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Research Article

IN-VITRO ANTHELMENTIC EFFICACY OF PUMPKIN SEED OIL (*CUCURBITA PEPO*) ON TOXOCARIOSIS (*TOXOCARA CATI*)

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ABSTRACT: The present study was conducted to evaluate the efficiency of pumpkin seed oil on embryonic and larval development of *Toxocara cati* eggs. The eggs were extracted from uterus of female *T. cati* worms. For embryonic and larval development, the eggs were suspended with 2.5% formalin-ringer solution maintained at 25°C and 80% humidity. After 35 days of incubation, 83.5% of the eggs developed into larvae. Twenty-five ml of pumpkin seed oil added into each suspension containing eggs and developed larvae, separately. The result showed that, the eggs were only developed into two cells stage in rates of 61.9% and 43.2% at first and fourth week of incubation, respectively. The rate of undeveloped eggs was 35.7% and 56.75% at first and fourth week, respectively. The effect of pumpkin seeds oil on second stage developed larvae in showed that, the rate of live larvae was 38.2% and dead larvae was 61.75% at first week and 12.8% and 87.17% at second week. In control group, 84% of the eggs developed into larvae at the fourth week of incubation period. The results revealed that, pumpkin seed oil has a high anthelmintic efficacy against *Toxocara cati* and it can be used as an effective traditional herbal medicine for treatment of helminthiases.

Key words: Toxocariasis, Pumpkin seed oil, Ovicidal, Larvicidal.

INTRODUCTION

Pumpkins including *Cucurbita pepo*, *Cucurbita maxima* and *Cucurbita moschata*, are gourd squashes that belong to the genus *Cucurbita* and the family *Cucurbitaceae* (Chevallier 2000). Although *Cucurbita pepo* which known as summer squash, is indigenous to the plateaus of South and Central America, it is cultivated worldwide. Pumpkin is an important leaf and seed vegetable tropical vine of high traditional nutritional and medicinal values (Nkang *et al.* 2004). Its flowers, fruits, and seeds have been used as vegetables, that are very rich in vitamins and antioxidants including C, A, and E. The oil of pumpkin seeds is dark green in color that contain a high amount of free fatty acids including four dominant, linoleic, oleic, stearic and palmitic which are existent with the relative distribution of 33.1%, 43.8%, 7.8% and 13.4% respectively, representing 98 + 0.1% of the total fatty acids amount (Badr *et al.* 2011).

Pumpkin is not only known as a fruit, extracts of the pumpkin ingredients (flesh, rind seeds oil and defatted seeds meal) possess various biological activities. Pumpkin seeds have been used for a long time in traditional medicine throughout the world for treatment of several maladies of gastrointestinal parasites as anthelmintic, prostate hyperplasia, urinary dysfunction and dysuria, diabetes, cardiovascular diseases, and hypertension (Dreikorn *et al.* 2002).

Moreover, pumpkin seed oil is consumed as nutritional supplements due to the natural source of proteins, polyunsaturated fatty acids, essential fatty acids, omega 9, 6, and 3, lutein, carotenes, vitamins such as carotenoids and phytosterols, β- and γ-tocopherols, chlorophyll, and trace elements including selenium and zinc (Gossell-Williams *et al.* 2006, Tsai *et al.* 2006). Importantly, the pumpkin seed oil has been found to show pharmacological activities such as antibiotic antibacterial, antifungal, anti-inflammatory activities and antioxidant effects (Jafarian *et al.* 2012); antiviral, cytotoxic and

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antitumor activities (Badr *et al.* 2011). The powder of pumpkin seeds and betel nut decoction have been used for treating hookworms, tapeworms and roundworms through expelling and killing the parasites (Liu *et al.* 2005).

The current study was aimed to evaluate the anthelmintic activity of pumpkin (*Cucurbita pepo*) seeds oil as plant origin on eggs (ovicidal effect) and on larvae (larvicidal effect) of *T. cati, in vitro*.

MATERIALS AND METHODS

Laboratory investigation

This study was conducted at the department of Surgery and Medicine, College of Veterinary Medicine, University of Duhok. The collected fecal samples from an infected cat with toxocariasis were submitted to laboratory for clinical examination in College of Veterinary Medicine, University of Duhok, to be examined by Sheather's flotation method and McMaster technique for demonstration of eggs of the parasite and calculating the number of eggs per gram of feces. The experimental design of this study is shown in a flow chart (Fig. 1).

Ethical approval was obtained from ethics committee of the College of Veterinary Medicine, University of Duhok, Iraq.

Direct examination by direct smear was used as described by Coles (1986). A small amount of fecal sample was put on a clean microscopic slide and mixed with a drop of tap water by a wood stick. It is covered by a cover slip slide, and then examined under light microscope at X 10 and X 40 magnifications.

Sheather's flotation method was performed as per the methods described by Coles (1986). The solution is composed of 500 mg of ordinary or beet Sugar, 6.5 gm of phenol crystal melted in water bath and 320 ml of distilled water. Two grams of feces mixed with 20 ml of tap water in a clean baker. Cheesecloth and tea strainer were used to remove debris and solid materials. The mixture was taken in 15 ml test tube and was centrifuged at 3000 rpm for 5 minutes. This step repeated for three times with distilled water and after each time of centrifugation the supernatant was discarded. Then the sediment was suspended with sheather's solution and fecal suspension was centrifuged at 3000 rpm for 5 minutes. A clean cover slide slip which put on the top of the filled tube was removed and examined under the compound microscope at X 10 and X 40 of lens power (Fig. 2).

In this study, McMaster Technique was used according to Australian standard diagnostic technique for animal diseases to identify the intensity of parasitic infestation by calculating the number of eggs per gram (epg) of faeces. The Whitlock McMaster egg counting slide has 3 chambers each 0.3 ml volume and each chamber sub-divided into two counting areas of 0.15 ml volume.

Two grams of faeces in a clean glass container was thoroughly mixed with 2.5 ml of distal water and 55.5 ml of Sheather's solution, and left for 5 minutes at room temperature. The two chambers of the Slide were filled with the mixture and then left for 10 minutes before examined under the light microscope at X10 magnification power for calculation the number of eggs per gram of faeces.

The numbers of eggs per gram of feces were calculated using the following equation (Gordon and Whitlock 1939): the number of eggs counted in scanned area of the slide multiplied by

$$\frac{\text{Volume of (water + faeces sample) + Flotation solution}}{\text{Volume of faeces sample} \quad X \quad \text{Measurement volume used}}$$

Source of eggs collection

Six live *Toxocara cati* adult female worms were collected from the small intestine of recently dead cat. The mature female of *T. cati* worms were identified according to the morphological features of the adult female worms (short cephalic alae at the anterior end, and narrow tail at the posterior end), the length (from 5.5 to 6.7 cm), the shape of eggs (spherical with pitted shell and egg has dark center), and the thickness of shell measurement (2.5 to 5.5 ìm) as described by Soulsby (Soulsby 1982). The collected gravid female worms were washed by normal saline (0.95% NaCl) to remove the tissue debris (Zibaei *et al*. 2007). The worms were divided into two groups (group 1 and group 2) each group with 3 female worms. Following disserving and dissecting the uterus of the gravid female worms, the eggs were collected (the intensity of eggs in group 1 was 10520 eggs/gram, and in group 2 was 10670 eggs/gram) and their size were measured (62.6 µm - 75.7 µm x 52.6 µm – 67.8 µm) (Table 1).

Pumpkin seeds oil sample

The applied oil of pumpkin seeds was obtained from College of Agricultural Engineering Sciences, University of Duhok according to method explained by Martinez *et al.* (2013). Briefly, the seeds were obtained from local natural pumpkins were washed and dried. The oil samples were achieved by pressing the raw dried and well cleaned seeds with a screw press at a low temperature (cold pressing). The seeds oil was mechanically obtained by

Table 1. Groups of *Toxocara cati* adult female worms and intensity rate of their eggs.

Groups	No. of Worms	Intensity of Eggs/gram
Group 1	3 Female	10520
Group 2	3 Female	10670

using a Komet DD 85 G Screw Press, named as PSO 1 (IBG Monforts Oekotec GmbH and Co. KG, Monchengladbach/ Germany). Before starting the pressing procedure, the head of the screw press was heated to a temperature ranging from 55°C to 65°C in order to avoid risk and achieve greater efficiency. For that reason, a designed heating ring attached to an automatic temperature control was connected to the head of screw-press. The temperature of the extracted oil was

Table 2. The percentage rate of developed and non-developed larvae of *T. cati* in group I.

Week	Percentage	Total	
1st		35	No. of eggs
	51.4	18	2 cells in egg
	0	0	4 cells in egg
	0	0	Morulla
	0	0	Developed larvae
	48.5	17	Non-developed larvae
2nd		49	No. of eggs
	71.4	35	2 cells in egg
	18.3	9	4 cells in egg
	0	-	Morulla
	0	-	Developed larvae
	10.2	5	Non-developed larvae
3rd		48	No. of eggs
	6.25	3	2 cells in egg
	10.4	5	4 cells in egg
	31.25	15	Morulla
	43.75	21	Developed larvae
	8.3	4	Non-developed larvae
4th		44	No. of eggs
	2.2	1	2 cells in egg
	2.2	1	4 cells in egg
	6.8	3	Morulla
	84	37	Developed larvae
	4.5	2	Non-developed larvae

Table 3. The percentage rate of larval development of *T. cati* at 2nd. stage.

	No. of Eggs	Fully developed larvae	Non-developed larvae
Total	79	66	13
Percentage	-	83.5%	16.4%

not affected by screw press head pre-heating, because the heaters were always turned off when optimal seeds flow had already been achieved. The extracted pumpkin seeds oil was then separated from the sediment through centrifugation at 3000 rpm for 15 minutes at 24°C and then stored in a freezer under -20°C until used in the study.

Experimental design

The collected eggs from worms in both group 1 and group II with equal number and intensity were put separately in two clean dry screwed tubes. In each group a different method was prepared and used for promoting the maturation and arresting the development of eggs and larvae of *Toxocara cati*. In group 1, 2.5% formalin ringer solutions added onto suspension of *Toxocara cati eggs* for accelerating the development of eggs. In group II, twenty-five ml of pumpkin seed oil were added into eggs for arresting and deactivation the development of eggs (Fig. 1).

Method of accelerating the maturation of Eggs

The modified method of Zibaei *et al*. (2007) was used for this purpose. The extracted eggs from the uteri of female *T. cati* worm, was suspended with 2.5% of formalin ringer solution (2.5 ml of formalin was added to 97.5 ml of ringer solution) maintained at 25°C under 80% of humidity in order to obtain the developed infective larvae in the eggs. The incubated suspended solution was monitored and oxygenated by using 5 ml of disposable syringe every day for a total of 4 weeks for development of eggs. At the end of incubation time, the concentration of infective eggs and percentage of embryonation were determined by microscopic examination.

Method of arresting the development of eggs

For arresting the eggs development, the modified method of Yildiz *et al*. (2011) was used. Twenty-five ml of pumpkin seed oil added onto extracted eggs from the uteri of female *T. cati* in group 2 and incubated at 25°C

Table 4. Effect of pumpkin seeds oil on the rate of developed and non-developed larvae of *T. cati, in virto* in group II.

Week	Percentage	Total	
1st		42	No. of eggs
	61.9	26	2 cells in egg
	2.3	1	4 cells in egg
	0	0	Morulla
	0	0	Developed larvae
	35.7	15	Non-developed larvae
2nd		31	No. of eggs
	61.2	19	2 cells in egg
	0	-	4 cells in egg
	0	-	Morulla
	0	-	Developed larvae
	38.7	12	Non-developed larvae
3rd		41	No. of eggs
	48.7	20	2 cells in egg
	7.3	3	4 cells in egg
	0	-	Morulla
	0	-	Developed larvae
	43.9	18	Non-developed larvae
4th		37	No. of eggs
	43.2	16	2 cells in egg
	0	-	4 cells in egg
	0	-	Morulla
	0	-	Developed larvae
	56.75	21	Non-developed larvae

under 80% humidity. This group was also monitored and oxygenated every day for 4 weeks.

Method of deactivation of larval development

After four weeks of egg development to larvae in group 1, the suspension was washed for three times by phosphate-buffered saline (PBS, PH 7.4) and centrifuged at 3000 rpm for 5 minutes. Then, it was equally sub-divided into two new groups (group A and group B). The content in group A was diluted with 25 ml of 2.5% of phosphate buffer formalin, and the content in group B was diluted with 25 ml of pumpkin seed oil. Then, the two groups separately were put into two clean dry screw test tubes. The two groups were incubated for two weeks at 25°C under 80% of humidity. The viability of developed eggs was monitored and oxygenated daily for observing the spontaneous motility and deactivation of the larvae inside the eggs.

RESULTS AND DISCUSSION

Development of eggs

In Group one the eggs of *T. cati* with suspension of 2.5% formalin ringer solution maintained at 25 °C under 80% of humidity during the period of 4 weeks, 8 microscopic fields of the incubated eggs were examined by light microscope (eggs/field) after Sheather's flotation examination. The rate of eggs which developed to larvae in group 1 was 21/48 (43.75%) and 37/44 (84.0%) at third and fourth week of incubation period, respectively (Table 2). The fully developed larvae were observed at third and fourth week of incubation, while other stages of egg development (1 cell stage, 2 cells stage, 4 cells stage, early morula stage, blastula stage and full larval stage) were found in first, second, third and fourth week of incubation, respectively (Fig. 4).

Development of larvae

The larval development of *T. cati* was observed within the period of incubation of non-embryonated eggs (Fig. 4). It was observed that, 83.5% of the eggs have developed into fully larvae at the period of 5 weeks of incubation (Fig. 5). The process was performed through examination of 10 microscopic fields at X10 magnification (Table 3).

Effect of pumpkin seeds oil on eggs

Effect of pumpkin seeds oil on the eggs of *T. cati, in vitro* in group two was estimated by weekly examination. Eight microscopic fields were regularly examined for estimation of the rate of development and non-developed larvae during the period of four weeks of incubation. The eggs were developed into two cells stage at a rate of 26/42 (61.9%) and 15/42 (35.7%) remained undeveloped at first week. While at 4th week the rate of the two cells

Table 5. The effects of pumpkins seeds oil on the activity of 2$^{nd.}$ stage larvae in group B.

	1st week			2nd week		
	No of exa-mined eggs	Live larvae	Dead larvae	No of examined eggs	Live larvae	Dead larvae
Total	34	13	21	39	5	34
Percentage	—	38.2	61.7	—	12.8	87.17

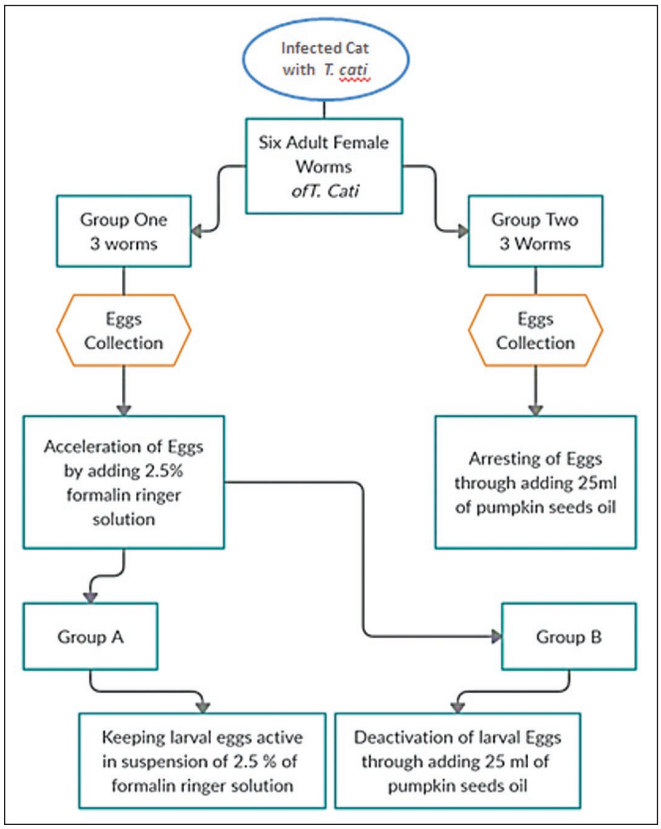

Fig. 1. The flow chart shows the experimental design of the study.

stage was 16/37 (43.2%) and non-developed rate was 21/37 (56.75%). However, no fully developed larvae within the eggs were seen in this period (Table 4).

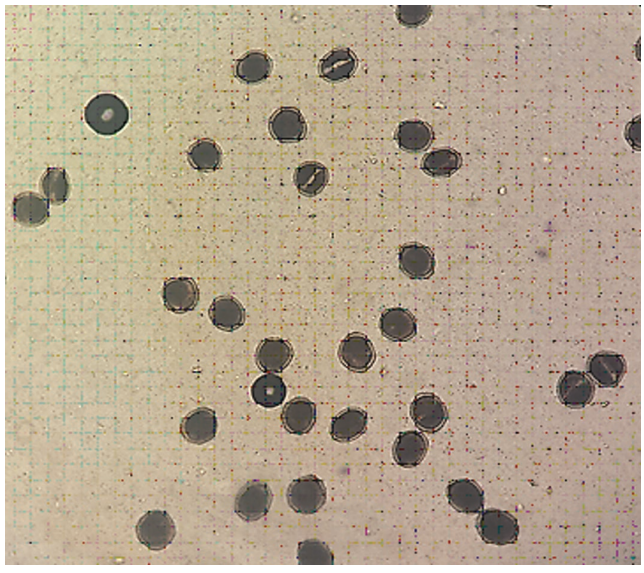

Fig. 2. Spherical and pitted eggs of *T. cati* by faecal flotation method X 10.

Effect of pumpkin seeds oil on larvae

Effects of the pumpkins seed oil on the second larval stage was also estimated for 2 weeks. Eight microscopic fields were examined for observation of live (Fig. 6) and dead (Fig. 7) larval rate. The rate of live larva was 13/34 (38.2%) and dead larvae was 21/34 (61.7%) at first week, while live larvae was 5/39 (12.8%) and dead larvae was 34/39 (87.17%) at second week (Table 5).

Pumpkin seeds are considered as medicinal plant and have widely been used in traditional treatment worldwide for prevention of several diseases especially prostatic diseases, urinary diseases, diabetes and parasitic infections (Shaban and Sahu 2017). In the last decade, pumpkin seeds found to show pharmacological and physiological activities such as anti-diabetic, antifungal, anti-inflammatory, antibacterial, antioxidant and antitumor (Jafarian *et al.* 2012); as well as improve fertility in animal models (Oyeyemi *et al.* 2008), prevent arteriosclerosis, high blood pressure, cardiac protection, immunostimulant and analgesic activity (Paulauskiene *et al.* 2005, Jariene *et al*. 2007).

In this study, the efficiency of pumpkin seeds oil on eggs and larval development of *T. cati* was investigated *in vitro*. In our knowledge, this is the first experimental *in vitro* study with *T. cati* for evaluation of efficacy of pumpkin seed oil on eggs and larval development.

Several studies have been reported on evaluation of the efficiency of pumpkin seeds extract on distractions, expelling and killing the helminths (Shaban and Sahu 2017), but rare have been reported on the efficiency of oil extraction from pumpkin seed. According to a study in Turkey by Yildiz *et al.* (2011), on the efficiency of *Artemisia absinthium* plant oil as anthelmintic agent

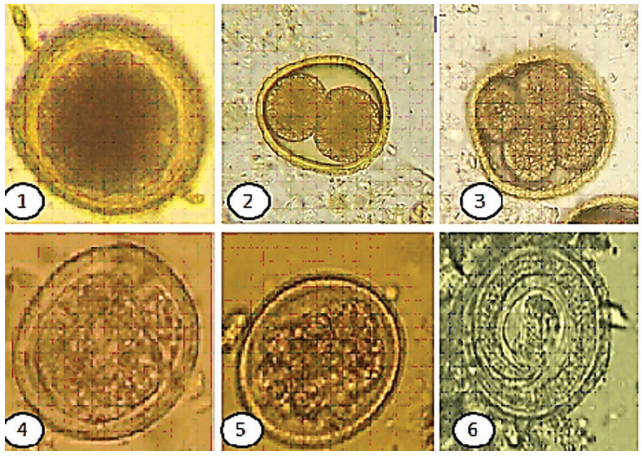

Fig. 3. Stages of *T. cati* eggs development (1. One cell stage. 2. Two cells stage. 3. Four cells stage. 4. Morula. 5. Blastula. 6. Full larval stage) X 40.

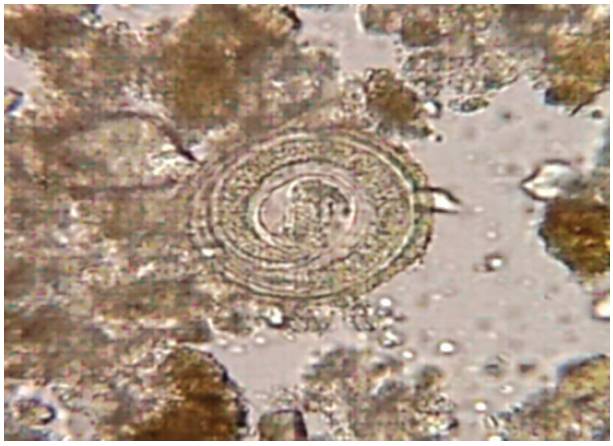

Fig. 4. Non- embryonated egg of *T. cati* by Sheather's flotation technique X 40.

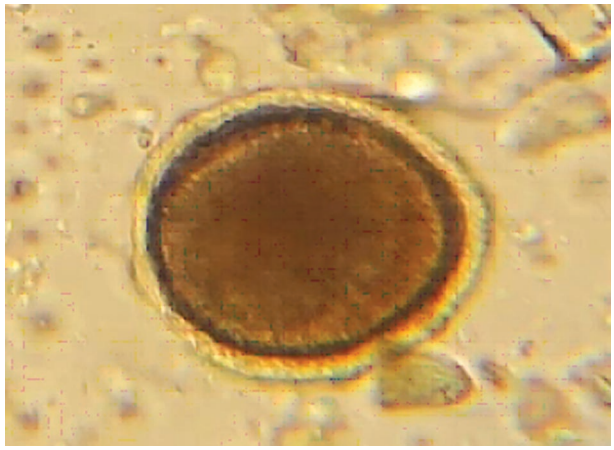

Fig. 5. Larval development of *T. cati* within the period of incubation X 40.

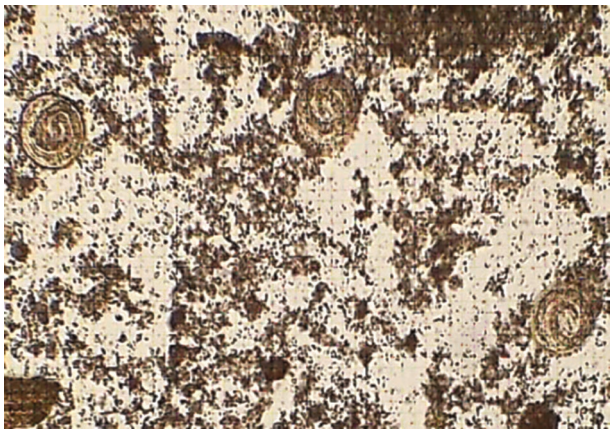

Fig. 6. Fully developed eggs to larvae at 2nd stage in control group by fecal flotation method X 10.

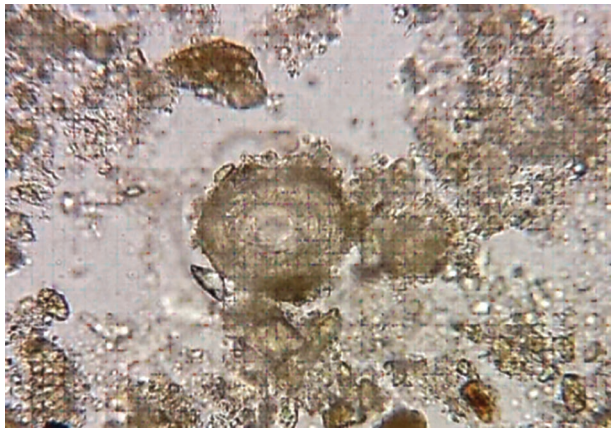

Fig. 7. Dead larvae on 2nd stage of pumpkin seed oil effect, by flotation method X 40.

against *T. cati, in vitro*. They found that, only the number of the parasitic eggs was decreased, but it didn't inhibit the larval development. So, it can be suggested that pumpkin seed oil is more efficient than *Artemisia absinthium* plant oil against *T. cati*, because depending on the results of the current study, pumpkin seed oil can inhibit development of both eggs and larvae of *T. cati.*

In Brazil, in a study by Feitosa *et al.* (2012), two groups of naturally infected Ostrich were treated with gastrointestinal nematodes (*Libiostrongylus douglassii* and *Libiostrongylus dentatus*) by 0.5gm/kg and 1gm/kg live weight of pumpkin (*Cucurbita pepo* L.) seed meal orally for 3 occasions at interval of 7 days. They found that the two groups showed a significant decrease in the number of shedding eggs at the range of 68.5% and 90% (Feitosa *et al.* 2012). In addition, a report by Alhawiti *et al*. (2019) has suggested that, pumpkin seed aqueous extract (*C. pepo*) has a significant effect in reduction the number and length of *Hymenolepis nana* adult worm and number of eggs in experimentally infected albino mice. Similar to these researches, the anthelmintic effect of pumpkin seed has clearly been shown in the current study.

Moreover, several studies have investigated the role of pumpkin seed as anthelmintic therapeutic in treatment of parasitic infection. A study by Diaz *et al.* (2004) revealed that, 23 g of pumpkin seeds in 100 ml of distilled water showed anthelmintic activity by inactivating the parasitic movements and destruction of the eggs. In addition, Ayaz *et al.* (2015) found that, the anthelmintic activities of water and ethanol extract of pumpkin seeds (*Cucurbita maxima*) on *Aspiculuris tetrapetra* in naturally infected mice was 81 and 85% respectively. In Italy, a study conducted by Lans *et al.* (2007) showed that one gram of meshed pumpkin seeds (*Cucurbita pepo*) mixed with 250 ml of distilled water showed anthelmintic activity against *Taenia* infection in dogs. Similarly, oral administration of pumpkin seeds extracts with boiled water in experimentally infected puppies with *Heterophyes heterophyes* showed distraction of parasitic eggs (Mahmoud *et al.* 2002). It seems that different types

of pumpkin seeds extracts play important role in treatment of parasitic infection.

Similar anthelmintic therapeutic effect has been reported for pumpkin seed by several *in vivo* experiments beside the *in vitro* studies. In Egypt a report which conducted by AbdelAziz *et al.* (2018) showed that, the pumpkin seeds ethanolic extract has a lethal anthelmintic effect on *Ascaridia galli* parasite in chicken *in vitro* and *in vivo*. Similar anthelmintic activity of the plant was observed on larval stages of *Haemonchous contortus, in vitro* (Marie-Magdeleine *et al*. 2009) and also on *Heligmosides bakeri, in vitro* and *in vivo* (Grzybek *et al.* 2016). Further studies are required to reveal the exact role, dose and effect of various extracts of pumpkin seeds in *in vivo* treatment of parasitic infection.

It has been reported that, the types of extractions play an important role in the activity of this medicinal plant as anthelmintic against parasites. Amorim *et al.* (1991) investigated the effect of three types of pumpkin seeds extractions (water, methanol and dichloromethane) against *Plasmodium berghei* in rats *in vitro.* They found that, only water extract inhibited the larval development of the parasite *Plasmodium berghei*.

CONCLUSION

It's clear from the current study that, the oil extract from pumpkin seeds (*C. pepo*) has an efficient anthelmintic activity against *T. cati* and it can be used as an effective anthelmintic against *T. cati* in cats. It's recommended to investigate pumpkin seed oil efficiency as anthelmintic herbal medicine in experimentally infected cats and dogs with *Toxocara cati* and *Toxocara canis, in vivo,* respectively.

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