STUDY THE PROTECTIVE AND THERAPEUTIC EFFECTS OF CRUDE GARLIC ON MORTALITY, OOCYST OUTPUT AND HEPATIC LESIONS IN EXPERIMENTAL INFECTION WITH *EIMERIA STIEDAE* IN DOMESTIC RABBITS.

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ABSTRACT

A study was conducted to investigate the protective and therapeutic effects of crude garlic (Allium sativum) against experimental infection with Eimeria stiedae in rabbits. Forty- two rabbits Divided into four groups : six rabbits a healthy control group (HC); 12 rabbits as challenged -garlic -protected group (CGP) which received a daily dose of 0.5 g/kg body weight crude garlic for five days before challenged with E. stiedae ;12 rabbits as challenged -garlic -treated group (CGT) which received a daily dose of 0.5 g\kg body weight crude garlic for five days after challenged with of *E*.*stiedae* and 12 rabbits as infected group (IG). The challenge dose was 4×10^4 sporulated E. stiedae oocyst per rabbits .Mortality rate, fecal oocyst count were elevated throughout the experiment. At the end of the experiment ,all rabbits were killed and histopathological examination was performed. Mortalities were recorded in HC group, while mortality was found to be 8% ,25% and 50% in the CGP,CGT and IC groups, respectively. CGP rabbits have the lower numbers of oocysts than those in the CGT than those in IC groups. Hepatic lesion were less severe in the CGP and CGT groups than in IC group. The results showed that oral administration of crud garlic ameliorated the adverse impacts of hepatic coccidiosis on rabbits as prophylactic and therapeutic, but garlic was more effective as prophylactic.

INTRODUCTION

Coccidiosis is a ubiquitous protozoan infection of animals seriously impairing their growth and food utilization ,it causes significant mortality in rabbits the Coccidia of rabbits have not been studied to the same degree as the species which occur in other hosts(1).

Kids are most susceptible; however, infected adults become carriers. There are 2 anatomical forms: Hepatic caused by *Eimeria stiedae* (2), and intestinal caused by the most important species are *probaply E.intestinalis*, *E. irresidua* and *Eimeria magna* and other eimeria species, transmission of both hepatic and intestinal coccidian is by ingestion of sporulated oocysts usually in contaminated feed or water, Eimeria stiedae is an inhabitant of the epithelial cells of the bile ducts and is the cause of severe liver damage in rabbits (3,4). Conventional disease control strategies have relied mainly on chemoprophylaxis, which is very expensive. Furthermore, the

continuous use and misuse of anticoccidial drugs has led to the emergence of drugresistant *Eimeria strains*. In recent time herbal medicines have become indispensable and are forming an integral part of the primary health case system of many nations (5,6). Garlic (*Allium sativum*) has the broadest spectrum of any antimicrobial substance that we know of it, is antibacterial, antifungal, antiparasitic, antiprotozoan and antiviral (7).

MATERIAL AND METHODS

Experimental animals

42 healthy rabbits (males), aged between 4 and 8 weeks and weighing between 500 - 1000 gm, were used. During the experimental period, the animals were kept in an experimental animal house of veterinary collage of Bagdad university. Rabbits individually housed in metal cages with grid on the bottom keeping rabbits from coming in contact with their feces. They were kept at 15-20 ° C. The rabbits were fed with commercial pellet food and water were supplied *ad libitum*. The absence of *E. stiedae* and other coccidian oocyst prior to the experiments was confirmed by fecal examination in rabbits by flotation method daily for 3 days prior to infection.

Isolation and speculation of *Eimeria stiedae* oocysts:

Eimeria stiedae oocyst parasite were obtained directly from the gall bladders and lesions of livers of naturally infected rabbits, the gallbladder and liver were removed and washed with normal saline to obtained oocysts then transferred to a clean sterile Petri-dish containing (2.5%) potassium dichromate solution at depth 3-5 mm for 3 days in room temperature 26 °C for sporulation. The content of the Petridish which have oocyst were centrifuged at 2000 rpm for 5 minute where the supernatant was discarded and the sediment was resuspended in a saturated salt (NaCl) solution and mixed well by stick for flotation and re centrifuged at 2000 rpm for 7 minute and draw of the supernatant which contain the oocyst by pasture pipette and put in glass beaker and retained the flotation 3 times with draw the supernatant each time and add it to the beaker ,distilled water was added to the beaker in 1:10 ratio, then placed the supernatant in plastic centrifuge bottles with tight-fitting lids are preferred for this experiment ,centrifuged at 3000 rpm for 7 minute for sediment . the sediment which have the sporulated oocyst where collected by pasture pipette in and resuspended in 2.5% potassium dichromate solution and stored at 4 °C in refrigerator until use (8).

propagation of Eimeria stiedae :-

Ten Eimeria – free rabbits of one month old were inoculated with 6000 freshly sporulated *E. stiedae* oocyst. Feces were examined daily until shedding of the first oocyst. Infected rabbits were then killed and oocyst were collected from their gallbladders. Oocyst were purified and allowed to sporulate in 2.5% potassium dichromate solution as mentioned before. Sporulated oocysts were used as a challenge material in the experiment.

Garlic (A. sativum) administration :-

Freshly crushed garlic bubbles were orally administered at a daily dose of 0.5 g/kg body weight (9) to the protected and treated groups of rabbits using a gastric tube for five successive days. garlic bulbs were purchased from a local market and crushed in a mortar on the day of administration.

Parameters evaluated

Mortality rate:- The mortality rate was recorded daily for each group.

Counting of oocyst in feces :-

Fecal sample were collected from different parts of ground of each group for parasitological examination beginning on day 16 post- challenge(PC) , When oocysts were detected in the feces until day 32 pc. Which are stored in plastic continuers labeled with group identification and date and transport to the laboratory for estimation the oocyst per gram of feces (OPG) was counted using the McMasster method (10).

3.12. Histopathology of liver and gall bladder changes:-

At the end of the experiment ,the animals were killed and postmortem examination done. The macroscopic appearance was recorded and any abnormal gross changes in internal organs were observed. Specimens were taken from all livers and gall bladders , the tissues have been kept in 10% formalin immediately after removal and 48 hours of fixation , tissue slide processing was made routinely using a set of increasing alcohol concentrations ,tissue sections were embedded in paraffin blocks, and sectioned by microtome at 5 um. All tissues were stained with hematoxylin and eosin stain and the histopathological changes were examined under light microscopic (11).

Experimental design:-

Forty two rabbits were divided to 4 groups of: 6 rabbits as healthy control group. 12 rabbits as challenged –garlic –protected group (CGP) which received a daily dose of 0.5 g\kg body weight freshly crushed crude garlic for five days just before challenged with *E. stiedae* .challenge was performed on the sixth day after administration of garlic. 12 rabbits as challenged –garlic –treated group (CGt) which received a daily dose of 0.5 g\kg body weight freshly crushed crude garlic for five days in the 16th day post - challenged ,when oocyst was detected in feces. 12 rabbits as infected group .the challenge dose was 4×10^4 sporulated oocyst of *E. stiedae* per rabbit .All groups are kept under observation for 32 days post challenged.

Statistical analysis :-

Statistical analysis of data was performed on the basis of two-way analysis significant level of (p < 0.05). specific group differences were determined using leas described by (12).

RESULTS

Clinical sings :-

No clinical abnormalities were observed in rabbits in the HC group. Rabbits in the CGT and CGP groups exhibited slight dullness and abdominal distension but rabbits in IG show depression, aneroxia, diarrhea, emaciation, abdominal distension.

Mortality rate in groups of experiment :-

The result of mortality rate in table (1). The result showed there is a significant difference (P<0.05) in mortality rate between groups of experiment. There was no mortality in 1^{st} and 2^{nd} weeks after infection in all groups of experiment. In 3^{rd} week there was no mortality in HC and CGP but it was (16%)in CGT group and (33%) in IG group, at the end of experiment the mortality was (8%),(25%)and (50%) in CGP, CGT and IG groups ,respectively. In this study result showed that the mortality rate was more in CGT and IG groups than CGP group.

Table (1) mortality rate in control and experimental groups in the periods of experiment .

WEEKS Groups	1	2	3	4
HC	0%	0%	0%	0%
CGT	0%	0%	16%	25%
CGP	0%	0%	0%	8%
IG	0%	0%	33%	50%

Values are means <u>+</u> standard errors .significantly (P<0.05).

HC healthy control, *CGP* challenged –garlic-protected, *CGT* challenge-garlic – treated, *IC* infected control.

Oocyst count in groups of experiment :-

The first oocyst output in feces was observed on 16 days (pre-patent period is 16 days), and oocyst were shed in considerable amounts until the end of the study. The table (2) show there was a significantly (P< 0.05) high oocyst output in IG group throughout the pre-patent period compared to the CGT group and the CGP group. A significant more oocyst out put in the CGT group throughout the entire pre-patent period was observed compared to the CGP group revealing a valuable effect of garlic on reducing the number of oocyst per gram in feces.

WEEKS	1	2	3
Groups			
НС	0%	0%	0%
CGT	12866±1387 Aa	7558.6±598.3 Ab	8530.4±376 Ab
CGP	8620.2±959.3 Ba	4796±925 Bb	6518.8±841.4 Bb
IG	78363±33442 B	106997±33868 A	111459±29573B

Table(2) show mean oocyst count at one per gram of feces

Values are means <u>+</u> standard errors .significantly (P<0.05).

HC healthy control, *CGT* challenge-garlic –treated ,*CGP* challenged –garlic-protected, *IC* infected control.

Histopathological study :

A-grossly

Gross lesions of liver in infected group rabbits with *E. stiedae*, hepatomegaly with multiple variable sized irregular yellowish–white nodules raised above the visceral surface of the liver resembling the enlarged bile ducts and pale yellowish fluid which comparative with liver of control rabbit The livers of the CGT group were less enlarged ,appeared pale in color and were studied with irregular yellowish white nodules. In the CGP group ,the livers were slightly or moderately enlarged with the presence of minute whitish necrotic foci. In CGT and CGP The gallbladders of groups were moderately distended with pale greenish fluid (fig. 1,2,3).

B-Histopathological finding

The livers of control rabbits were within the normal histological limits. The IG group lesions are mainly confined to the bile ducts consisted primarily of extensive hyperplasia of epithelial linings. The bile ducts are markedly enlarged and lined by hyperplasic columnar epithelium thrown into multiple arborizing papillary fronds extending into the ductal lumina, resembling adenomatous hyperplasia with presence of developmental stages of the parasitic ,large numbers of protozoal stages including microgametocytes, macrogametocytes and oocysts (figs 4,5). Other sections showed that the bile ducts were highly dilated with flattened epithelial lining having no or minimum projections to the lumen which are filled with large numbers of thin walled, ovoid oocysts (fig 6). In addition the lumina of bile ducts mucin, inflammatory cells mainly neutrophils and sloughed epithelial cells. The hyperplastic bile ducts were surrounded by thick band of fibrous connective tissue capsule infiltrated with

inflammatory cells mainly mononuclear cells. The affected bile ducts caused pressure atrophy of hepatic parenchyma leaving a thin rim of hepatocytes. The liver parenchyma showed extensive areas of necrosis and hemorrhage with deposition of bile pigment fig (7). Other areas showed severe fatty degeneration of hepatocytes .The hepatic cells contain large vacuoles pushing the pyknotic nuclei aside fig(8). Marked hepatic fibrosis with mononuclear cells infiltration was seen in all examined sections fig(9).Fibrin network infiltrated with neutrophils were also seen. The central veins and sinusoids were greatly dilated and congested containing serum protein and inflammatory cells in their lumina .Furthermore there is fibrinous thrombosis of the blood vessels fig(10). The main histopathological appearance was the extensive hyperplasia of the epithelial lining forming long papillary projections leading to blockage of the lumen. The hyperplastic epithelial cells contain large numbers of different stages of the parasite while oocysts was clearly seen in the lumen (fig 11).

In the CGT group it was lesser lesions, the bile ducts showed marked distraction of the different stages of the life cycle of the parasite with release of their contents in the lumen of the bile duct appeared as fine eosinophilic granules with few numbers of the oocysts (fig 12,13), infiltration of mononuclear cells in the lamina propria of mucosa with presence of few numbers of oocysts in the lumen of gallbladder fig(14). The main microscopical findings of livers tissue sections of GGP group are the mild pathological changes affecting the intra-hepatic bile ducts characterized by mild hyperplasia of the epithelioid lining with few oocysts in their lumina fig(15). Hepatic parenchyma showed formation of multiple granulomatous reaction characterized by epithelial cells aggregation with formation of multinucleated giant cells (fig 16).Furthermore there is increase in mitosis of hepatocytes ,tow nuclei appeared in each hepatic cell (fig 17),The gall bladder section showed nearly normal histological structure as compared with control (fig 18).



Macroscopic fig (1) liver of a rabbit in the challenged-garlic-treated group.





Fig (4) liver of IC showing large numbers of papillary fronds due to extensive hyperplasia of biliary columnar epithelial lining (



Fig (5) liver of IC showing different stages of the protozoal stages including microgamtocytes(), macromametocytes () and oocysts () (H&E 400X).



Fig (6) liver of IC showing highly dilated with flattened epithelium having no or minimum projection to the lumen (→) which are filled with large numbers of oocysts (→) (H&E 100X).



Fig (7) liver of IC showing severe hemorrhage oozing to the necrotic area



Fig (8) liver of IC showing severe fatty degeneration the hypatocytes contain large vacuoles pushing the pyknotic nuclei aside () (H&E 400X).



Fig (9) liver of IC showing marked fibrosis of hepatic parenchyma () with mononuclear cells infiltration () (H&E 100X).



Fig (10) liver of section IC showing large fibrinous thrombus inside the lumen of blood vessel () (H&E 100X).





Fig(12)liver of GGT group rabbit : Bile duct showing marked distruction of different stages of the parasite (macrogametocytes and microgametocytes) appeared as fine eosinophilic granules (\longrightarrow) with few oocysts appeared in the lumen (\rightarrow) (H&E 100X).



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fig (14) Gall bladder of GGT group rabbit showing mononuclear cells infiltration in the lamina propria of mucosa () with presence of few numbers of oocysts in the lumin () (H&E 400X).



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Fig (16) liver of GGP group rabbit showing granuloma consisting of neutrophils and epithelioid cells aggregation () with formation of multinucleated giant cells () (H&E100X).



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Fig (18) Gall bladder of GGP group rabbit showing nearly normal histological

DISCUSSION

The present study revealed that 8% Mortality rate in the CGP group .However , the mortality in the CGT and IC groups was 25% and 50%, respectively .This could be attributed to severe damage of liver induced by hepatic coccidiosis and the severity of infection(13).The number of oocysts affected badly the immune status of rabbits (14), so the reduce in mortality rate in groups it return to natural immunity booster of garlic. Garlic has an abundance of sulphur containing amino acids and other compounds that seem to initiate increased activity in the immune system. For example, macrophages, which kill invading pathogens are stimulated by the presence of garlic. Several studies have shown that garlic stimulates immune function by making macrophage or killer cells more active.

The result of this study showed reduction in oocyst per gram of feces in animals treated and protective with crude garlic at dose (0.5mg\kg B.W orally) and this is in agreement with (15) who showed there is a reduction in oocyst per gram of feces of animals treated and protective with garlic. Other study (16) whom studied the effectiveness of garlic for controlling gastrointestinal parasites in adult female goats which naturally infected with haemonchus contortus and coccidian by measuring fecal egg count, they Founded significant decrease in the numbers of coccidia oocyst was observed in goats which received high dose (10 ml) of garlic extract and reduction in eggs per gram of feces for h. contortus. The inability of garlic to reduce the number of oocyst per gram in the CGT group could be explained by the fact that garlic was used after the shedding of oocyst ,indicating that it may be ineffective for the treatment of hepatic coccidiosis .Although oocyst count in the CGP rabbits was reduced ,shedding of oocyst did not stop until the end of the experiment, which may necessitate the administration of repeated doses of garlic. And it is important to study possible other targets of garlic in light of its reported anti-nematode activities in mice (17). The severity of disease related to dose of oocyst (18). Dilated bile ducts filled with numerous unsporulated oocysts, papillary hyperplasia and atrophy of hepatic parenchyma, fibrosis, mononuclear cells infiltration and different development stages of E. stiedae present in the biliary epithelial or free in the ductal lumen ,all these observations in this study are in agreement with those described by others (19, 20, 21, 22).

These epithelial lesions may be due to liberated toxins or mechanical irritation evoked by protozoan (23). A sever peribilary chronic inflammatory reaction was represented by extensive fibrosis and mononuclear cell infiltration (1,22). *E.stiedae* causes lipid peroxidation resulting from the destruction of the bile duct and subsequently the hepatic parenchyma. The peroxidation of cell membrane causes impairment in its permeability and tiggers a series of reactions that my result in cell death (24,25).

Hepatomegally with irregular white yellowish nodules is a characteristic lesion of E. stiedae infection (19) marked proliferation and distention of bile ducts forming nodules raised above the surface of liver. Histopathological examination of the liver revealed characteristic lesions of hepatic coccidiosis which were less in severity and distribution in the GGP group compared to the CGT group .the lesions consisted of papillary hyperplasia ,infiltration mononuclear cells infiltration in the lamina propria of mucosa with presence of numbers of oocysts in the lumin .These epithelial lesions may be due to liberated toxins or mechanical irritation evoked by the protozoan (23).

In the CGT, highly dilated with flattened epithelium and filled with numbers of oocysts. A peribiliary chronic inflammatory reaction was represented by extensive fibrosis and mononuclear cells infiltration (1, 22). The liver of the CGP group exhibited minimal involvement of the bile duct compared to the CGT group, consequently, the hepatic parenchyma was less affected, this result agreed with (15) who found that oral administration of crude garlic as a protection against *E.stiedae* in rabbit are less effective as a treatment.

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الخلاصة:

Eimeria.stiedae استهدفت هذه التجربة دراسة التأثير الوقائي والعلاجي لنبات الثوم على طفيلي Eimeria.stiedae في الأرانب المصابة تجريبيا بتم استخدام 42 أرنب ذكر , قسمت إلى أربع مجاميع : مجموعة سيطرة ضمت 6 أرانب ومجموعة الوقاية ضمت 12أرنب جرعت ب (0.5 g\gm) من الثوم لمدة خمس أيام قبل الإصابة بجرعة التحدي ومجموعة العلاج ضمت 12أرنب جرعت ب (0.5 g\gm) لمدة خمس أيام بعد يوم 16من الإصابة (طعابة (محموم أي أربب محموعة العلاج ضمت 12أرنب جرعت ب (المحموم أي أربع مجاميع بي أي أربع مجامع الوقاية ضمت 6 أرانب ومجموعة الوقاية ضمت 12أرنب جرعت ب (10.5 g\gm) من الثوم لمدة خمس أيام قبل الإصابة بجرعة التحدي ومجموعة العلاج ضمت 12أرنب جرعت ب (10.5 g\gm) لمدة خمس أيام تبارع الإصابة الإصابة (10.5 g\gm) ألف كيس بيضة ناضج .

تمت دراسة معدل الهلاكات وعدد أكياس البيض المطروحة في البراز والتغييرات المرضية النسيجية في أكباد أرانب عند نهاية التجربة وكانت النتائج كالأتي : لم تسجل أية هلاكات في مجموعة السيطرة ، في حين وجد إن نسبة الهلاكات كانت8٪، 25٪ و 50٪ في مجوعة الوقاية ،مجموعة العلاج و مجموعة الإصابة ، على التوالي سجلت مجموعة الوقاية اقل معدل في أعداد البيوض المطروحة في البراز من مجموعتي العلاج والإصابة. أما بالنسبة للتغيرات النسيجية فقد كانت اقل ضررا في مجموعتي الوقاية والعلاج ومجموعة إلى الترابي الأصابة. أظهرت النتائج إن الثوم تأثيرا ايجابيا على الكوكسيديا الكبدية في الأرانب كعلاج ووقائية, لكان التأثير الوقائي للثوم كان أكثر تأثيرا.

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