using Microsoft Excel version 2010 and Statistical Package for Social Sciences (SPSS) for Windows version 15.0. Categorical variables were presented as number and percentage. The inhibition percentage caused by chlorhexidine 0.02% was taken as the reference values in different incubation periods (24, 48 and 72 hours). Differences between inhibition percentages of different concentrations of tested medications were compared to the reference drug using Yates' corrected chi-squared test. Significance level was set at 0.05 while the highly significance level was set at 0.001.

Results

The results of the present study are shown in the **Tables (1-6)**.

Discussion

Acanthamoeba was first recognized as an ocular pathogen in 1973 and was the cause of an epidemic in the 1980s due to contaminated contact lenses [21].

A variety of drugs have been used for treatment of AK including chlorhexidine, polyhexamethylene biguanide, propamidine isethionate, dibromopropamidine isethionate, neomycin, paromomycin, polymyxin B, clotrimazole, ketoconazole, miconazole and itraconazole [22]. Miltefosine, a hexadecylphosphocholine, has also been shown to have amoebicidal potential [23].

Treatment failures occur and may be due to inherent or acquired resistance (perhaps attributed to strain and species susceptibility differences) or to advanced corneal disease [21]. The authors assumed that failures may also be due to the inability to achieve cysticidal concentrations of anti-amoeba agents in the cornea.

Interest in medicinal plants has burgeoned due to increased efficiency of new plant-derived drugs and the growing interest in natural products. Because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades. The use of plants as medicines dates from the earliest years of man's evolution [24]. Medicinal plants serve as therapeutic alternatives, safer choices or in some cases, as the only effective treatment. A larger number of these plants and their isolated constituents have shown beneficial therapeutic effects including anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial and immuno-modulatory effects [25].

Among the promising medicinal plants is *Nigella sativa*, a

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Table 1: Mean Number and Percentage of Inhibition of Viable *Acanthamoeba* Trophozoites Incubated with *Nigella sativa* Aqueous Extract for 24, 48 and 72 hrs.

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x10^4)	Percentage of Inhibition (%)
Parasite control	10	0	10	0	10	0
Drug Control (Chlorhexidine 0.02%)	0	100	0	100	0	100
Nigella sativa aqueous extract concentrations						
500 µg/ml	2	80	0	100	0	100
1 mg/ml	1	90	0	100	0	100
5 mg/ml	0	100	0	100	0	100
10 mg/ml	0	100	0	100	0	100
15 mg/ml	0	100	0	100	0	100
20 mg/ml	0	100	0	100	0	100
25 mg/ml	0	100	0	100	0	100
30 mg/ml	0	100	0	100	0	100

Table 2: Mean Number and Percentage of Inhibition of Viable *Acanthamoeba* Cysts Incubated with *Nigella sativa* Aqueous Extract for 24, 48 and 72 hrs

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)
Parasite control	25	0	25	0	25	0
Drug Control	11	56	9	64	5	80
(Chlorhexidine 0.02%)						
Nigella sativa aqueous extract concentrations						
500 µg/ml	25	0 *	16	36 *	15	40 *
1 mg/ml	20	20 **	15	40 **	8	68 **
5 mg/ml	18	28 ***	12	52 ***	7	72 ***
10 mg/ml	11	56 ****	10	60 ****	5	80 ****
15 mg/ml	9	64 *****	8	68 *****	4	84 *****
20 mg/ml	3	88 ******	2	92 ******	2	92 ******
25 mg/ml	1	96 *******	0	100 *******	0	100 *******
30 mg/ml	0	100 ********	0	100********	0	100 *******
P value & Significance	X2*=19.44, p=0.001 (S)		X2*=3.92, p=0.047 (S)		X2*=0.33, p=0.003 (S)	
	X2**= 6.88, p=0.008 (S)		X2**=2.88, p=0.089 (NS)		X2**=0.94, p=0.333 (NS)	
	X2***=0.33, p=567 (NS)		X2***=0.74, p=0.390 (NS)		X2***=0.44, p=0.507 (NS)	
	X2****= 0.08, p=0.775 (NS)		X2****=0.08, p=0.770 (NS)		X2****= 0.13, p=0.723 (NS)	
	X2***** = 0.08, p= 0.772 (NS)		X2*****= 0, p=1 (NS)		X2***** = 0, p= 1 (NS)	
	X2****** = 4.86, p= 0.027 (S)		X2****** = 4.19, p= 0.041 (S)		X2****** = 0.664, p= 0.415 (NS)	
	X2******* = 8.88, p= 0.002 (S)		X2******* = 8.67, p= 0.003 (S)		X2******* = 3.56, p= 0.059 (NS)	
	X2******** = 11.66, p< 0.001 (HS)		X2********= 8.67, p= 0.003 (S)		X2******** = 3.56, p= 0.059 (NS)	

*= Statistically significant difference in comparison to drug control in the same time interval (p<0.05)
**= Statistically highly significant difference in comparison to drug control in the same time interval (p<0.001)

dicotyledon of the Ranunculaceae family. It is an amazing herb with rich historical and religious background. It has been employed since long time as a spice and food preservative [7].

The black seed was referred to by the prophet Mohammed (PBUH)as having healing powers, he advised his followers "Hold on to use the Black cumin, for it has a remedy for every illness

Table 3: Mean Number and Percentage of Inhibition of Viable *Acanthamoeba* Trophozoites Incubated with *Nigella sativa* Alcoholic Extract for 24, 48 and 72 hrs.

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)
Parasite control	10	0	10	0	10	0
Drug Control	0	100	0	100	0	100
(Chlorhexidine 0.02%) Nigella sativa Alcoholic extract concentrations						
500 µg/ml	2	80	0	100	0	100
1 mg/ml	1	90	0	100	0	100
5 mg/ml	1	90	0	100	0	100
10 mg/ml	0	100	0	100	0	100
15 mg/ml	0	100	0	100	0	100
20 mg/ml	0	100	0	100	0	100
25 mg/ml	0	100	0	100	0	100
30 mg/ml	0	100	0	100	0	100

Table 4: Mean Number and Percentage of Inhibition of Viable *Acanthamoeba* Cysts Incubated with *Nigella sativa* Alcoholic Extract for 24, 48 and 72 hrs.

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)
Parasite control	10	0	10	0	10	0
Chlorhexidine 0.02% (Drug Control)	0	100	0	100	0	100
WGA concentrations						
50 µg/ml	0	100	0	100	0	100
100 µg/ml	0	100	0	100	0	100
150 µg/ml	0	100	0	100	0	100
250 µg/ml	0	100	0	100	0	100
500 µg/ml	0	100	0	100	0	100
1 mg/ml	0	100	0	100	0	100
2 mg/ml	0	100	0	100	0	100

*= Statistically significant difference in comparison to drug control in the same time interval ($p<0.05$)
**= Statistically highly significant difference in comparison to drug control in the same time interval ($p<0.001$)

except death" [25]. Historically, it has been recorded that *N. sativa* seeds were prescribed by ancient Egyptians and Greek physicians to treat headache, nasal congestion, toothache and intestinal worms as well as a diuretic and increase milk production [26]. The seeds of *N. sativa*, known as black seed, black cumin or "Habatul-Barakah," have long been used in folk medicine in the Middle and Far East as a traditional medicine for a wide range of illnesses including bronchial asthma, headache, dysentery, infections, obesity, back pain, hypertension and gastrointestinal problems [27-28].

The seeds of *N. sativa* are the source of the active ingredients of this plant [26]. By analysis of *N. sativa* oil, thymoquinone (TQ), dithymquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) are considered the main active ingredients [29]. The seeds also contain other ingredients including nutritional components such as carbohydrates, fats, vitamins, mineral elements (calcium, iron and potassium) and proteins including eight of the nine essential amino acids [30]. They contain carotene which is converted by the liver to vitamin A [31].

Nigella sativa seeds were found to have phospholipids. The major separate individual phospholipid classes are phosphatidylcholine followed by phosphatidyle-thanolamine, phosphatidylserine and

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Table 5: Mean Number and Percentage of Inhibition of Viable Acanthamoeba Trophozoites Incubated with WGA for 24, 48 and 72 hrs.

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)
Parasite control	10	0	10	0	10	0
Chlorhexidine 0.02% (Drug Control)	0	100	0	100	0	100
WGA concentrations						
50 µg/ml	0	100	0	100	0	100
100 µg/ml	0	100	0	100	0	100
150 µg/ml	0	100	0	100	0	100
250 µg/ml	0	100	0	100	0	100
500 µg/ml	0	100	0	100	0	100
1 mg/ml	0	100	0	100	0	100
2 mg/ml	0	100	0	100	0	100

*= Statistically significant difference in comparison to drug control in the same time interval (p<0.05)

Table 6: Mean Number and Percentage of Inhibition of Viable *Acanthamoeba* Cysts Incubated with WGA for 24, 48 and 72 hrs.

Study and control groups	Duration of Incubation					
	24 Hours		48 Hours		72 Hours	
	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)	No. (x 10^4)	Percentage of Inhibition (%)
Parasite control	25	0	25	0	25	0
Drug Control (Chlorhexidine 0.02%)	11	56	9	64	5	80
WGA concentrations						
50 µg/ml	25	0	20	20	18	28
100 µg/ml	25	0	20	20	17	32
150 µg/ml	20	20	15	40	15	40
250 µg/ml	19	24	13	48	13	48
500 µg/ml	14	44	10	60	10	60
1 mg/ml	10	60	8	68	7	72
2 mg/ml	6	76	5	80*	5	80

*= Statistically significant difference in comparison to drug control in the same time interval (p<0.05)

phosphatitdylinisitol, respectively [32]. The seeds also contain monosaccharides in the form of glucose, rhamnose, xylose and arabinose. They are rich in the unsaturated and essential fatty acids. The major unsaturated fatty acid is linoleic acid followed by oleic acid [33].

Lectins (agglutinins) are naturally occurring proteins recognized by their ability to bind, with high specificity, to glycosylated residues on parasite membrane. Many lectins are derived from common human foods such as beans and wheat [34]. No side effects were reported by human volunteers who were fed wheat germ agglutinin (WGA) at a large dose (200 mg) daily for 4 days [35]. Many trials were done to target carbohydrate residues on the surface membrane of protozoa by the use of lectins [14, 36]. A wide range of biological functions are mediated by lectin interaction with cell surface glycoproteins, including cellular differentiation and adherence [10].

In the present work, the *in vitro* effects of *N. sativa* aqueous and alcoholic extracts as well as WGA were studied in comparison to chlorhexidine (0.02%) as a drug control. Results of the present study can be considered as a preliminary report on the amoebicidal activity of those agents on *Acanthamoebae*.

Chlorhexidine (0.02%) showed a trophozoite inhibition percentage of 100% after incubation for 24, 48 and 72 hrs. However, it produced cyst inhibition percentages of 56%, 64% and 80% after the same durations respectively. Similar results were reported by [37] who stated that chlorhexidine had a great *in vitro* anti-*acanthamoebal* activity for several *Acanthamoeba* strains, both for cysts and trophozoites.

The MLC of *N. sativa* aqueous extract that had 100% inhibition percentage on trophozoites was 5 mg/ ml (after incubation for 24 hrs) and 500 µg /ml (after 48 hrs).

These results are nearly similar to those reported by several authors working on other protozoa. El Wakil [9] found that *N. sativa* aqueous extract (at concentrations of 100 and 500 µg /ml) had a potent lethal effect on two *Blastocystis hominis* isolates, but with different extent. The author also found that the concentration of 500 µg/ml had a significant inhibitory effect on

the living cell rate of both isolates.

Also, the *in vitro* inhibitory activity of *N. sativa* aqueous extract was tested on the growth and motility of *Trichomonas vaginalis* in comparison to metronidazole [10]. The MLC was found to be 10 mg/ml after 48 hrs with a remarkable effect on *T. vaginalis* growth and motility.

Results of the present work showed that the MLC of *N. sativa* aqueous extract on *Acanthamoeba* cyst stage was 30 mg/ml after 24 hrs incubation and 25 mg/ml after 48 hrs. These results show that higher concentrations are required to cause 100% inhibition of cysts compared to trophozoites. In addition, chlorhexidine (0.02%) caused inhibition percentages of 56%, 64% and 80% on *Acanthamoeba* cysts after incubation for 24, 48 and 72 hrs respectively. Likewise, [22] stated that *Acanthamoeba* trophozoites are sensitive to most available chemotherapeutic agents. Persistent infection is related to the presence of *Acanthamoeba* cysts, against which very few of these agents are effective. Also, the same finding was reported by Polat et al. [18] who stated that the sensitivity of *Acanthamoeba* trophozoites was higher than that of cysts.

Results of the present study reveal a more potent cysticidal activity of *N. sativa* aqueous extract on *Acanthamoeba* cysts compared to chlorhexidine (0.02%). The highest inhibition percentage achieved by chlorhexidine (0.02%) on *Acanthamoeba* cysts was 80% after 72 hrs incubation. The same result was obtained using *N. sativa* aqueous extract at a concentration of 10 mg/ml after the same duration. Moreover 100% inhibition was obtained by *N. sativa* aqueous extract after 48 hrs on increasing the concentration to 25 mg/ml or to 30 mg/ml after 24 hrs with a significant ($p<0.05$) and a highly significant ($p<0.001$) differences respectively.

The present work showed that the MLC of *N. sativa* alcoholic extract that gave 100% inhibition of *Acanthamoeba* trophozoites was 500 µg /ml (after incubation for 48 hrs) which was similar to that of the aqueous extract. After incubation for 24 hrs, the MLC was 10 mg/ml while that of *N. sativa* aqueous extract was 5 mg/ml after the same duration.

Regarding the effect of *N. sativa* alcoholic extract on *Acanthamoeba* cysts, the MLC was 30 mg/ml after 24 hrs and 25 mg/ml after 72 hrs. These results are nearly similar to those obtained by *N. sativa* aqueous extract although 100% inhibition was achieved by 25 mg/ml concentration after 48 hrs incubation.

It is also evident that cysticidal activity of *N. sativa* alcoholic extract is more potent compared to chlorhexidine (0.02%).

The effect of *N. sativa* alcoholic extract on giardiasis was evaluated by Bishara and Masoud [9]. It was found to be as effective as metronidazole in the cure of giardiasis in experimental animals. Both gave a cure rate of 80% after a single oral dose of 40mg/kg body weight.

In a more recent study a strong anti-malarial activity of *N. sativa* ethanolic extract was reported by Abdulelah and Zainal-Abidin [38] against *Plasmodium berghe*i in mice. Both intra-peritoneal and oral treatments showed suppressive activities (decreased parasitaemia and increased survival times of the infected mice) with the highest values noted by the 100 µL/ kg dose (70.59% & 86.19% suppression by intraperitonial and oral routes respectively).

The anti-acanthamoebal activity of *N. sativa* can be attributed to the various beneficial effects of the crude and purified components of its black seeds including antimicrobial effect. They also have mast cell stabilizing, anti-inflammatory as well as immune stimulatory activities [39,40]. Most of these biological activities of *N. sativa* have been attributed to thymoquinone, the main active constituent of the volatile oil extracted from the seeds [41]. *Nigella sativa* seeds contain phenolic anti-oxidants which are well known for their anti-inflammatory and anti-parasitic properties [42]. The seeds also contain different classes of alkaloids that were believed to block protein synthesis of *Plasmodium falciparum* [43].

In the present work, the influence of WGA on pathogenic *Acanthamoebae* was studied *in vitro*.

The MLC of WGA which had an inhibition percentage of 100% on *Acanthamoeba* trophozoites was 50 µg /ml after 24 hrs incubation similar to chlorhexidine 0.02%.

Regarding the effect of WGA on *Acanthamoeba* cyst stage, concentration of 1 mg/ml caused more or less similar results to the drug control after all durations of incubation with statistically insignificant differences ($p>0.05$).

The anti-parasitic properties of WGA were investigated in several previous studies. Wheat germ agglutinin could inhibit *Giardia lamblia* excystation as effectively as monoclonal antibodies directed against cyst wall antigens. Excystation was induced *in vitro* by mimicking cyst passage through the stomach and upper small intestine. The *in vitro*-derived cysts were exposed to an acidic reducing environment followed by protease treatment at a slightly alkaline pH. Pre-exposure of cysts to polyclonal rabbit antiserum against purified cyst walls or to WGA (10ug/ml) inhibited excystation by >90% [36].

In the present study increasing the concentration of WGA to 2 mg/ml resulted in inhibition of *Acanthamoeba* cysts at a percentage of 76% after 24 hrs incubation (compared to 56% by the drug control) and a significantly higher inhibition percentage of 80% after 48 hrs compared to 64% by the drug control ($p<0.05$). So it is expected that increasing the dose of WGA may result in complete inhibition of the more resistant *Acanthamoeba* cysts.

Then, the low concentration of 50 µg /ml was found lethal to *Acanthamoeba* trophozoites after 24 hrs incubation, while the relatively higher concentration of 2 mg/ml could not achieve 100% inhibition of *Acanthamoeba* cysts. Starting with such low concentrations of 50 µg/ml, 100 µg/ml, 150 µg/ml, 250µg/ml and 500 µg/ml in the present study goes in accordance with similar concentrations used effectively in previous studies against other protozoal infections.

Ortega-Barria et al. [14] reported that a concentration of 100 µg /ml of WGA inhibited the growth of *Giardia* trophozoites by 80% after 72 hrs incubation *in vitro* and decreased cyst passage in mice infected with the closely related *Giardia muris*. Also, applying the same dose (100µg/ml) for the same duration (72 hrs) resulted

in 81.36% inhibition of *Trichomonas vaginalis* growth [10], while growth inhibition of 100% was produced at a concentration of 250µg/ml.

The *in vitro* inhibitory effect of WGA on *Acanthamoeba* may be explained on the assumption of some mechanisms suggested by Ortega-Barria et al. [14] for the *in vitro* inhibition of *Giardia* growth by WGA. They suggested that the lectin may agglutinate trophozoites preventing them from multiplying, may interfere with the function of surface glycoproteins or it may be cytotoxic to the parasites. Also, Mirhaghani and Warton [44] and Tonkal [10] suggested that growth inhibition may be attributed to the specificity of WGA for N-acetyl-D-glucosamine-containing residues in *T. vaginal is* membrane.

The effect of other natural products on *Acanthamoeba* growth was studied by other investigators. Ethanolic extract of propolis was found to have amoebicidal as well as cysticidal properties for *Acanthamoeba* trophozoites and cysts in a dose and a time dependant manner [45]. Also ethanol extracts of Arachis hypogaea L., Curcuma longa L. and Pancratium maritimum L. have cysticidal properties for *Acanthamoeba* castellanii [46].

In conclusion, preliminary evaluation of the anti-acanthamoebal activity of *N. sativa* aqueous and alcoholic extracts as well as WGA showed that these agents had considerable lethal effects on *Acanthamoeba* trophozoites and cysts. These effects were found to be dose and time dependent. They were also found comparable to or even superior than the effect of the commonly used chlorhexidine (0.02%) as a chemotherapeutic.

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