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Pathological study for effect of alcoholic extraction of *Nigella sativa* on inffected rabbits with *Fasciola gigantica.*

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Abstract

This study included of effect alcoholic extract of *Nigella sativa* seeds on Fasciola gigantica parasite used (24) adult rabbits which distributed randomly into four equal groups and give doses (200,400,600 mg / kg) and control group of body weight , after end of experiment was drugg and the blood drawing directly from heart to conducting immunologic tests (Total count of Red blood cells, Haematocrit value, Estimating concentration Hemoglobin, Total count of White blood cells and Differential count of White blood cells) and then the animals dissected and eradication of the liver, spleen and kidney for the preparation of tissue sections.

Results refer there are not significant difference of alcoholic extract of Nigella sativa seeds at level ($P<0.05$) in blood components of healthy animals while in infected animals and treated there are less significant effect when compared with the infected animals only which appeared increase significant in the count of White blood cells and significant decrease in the count of Red blood cell and Haematocrit value and amount of Hemoglobin.

Also this results showed the ability of alcoholic extract of Nigella sativa seeds at dose (600 mg/kg) Was more effective in reducing the numbers of worms F.gigantica in infected rabbits when compared with the untreated animals.

Introduction

Fascioliasis disease is one of the most dangerous health problems because of its infection by worms belonging to the genus *Fasciola* that cause economic losses in animals as cows, sheep and rabbits (Soliman, 2008). While humans infection are incidental, so this Fascioliasis is animal disease primarily (Hurtrez-Bousses *et al.*,2001).

These worms belong to the Phylum: Platyhelminthes, Class : Trematodes, Sub class: Digenea and genus: *Fasciola*, which includes two Species *F.hepatica* and *F.gigantica* , which are responsible for the Morbidity and Mortality in sheep and cattle (Mas-Coma *et al.*,2005).

The disease is distributed in medium and south iraq areas where there is Intermediate host which is one of the types of snails of the genus: *Lymnaea* that return to the Phylum: Mollusca the miracidum stage of *F.gigantica* when penetrated this snail needed about (5-7)weeks to development to cercariae which encysted on vegetation as metacercariae and transmitted to the (final host) which infected with acute and chronic phase characterized by weight loss in animals as well as the lack of milk production and cachaxia in severe infection (Saba *et al.*,2004). As well as the poor quality of the meat and damaged livers infected with the parasite and its effect on the fertility of animals in addition to the delay in the growth of small animals (Hossain *et al.*,2011).

The scientists developed many drugs to treatment the disease, but most of these drugs are toxic and ineffective against the disease in acute and chronic phases, most

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of drugs are effective against chronic phase only such Bithional drug which is not favor to use because of its side effects as vomiting, dyspepsia, swollen abdomen, headach (Knodell *et al.*,2003) and Praziquantal drug which is characterize by its effecting is not constant (Savioli *et al.*,1999).

The resistance of these worms against of these drugs is one of the most important problem to control distribution of this disease which requires continued research for more effective treatments and less toxic than previous treatments (Al-Beitawi and El-Ghousein,2008).

So researchers producing another of alternative treatments, such as plant extracts because of their effectiveness against the parasite and without any side effect (Sunita&Singh,2011).

Recent scientific studies reffer to the importance use of medical herbs and plant extracts in control of many different pathogens parasite that affects on humans and animals, from numerous studies of *Nigella sativa* extract proven the pharmacokinetics against many parasites because there are many active materials such as Alkaloids, Thymoq -uinone,Phenols, Tannins which have Medical importance (Al-Zubaidy,2002).

Materials and Methods

Sample collection:-

1-Preparation of alcoholic extract & stock solutions

Nigella sativa seeds brought from local markets of AL-Najaf city, purified from impurities and kept at laboratory temperature in a dry place and grind by blender to get on fine powder.

(20) gm of dry powder material put in Thimbles of soxhlet apparatus according the method (Qureshi *et al.*,1992) . (200) ml of ethanolic alcohol with concentration of 96% were added for (24) hours, After that concentrated extracted material by the rotary evaporator with temperature at (40-45)° C after evaporated of all ethanol in the mixed observed thick material textures (Gelatinous formed).

prepared the stoke solution by dissolving (60) gm of the extract in (100) ml of distill water, and thus became the basic concentration of the solution (stoke solution) 60% or equivalent 600mg /ml . Then prepared three different concentration (20%,40%,60%) of *Nigella sativa* extract to know its effect in the vitality of eggs and adult worms of *F.gigantica* which are prepared by mixing certain size of stoke solution according to the law C1V1=C2V2

2-Eggs and Adult worms of parasite collection

Eggs isolated from the cows and buffaloes feces according to the method (Hillyer *et al.*, 1996) (5) gm of infected animal feces placed in a glass beaker (500) ml with tap water (100)ml with mixing by the glass rod, then filtration by using a piece of gauze and collected the filtrate in other glass beaker, then leaved for 10 minutes, to precipitat pouring the upper part of the solution and remained (10) ml of sediment, added tap water and leaved for another 10 minutes to precipitat and poured the filtrate and stayed the sediment, repeated this process several times until they change the color of the liquid to the transparent color which contains only eggs of parasite, then used in subsequent experiments, The eggs diagnosed by depending

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on (Soulsby,1982). *F. gigantica* worms collected from the livers,bile ducts of cows and buffaloes infected and slaughtered in the abattoir of Al-Najaf livers and bile ducts placed in plastic containers and brought directly to lab.

Bile ducts dissecting by slicing blade and the worms isolated and washed several times with Normal saline solution to remove impurities, placed in glass bottles containing normal saline solution.

Adult worms of the parasite *F.gigantica* diagnosed by depending on (Lotfy & Hiller,2003), Then used in subsequent experiments.

3- **Collection and breeding of snails**

Snails from *Lymneae auricularia* species were collected from herding regions and drainage surrounding province of AL-Najaf and including Manathira , Mishkhab which is frequently in this regions more than any other type of snails where it was transferred by container on plastic bottles of water from the same river which collected samples and then transferred to the laboratory at the Animal house / College of Education for Girls. Diagnosed as a species *L. auricularia* depending on (Mansoorian,2001).

This a snail have right-wing shell, fragile and colored yellow or brown but not cover and the head distinctive and contains a pair of triangular tenticles and then placed in a glass basins container on the tap water, which put before (24) hours to disposal of the chlor as well as put in each basin un aerator and use Cmpelan plant *Ceratophyllum* collected from regions collecting snails herself and that was used as food for snails (Al-Ali,2002)taking into account the replacement of water twice week. Snails left for a week for the purpose of laying eggs and then collected egg masses and transferred to plastic basins for the purpose of obtaining on generations laboratory preparation to being infected by miracidia of the *F.gigantica.*

4-Laboratory Animals

(24) domestic strain rabbit *Lepus lepus arabica* were reared in the animal house of the College of Education for Girls / University of Kufa, weights ranged from 1-1.5 kg puts in the places in located to them it furnished ground with wood dust which replaced weekly in order to preserve on clear was also taking into account the environmental conditions as the animal house is equipped with an air vicious for ventilation, and the thermometer and oil heated to control the temperature between 25-22° C, as well as control the lighting daily (14) hour and were feed on fodder which used as feed for poultry bought from the local market and supplied with water by using special utensils and these rabbits were distributed to the four groups according to the research experiments.

5-Infection of the snails

(300) snail of *L.auricularia* species Infected by (20-25) miracidium of the parasite *F. gigantica* using the Tissue Culture Chambers which placed the snails in them. The modern hatching Miracidia by using the Pasteur Pipette and leave the snails with Miracidia for two-hours according to (Al-Ali,2002) then returned these snails into breeding basins and after one month notes the release of cercaria**e** from the snails and encyst**ed** on glass flasks walls and water surface (Al-Mayah,2004).

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6- Metacercariae collection

After one month from infection metacercariae placed on the surface of the basins small pieces of nylon sacks in order to adhesion of metacercariae and examined under Disecting microscope observation the presence of Metacercariae , the process of put pieces nylon and collected continued for three months to collected the sufficient quantity of metacercariae for infection (Al-Mayah,2004).

7- **Effect ethanolic extract with doses (200, 400 and 600 mg / kg) in worms number rate *In vivo*: -**

Rabbits in this experiment were divided into (4) groups each group contain(6) infected rabbits by (100) metacercariae / rabbit in (2) ml of distilled water. The metacercariae administration orally by use Stomach tube. the second group also administration orally by metacercariae and treated with ethanolic extract with dose (200 mg / kg), The third group ruminating by metacercariae and treated with ethanolic with dose (400 mg / kg), the fourth group ruminating by metacercariae and treated with ethanolic extract with dose (600 mg / kg).Then dissected animals after three months of infection and prepared histological sections to observed the histological changes(AL-Ali,2003).

8-Blood sample collection

Before rabbits numbed by chloroform material measured the total weight of the animal and pulled the blood directly from the heart by medical syringe with volume of (1) ml and measure needle (21) degrees and placed in a capillary tube contain on Heparin to examined the Blood Parameters.

9- **Statistics analysis**

Completely Randomized Design(CRD) is using with the experience of two factors (concentrations and time periods) and the results were tested by use Least Significance Differences(LSD) at the level of probability of ($P < 0.05$)(Al-Rawi and Khalafallah,2000).

Results

Results appeared that rate of the total number worms in all treatment rabbits with extract are lower than of the infected animals and the numbers rate of worms of the treatment groups with extract is (6.4) at dose (200mg/kg)and the therapeutic efficiency are 75% , but at dose (400mg/kg) the numbers rate of worms are (2) and the therapeutic efficiency are 82% , while the numbers rate of worms (0) at dose are (600mg/kg) and the therapeutic efficiency are 100 % and there are significant difference at the level ($P < 0.05$) when compared with
negative and positive control groups were (0) , (16.7) and therapeutic efficiency are 100% , 33 % respectively.as in Table (1) .

The results reached that there are significant differences at level ($P < 0.05$) for (RBCs), (PCV) and (Hb) in rabbits infected with *F.gigantica* (positive control group) and the infected and the treated rabbits groups after one hour , one week , four weeks and Negative control group as in Tables (2,3,4).

Also the results showed that the infected rabbits group with *F.gigantica* (positive control) give higher value of total number of white blood cells (WBCs) at level ($P < 0.05$) and the rate reached **(**10.41±0.21) when compared with the Negative control group which reached **(**5.52±4.8) and the infected and treated rabbits groups after four weeks followed that then and the treated rabbits groups after one week and later the infected and treated rabbits groups after one hour.

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Also showed there are significant higher in differential count of Eosinophils in the blood samples of infected rabbits (Positive control) at level ($P < 0.05$) and the rate reached (7.27±2.61) when compared with the normal rabbits (Negative control) which reached (4.76±1.09) while there are significant differences between treats when compared with the Negative control.

Also noted from these results there are significant lower at level ($P < 0.05$) in the differential count of Mononcytes, Lymphocyts, Neutrophiles and Basophiles when compared with the Negative and Positive control groups.

Table(1): Total rate number of the worms in organs of the rabbits treated groups with different doses after three months from the infection.

Organs / Treated rabbit groups	The numbers of the worms in the organs			Total rate numbers of the worms	Therapeutic efficiency
	Liver	kidney	spleen		
Negative Control	–	–	–	0	100%
Positive Control	8.4	5.2	3.1	16.7	33%
Alcoholic extract of *Nigella Sativa* 200mg/kg	4.3	2.1	–	6.4	75%
Alcoholic extract of *Nigella Sativa* 400mg/kg	2	–	–	2	82%
Alcoholic extract of *Nigella Sativa* 600mg/kg	–	–	–	0	100%

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Table(2): Effect of ethanol extract for *Nigella sativa* seeds with dose (200 mg / kg) in the blood parameters of infected rabbits with *F. gigantica*.

Animal Group	RBC (10^{12}/L)	PCV (%)	Hb (gm/dl)	WBC (10^{9}/L)	differential count of white blood cells %				
					Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
Infected animals and treated after one hour	5.41 ± 4.8	31.21 ± 1.1	11.33 ± 0.14	5.65 ± 5.2	1.92 ± 9.5	65 ± 0.4	20.62 ± 0.6	4.88 ± 1.20	0.21 ± 0.20
Infected animals and treated after one weeks	4.21 ± 3.7	29.10 ± 0.9	9.20 ± 0.12	9.23 ± 7.5	1.02 ± 8.90	65.1 ± 0.41	20.62 ± 0.6	5.54 ± 2.1	0.11 ± 0.1
Infected animals and treated after Four weeks	6.43 ± 5.8	33.11 ± 2.5	12.52 ± 0.29	6.52 ± 5.61	0.18 ± 7.5	63.10 ± 0.30	19.60 ± 0.5	6.43 ± 2.0	0.10 ± 0.1
(Positive control)	4.41± 5.12	24.71± 1.20	8.53 ± 0.1	10.41± 0.21	2.36± 0.61	68.23± 0.62	26.97± 0.42	7.27± 2.61	0.32± 0.20
(Negative control)	5.51± 4.9	32.21± 1.31	11.43± 0.15	5.52± 4.8	1.83± 9.23	65.07± 0.58	20.57± 0.53	4.76± 1.09	0.34± 0.24

Values are Means ± SE
* Significant differences ($P < 0.05$).

Table(3): Effect of ethanol extract for *Nigella sativa* seeds with dose (400 mg / kg) in the blood parameters of infected rabbits with *F. gigantica*.

Animal Group	RBC (10^{12}/L)	PCV (%)	Hb (gm/dl)	WBC (10^{9}/L)	differential count of white blood cells %				
					Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
Infected animals and treated after one hour	5.30 ± 4.5	31.33 ± 2.8	11.51 ± 0.16	5.54 ± 4.30	1.80 ± 8.50	63 ± 0.51	20.52 ± 0.5	4.95 ± 2.23	0.20 ± 0.1
Infected animals and treated after one week	5.91 ± 4.8	31.98 ± 2.98	11.52 ± 0.17	5.32 ± 3.21	1.20 ± 8.33	62.91 ± 0.50	20.43 ± 0.46	5.93 ± 3.10	0.1 ± 0.13
Infected animals and treated after Four weeks	6.51 ± 5.9	33.95 ± 3.01	12.67 ± 0.20	5.30 ± 3.20	1.10 ± 7.01	60.52 ± 0.33	19.33 ± 0.14	6.76 ± 2.32	0.1 ± 0.12
(Positive control)	4.41± 5.12	24.71± 1.20	8.53 ± 0.1	10.41± 0.21	2.36± 0.61	68.23± 0.62	26.97± 0.42	7.27± 2.61	0.32± 0.20
(Negative control)	5.51± 4.9	32.21± 1.31	11.43± 0.15	5.52± 4.8	1.83± 9.23	65.07± 0.58	20.57± 0.53	4.76± 1.09	0.34± 0.24

Values are Means ± SE
* Significant differences ($P < 0.05$).

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Table(4): Effect of ethanol extract for *Nigella sativa* seeds with dose (600 mg / kg) in the blood parameters of infected rabbits with *F. gigantica*.

Animal Group	RBC (10^{12}/L)	PCV (%)	Hb (gm/dl)	WBC (10^{9}/L)	differential count of white blood cells %				
					Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
Infected animals and treated after one hour	6.65 ± 5.84	34.76 ± 3.56	13.55 ± 0.31	5.10 ± 3.21	1.75 ± 8.4	62.96 ± 0.50	19.62 ± 0.43	5.56 ± 3.52	0.11 ± 0.1
Infected animals and treated after one week	6.73 ± 5.86	34.88 ± 3.85	13.67 ± 0.33	4.91 ± 2.80	1.65 ± 7.5	62.75 ± 0.49	19.10 ± 0.39	5.91 ± 3.66	0.10 ± 0.1
Infected animals and treated after Four weeks	7.12 ± 6.10	35.51 ± 4.65	15.50 ± 0.50	4.72 ± 2.68	1.44 ± 7.10	60.95 ± 0.43	17.50 ± 0.20	6.54 ± 3.96	0
(Positive control)	4.41± 5.12	24.71± 1.20	8.53 ± 0.1	10.41± 0.21	2.36± 0.61	68.23± 0.62	26.97± 0.42	7.27± 2.61	0.32± 0.20
(Negative control)	5.51± 4.9	32.21± 1.31	11.43± 0.15	5.52± 4.8	1.83± 9.23	65.07± 0.58	20.57± 0.53	4.76± 1.09	0.34± 0.24

Values are Means ± SE
* Significant differences ($P < 0.05$).

Discussion

Through observing results of a study decrease rate for number worms in infected rabbits by liver giant worms, showed presence on both liver and internal viscera where animals differ in sensitivity to infection by liver giant worm and this sensitivity depend on several factors such as type of animal , strain , age , type of nutrition and nature of the histological structure to liver as well as immune state and number metacercariae which eaten (Alhaidary *et al*.,2010).

Al-Ali,(2003) found that infection percentage by *F.gigantica* in local rabbits is 100% when injected by (100) metacercariae, where current study showed that infected rabbits and non-treated by extract were infection percentage is equal 100% and represent with more paths migration worms in livers these rabbits (positive control group) and this consistent with referred (Cervi *et al*.,2004) that rabbits, mice and sheep hosts very sensitive direction infection with this parasite.

The rabbits in infected groups and treated show that ethanolic extract clear effected in reducing from numbers and can explained inhibitory mechanism action to this extract through contains on effective substances such as : Alkaloids , Phenols, Tannins, Glycosids.

Especially Alkaloids compounds which behave as catalysts factors for cellular and humoral immunity in infected rabbits with *F.gigantica* by stimulating lymphocytes lead to raising efficiency immune system and increases ability phagocyte on attack foreign objects and may lead reason to that alcohol extract for *Nigella Sativa* seeds works on increase body response and formation antibodies in laboratory animals infected (Xingming *et al.*,2009).

This compliant with referred researchers(Salem,2005; Ismail *et al.,* 2009) about role *Nigella sativa* as an immune an enhanced in local rabbits through increasing

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Cytokines level in body, which in turn contributes in stimulate B-cell lymphocytes on divide and formation plasma cells, leading to increased production of antibodies, as well as help regulate immune response.

May lead reason to that Phenols be effected on Acetyle -choline estrase enzyme control on flexibility and permeability cell membrane,

phenols losing membrane permeability property, which lead to entry various and toxic materials without regulation and then death of the parasite (Naguleswaran *et al*.,2006). This compliant with referred (Al-Kwori ,2006; Ozdemir *et al*.,2012) that Phenols existing in *Nigella sativa* seeds have active role where contributing with Ascorbic acid also existing in *Nigella sativa* seeds in treatment parasitic infections.

Current study found that eggs and worms isolated from untreated rabbits were smaller than isolated from natural hosts such as cows and buffaloes.

The morphological difference phenomenon in Trematodes normal state

adapted through these worms to live and mature in different hosts

(Nongenetic modification) where refered (McConville *et al*.,2009) that parasite *Fasciola* appear great morphological differences depending on isolated host as (cows, rabbits and guinea pigs).

Found(Al-Ali,2003) that eggs and worms *F.gigantica* isolated from rabbits were smaller than isolated from natural host such as cows and buffaloes.

Current study found that rabbits treated by alcoholic extract of *Nigella sativa* gave protection represented with reduced worms migration paths in liver addition to change in shape and size worms, also failure find any eggs for parasite lead reason to contain this extract on previous mentioned compounds which act on reduced worms and eggs numbers in animals treated through influence on natural evolution for growth Trematodes and thus on eggs producing process (Toama,2011).

Current study noted significant differences in blood samples for infected rabbits and treated compared with positive control animals with respect red blood cells noted decrease significant in infected animals may showed (Kowluru *et al.*,1989) to low activity enzyme Na-K-ATPase in covers red blood cells in infected rats with *F.gigantica* and this leads to increase in size cells and Osmotic fragility and decrease in Filterability this leads to disturbances in capillary circulation resulting in analysis some of globules and occurrence of Anemia, In addition changes in components membrane lipids which lead to change in elasticity of red blood cells, causing degradation easily (Ishimura *et al* , 1998).

He explained (Vlassara *et al.*,1987) red blood cell easily Phagocytosis by Macrophage in mice infected with *F.gigantica*, which shorten life.

That decline in number of red blood cells in infected animals also accompanied decrease in size (PCV) compared with infected animals and treatment and this illustrated results of the study.

Same case to amount hemoglobin (Hb) in blood where results of the study noted lower amount (Hb) in infected animals. These results are conform with finding (Babu *et al.*,2003).

May lead reason to sensitive red blood cells for infection by *F.gigantica* being non-defense cells in body compared to white blood cells (Al-Nzal,2005).

In groups infected and treatment noted increase significant in red blood cells and this accordance with (El-Sarha *et al.*, 1997) that giving goats

(3gm) from *Nigella sativa* grinded with parasite daily for (4weeks), causing increase in RBC amount and size PCV.

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White blood cells, the study noted High significant in number of white blood cells in infected animals compared with normal control group may attributed to negative impact on function of the immune system (Serradell *et al.*,2007).

Also noted existence significant differences in blood of infected animals and treated by extract after three months from infection compared to positive control group seen increase significant in white blood cells may due to infiltration cells and their migration to sites infection (Sako *et al.*,2009).

Can be attributed cause of contrast to occurred acute inflammation associated for infection with this parasite, which leads to stimulate bone marrow to produce large amount of Neutrophiles and then get rise in total number to white blood cells and this compliant with (Gajewska *et al.*, 2005).

Can be increase in total number of white blood cells caused by degeneration parasite and elimination upon in treated groups by extract not being able from stability in tissues and occurs infection in them (Movassaghi-Ghazvini *et al.*,2008).

While Infected and treatment groups accompanied decrease in white blood cells which behavior same pattern with increasing dose and seems that these results according with (Ayoub, 1999) when give rat (8gm / kg) from aqueous extract for oil *Nigella sativa* seed and for period (15days), observed decrease in total number to white blood cells.

Also found (El-Sarha *et al.*, 1997) giving goats (3gm) from *Nigella sativa* grinded daily for (4weeks) occurs decrease in number of white blood cells.

Results differential count to white blood cells, where Showed changes in appear in numbers where appear High significant in Eosinophile numbers accompanied lower significant in number Neutrophils and monocytes which may lead to effect Glycosids existing in *Nigella sativa* seeds in Mitosis process to these cells (Swami&Tan,2000).

As *Nigella sativa* seed contain on Lectins (Matanovic *et al.*,2007),

Lectins are globular proteins able on link with sugar and general, considered one of substances which stimulate division lymphocytes.

or may lead to that extract user may stimulate migration Neutrophils to sites presence parasite to eliminate upon by phagocytosis (Roitt *et al.*, 1998; Zeweil *et al.*,2008) Compared to positive control group note increase in lymphocytes which induce on production of lymphokines which turn activated phagocytosis and attract inflammatory cells such as Neutrophils to sites infection (Zainal-Abidin,2007).

Decrease in rates monocytes numbers, may lead to migration some these cells to parasite presence areas with compensation peripheral blood by new numbers add to him and because need continues to inhibit entrenched process infection note fluctuations between decrease and increase for continuing migrating process to infected part although compensate shortfall in numbers in peripheral blood(Edith *et al.*,2010).

Basophils note a great lower significant in infected animals and treatment, and this attributed to activation by alkaloids and phenols in stimulating migration Basophiles to sites presence of the parasite, which enhance its role in phagocytosis process and this accordance with (Aslan *et al.*,2009).

As observed increase significant in Eosinophils number may lead cause to infection by some allergic diseases and parasitic infections especially parasites ability on invade and stability in tissues (Munguía-Xóchihua *et al.*,2007) or to sensitive these cells to parasite antigens spread in blood which due to increased numbers in peripheral blood because stimulate bone marrow to produce this type of cells (Mellau *et al.*,2010) and these results according with several studies which

confirmed increased Eosinophils when infection with parasites and its role in destruction larvae stages for many from parasites by adhesion these cells in parasite wall and secretion granules act on damage outer wall of the parasite and may due damage to action of peroxide hydrogen (H2O2), which accompanied liberation with release granules Eosinophils (El-Ridi *et al.*,2007).

As mention both (Mclaren & Boros,1983) occurs increase in Eosinophils number in mice experimentally infected by *Schistosoma mansoni.*

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