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Protective Effects of Palm Pollen Aqueous Extract Against *Eimeria papillata* Induced Intestinal Damage in Mice

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Abstract.- Coccidial infections are known to cause cytotoxicity and oxidative damage within intestinal tissues in their hosts. The current work was designed to study the protective effects of palm pollen aqueous extract (PPE) against *Eimeria papillata*-induced intestinal damage in mice. Coccidiosis was induced in male albino mice via oral inoculation with 1.5×10^3 sporulated *E. papillata* oocysts. Infected mice were administered PPE as daily dose of 150 mg/kg for five successive days. On day 5 *p.i.*, animals were scarified and jejunum samples were prepared for paraffin embedded histological sections and jejunal homogenate was used for determination of oxidative damage biomarkers. The data show that *E. papillata* infection in mice induced marked histological alterations within jejunum tissue in the form of inflammation, vacuolation of the epithelium and destruction of some villi with concurrent decrease in goblet cell number. Upon treatment of infected mice with PPE, the histological injury score within infected jejunum tissue was reduced by 60% and goblet cell number was significantly restored near its control values. Also, the results showed that *E. papillata* induced a state of oxidative damage and disturbance in antioxidant system within jejunum tissue. The infection enhanced lipid peroxidation and protein oxidation processes as evidenced by the significant increase in hydrogen peroxide, malondialdehyde and protein carbonyl contents. The antioxidant enzymes, catalse and glutathione peroxidase were decreased in their activities as a consequence of the infection with concurrent reduction in reduced glutathione level and total antioxidant capacity within infected jejunum tissue. Moreover, mediators of nitric oxide pathway of inflammation (tumor necrosis factor-α, inducible nitric oxide synthase and nitric oxide) were significantly increased after infection. Collectively, treatment of *E. papillata* infected mice with PPE led to reduction in lipid peroxidation and protein oxidation processes, with concureent decrease in the activities of mediators of nitric oxide pathway of inflammation; in addition to the significant enhancement of the jejunal antioxidant system.

Key words: Palm pollen extract, protective activities, murine coccidiosis, cytotoxic damage, oxidative damage**.**

INTRODUCTION

Coccidiosis is an enteric disease caused by protozoan parasites of the genus *Eimeria*. Such parasitic disease produces enteritis of varying severity, and is associated with many structural and functional changes to their hosts leading to nutrition imbalance, disturbance in food digestion and absorbance, and finally decreased weight and low performance (Gres *et al*., 2003; Dkhil and Al-Quraishy, 2012; Metwaly *et al*., 2013). Economically, intestinal coccidiosis is considered to be a threatening factor to the industrial farming of animals such as cattle, rabbits, and poultry (Naidoo *et al*., 2008; Veronesi *et al*., 2013).

Eimeria papillata is used as a model coccidian parasite that infects jejunum of mice causing many pathological changes and metabolic disturbances in the infected animals (Dkhil and Al-Quraishy, 2012).

Numerous broad-spectrum anti-coccidial drugs are currently in use for treatment and prophylactic control of eimeriosis. Currently, plant-based natural products are promising sources for novel anti-Eimeria agents. These products do not necessarily target only the parasites, but may also have organ-protective properties in the Eimeria-infected hosts. Recent studies showed that a number of plant products have protective activities against coccidiosis and its induced intestinal damage (Wunderlich *et al*., 2014; Masood *et al*., 2013).

Date palm *(Phoenix dactylifera* L.; *Palmae)* is widely growing tree in the Arab region. This tree has religious, social and historical importance (Baliga *et al*., 2011). Thousand tones of the palm tree male reproductive cells (pollen grains) are

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produced by millions of palm trees grown in the Arabic region per year (Elberry *et al*., 2011). Date palm pollen has been held for generations to be nutritive and therapeutic agent and its suspension is widely used as a folk remedy for curing many diseases in traditional medicines (Mahran *et al*., 1976; Bahmanpour *et al*., 2006). It has antimicrobial, anti-inflammatory, anti-toxicant, antioxidative and hepatoprotective activities (Baltrusaityte *et al*., 2007; Uzbekova *et al*., 2003; Choi, 2007; Eraslan *et al*., 2008; Campos *et al*., 2003; Leja *et al*., 2007).

The current work aims to study the protective effects of palm pollen extract upon the induced intestinal damage in mice infected with *Eimeria papillata* parasite.

MATERIALS AND METHODS

Preparation of palm pollen extract

Aqueous extract of palm pollens (PPE) was prepared by mixing 0.5 g of palm pollens within 10 ml of sterile saline with vigorous shaking, followed by warming at water bath, 60^{o}C for 90 min. Samples then were stored at 3^{o}C overnight, followed by centrifugation at 2500 rpm for 10 min. The clear supernatant was then separated into clear tubes and stored at 4°C until use.

Animals and experimental design

Eighteen male Swiss albino mice (9-11 weeks) were randomly divided into three groups, six mice per each group. The first group received saline. Second and third groups were orally inoculated with 1.5×10^3 sporulated *E. papillata* oocysts. Third group daily received PPE (150mg/kg) for five successive days (Al-Quraishy *et al*., 2014)). The experiments were approved by state authorities and followed Saudi Arabian rules for animal protection.

On day five post-infection, animals were anaesthetized and jejunum tissue was collected. Pieces of jejunum were used to prepare 10% homogenate solution in phosphate buffer saline. Other pieces were fixed in 10% neutral buffered formalin, and embedded in paraffin blocks.

Histopathological studies

Eosin/hematoxylin stained jejunal tissue sections were scored for inflammatory lesions, tissue destruction, and tissue repair according to previously discussed method (Dommels *et al*., 2007). A rating score between 0 (no change from normal tissue) and 3 (lesions involved most areas and all the layers of the intestinal section) was given for each aspect of inflammatory lesion, tissue destruction, and tissue repair. The sum of inflammatory lesions, tissue destruction, and tissue repair scores was used to represent the total histological injury score (HIS) for each intestinal section.

The number of goblet cells within periodic acid Schiff's stained jejunum sections were counted on at least ten well-orientated villous-crypt units (VCU) for each animal individually. Results were expressed as the mean number of goblet cells per ten VCU.

Biochemical studies

The homogenate of pieces of jejunum in phosphate buffer saline has been used for determination of lipid peroxidation products, malondialdehyde according to Ohkawa *et al*. (1979), hydrogen peroxide level according to Aebi (1984), nitrite/nitrate level according to Berkels *et al*. (2004), protein oxidation products according to Levine *et al*. (1990), tumor necrosis factor alpha and Inducible nitric oxide synthase activity with the help of solid phase sandwich enzyme linked immunosorbent assay (ALPCO Diagnostics, USA), reduced glutathione according to Prins and Loose (1969), catalase activity according to Aebi (1984), Glutathione peroxidase (GPX) according to Palgia and Valentine (1967), and total antioxidant capacity (TAC) according to Koracevic *et al*. (2001).

Statistical analysis

One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS version 17.0). All P values are two tailed, and $P<0.05$ was considered as significant for all statistical analyses in this study.

RESULTS

Light microscopic examination of haematoxylin and eosin-stained sections revealed

that jejunal epithelial cells contain the developmental stages of *E. papillata*. Infected crypts suffered disorganization and moderate inflammatory response as represented by the presence of large parasitophorus vacuoles that fill more than one third of the cytoplasm, cytoplasmic degenrations, nuclear enlargement with less condensed chromatin and degeneration of cell membrane. Moreover, lamina propria appeared with large number of infiltrating inflammatory cells (Fig. 1). These histopathological alterations within infected jejunum tissue were semi-quantified and represented as histological injury score before and after treatment of infected mice with the palm pollen extract.

Histological injury score was maximal in jejunum tissue infected with *E. papillata* parasite. Upon treatment of infected mice with PPE, the observed histopathological changes within infected mice jejunum were diminished and the histological injury score decreased by about 60 % (Figs. 1, 2). In addition, there was a significant reduction of goblet cell number seen at the site of *E. papillata* infection in the jejunum (Figs. 3, 4). Again, PPE treatment was associated with a significant increase in the *E. papillata*-induced decreases in goblet cell number (Figs. 3, 4).

Experimental *E. papillata* infection in mice induced significant elevation in protein carbonyl content and malondialdehyde by 70% and 100 % respectively, with concurrent duplication in hydrogen peroxide levels (Table I). Also, mediators of nitric oxide pathway of inflammation were significantly increased in their levels within jejunum tissue as a consequence of *Eimeria ppillata* infection as evidenced by: the threefold increase in tumor necrosis factor-α, duplication of inducible nitric oxide synthase and the twofold increase in nitrite/nitrate levels (Table II).

Upon treatment of *E. papillata* infected mice with PPE, oxidative damage biomarkers of malondialdehyde, protein carbonyl content and hydrogen peroxide levels were significantly diminished nearly to 66%, 32% and 70% respectively of their values in infected non-treated group (Table I). In addition, mediators of nitric oxide pathway of inflammation were significantly reduced in their levels after treatment of *E. papillata* infected mice with PPE (Table I).

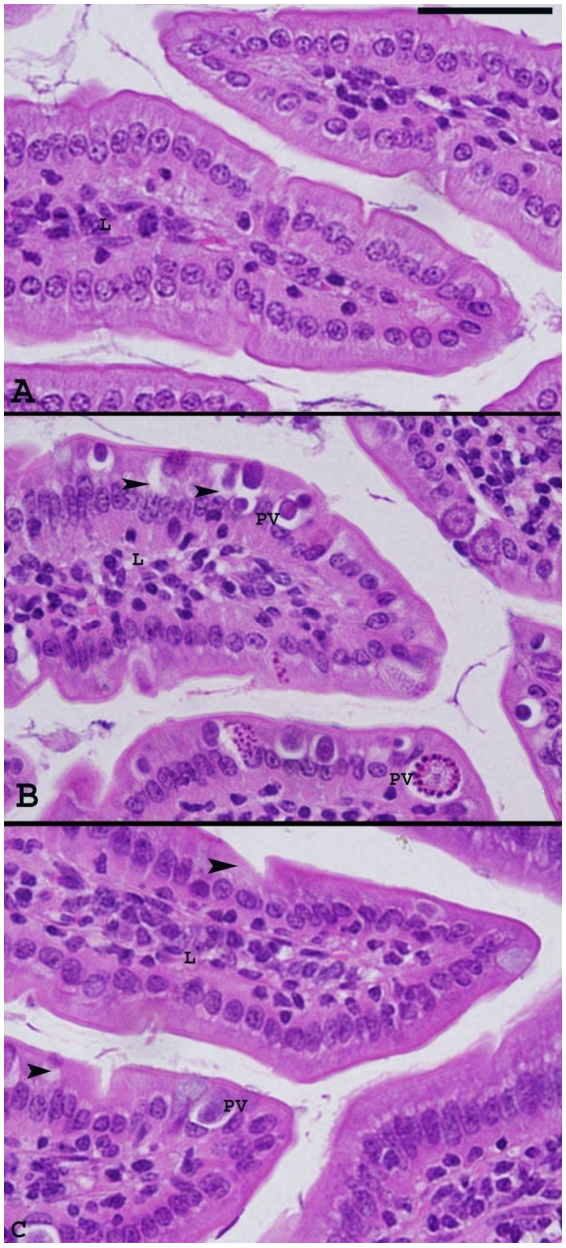

Fig. 1. Effect of palm pollen extract on *E. papillata*-induced jejunum damage on day 5. (A) Control non-infected jejunum with normal architecture of the lamina propria and absorptive epithelium. (B) Infected jejunum with several histopathological changes in lamina propria and absorptive epithelia. (C) Infected treated mouse with less histological alterations. The head arrow refers to cytoplasmic degenerations, PV refers to parasitic vacuoles and L refers to lymphocytic infiltrations. Sections are stained with hematoxylin and eosin. Bar=50 µm.

Table I.- Effect of PPE on oxidative damage biomarkers, nitric oxide pathway and anti-oxidant biomarks in jejunum of mice infected with *E. papillata* on day 5 *p.i.*.

	Non infected	Infected	Infected + Pollen
Oxidation damage biomarkers			
MDA	30.5±5	63.6±8.4[a]	42.1±8 [a,b]
H_2O_2	3.5±0.3	6.3±0.6[a]	4.4±0.3 [a,b]
Carbonyl	0.045±0.004	0.07±0.014[a]	0.037±0.002 [a,b]
Nitric oxide pathway			
MDA	305.1±46.3	577.5±65.7[a]	410.4±76.6[a,b]
H_2O_2	46±1.2	107.8±3.8[a]	47.8±4.9[b]
Carbonyl	109.5±5.7	345.8±20.6[a]	181.2±6.8[a,b]
Anti-oxidant biomarkrs			
Reduced Glutathione	1.3±0.3	0.78±0.15 [a]	1.5±0.24[b]
Catalase	0.5±0.09	0.14±0.02[a]	0.26±0.06 [a,b]
Glutathion perioxidation	86.5±12.1	38.2±5.5[a]	225.7±17.9 [a,b]
Total anti-oxidant	2.1±0.35	1.2±0.16[a]	1.7±0.2 [a,b]

Values are means ± SD; a, significance against non-infected control group at P≤0.05; b, significance against infected (- PPE) group at P≤0.05, (n=6).

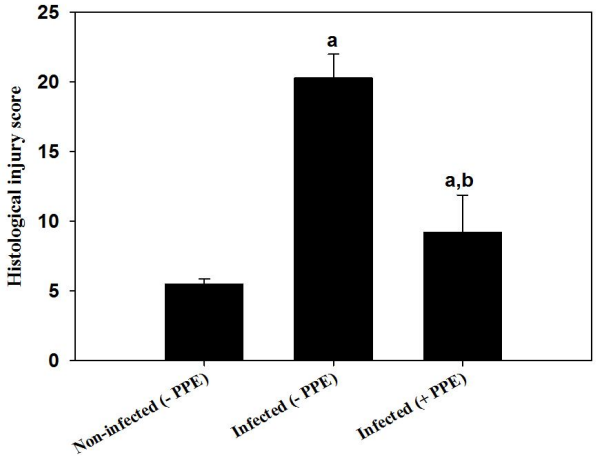

Fig. 2. Effect of palm pollen extract on *E. papillata*-induced histological injury scores in jejunum of mice on day 5 *p.i*. Values are means ± SD. a, significance against non-infected control group at P≤0.05; b, significance against infected group at P≤0.05.

The antioxidant system within infected jejunum tissue was significantly altered by *E. papillata* infection. The activity of glutathione peroxidase and catalase enzymes was decreased

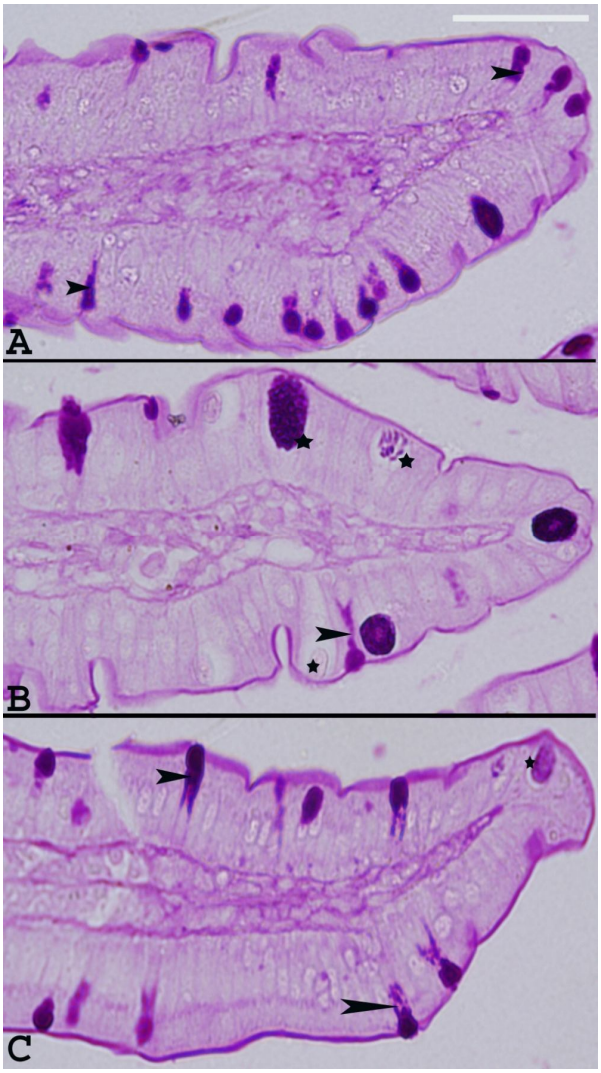

Fig. 3. Effect of palm pollen extract on *E. papillata*-induced decrease in goblet cells on day 5 *p.i*.. (A), Control non-infected mice jejunum showing normal content of goblet cells. (B), *E. papillata* infected mice jejunum showing depletion in their goblet cells. (C), PPE treated and infected mice jejunum showing increases in their goblet cell number. The head arrow refers to goblet cells and the star refers to intracellular eimeria stages. Sections were stained with Periodic acid Schiff's method. Scale bar = 50 µm.

from 86.5 and 0.5 mU/g to 38.2 mU/g and 0.14 U/g, respectively (Table I). In addition, the levels of reduced glutathione and total antioxidant capacity were decreased from 1.3 µg/g and 2.1 mM/g to 0.78 µg/g and 1.2 mM/g, respectively. Again, PPE

showed a great significant enhancement in the antioxidant system within infected jejunum tissue. Total antioxidant capacity of jejunum tissues was raised by 42%. Also, reduced glutathione level was increased to be 1.5 µM/g. In addition, the activity of the antioxidant enzyme GPX was sharply raised by about six folds and that of CAT by 86%.

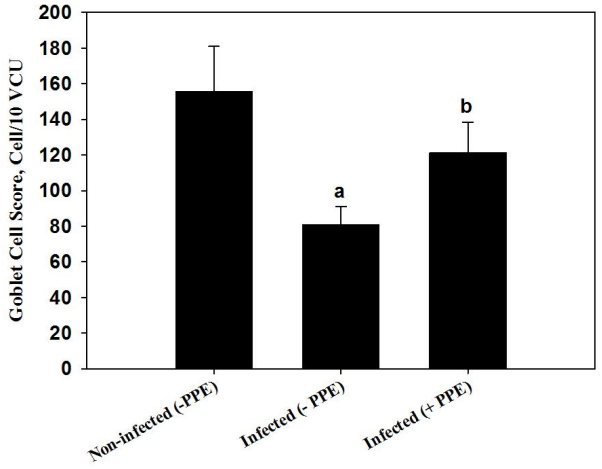

Fig. 4. Changes in jejunal goblet cell score in mice infected with *E. papillata* and after treatment with palm pollen extract. Data were obtained from Periodic acid Schiff's-stained sections as shown in Figure 3. Values are means ± SD. a, significance against non-infected control group at P≤0.05; b, significant against infected group at P≤0.05.

DISCUSSION

Intestinal coccidial infection is considered to be one of the most serious problems that face animal farming industry as it induces severe economic losses of the production of domestic and farm animals (Mehlhorn, 2008). Many anticoccidial drugs are currently in use to fight intestinal coccidiosis (Wunderlich *et al*., 2014; Masood *et al*., 2013), but the raising public distrust of drug treated animal products and the developing of drug resistance problems forced scientists to search for new alternatives of natural origins instead of synthetic drug therapy.

Date palm tree is a widely distributed in sub-tropic and tropical regions and its products are used as folk or traditional medicine. Our previous work proved that aquous extract of date palm fruit has anticoccidial and intestinal, and hepatoprotective activities (Metwaly *et al*., 2012a,b). Also, date palm pollen extract showed significant anticoccidial, anti-apoptotic and growth, and metabolic enhancement activities on mice infected with *E. papillata* parasite (Metwaly *et al*., 2014; Al-Quraishy *et al*., 2014).

In the current study, experimental coccidial infection was induced by oral inoculation of mice with 1.5×10^3 sporulated *E. papillata* oocysts. On day five post infection, the maximum rate of parasite oocysts within faeces of infected mice occur and hence maximum degree of intestinal damage associated with the discharge of fully formed oocysts. So, day 5 *p.i.* was chosen for animal scarifying.

The infection induced disorganization of intestinal villi, large vacuolations and cytoplasmic degenerations, inflammatory cell infiltration and depletion of goblet cell number. These findings are in agreement with previous studies, and may be due to the destruction of villous architecture by eimeria stages and its toxic metabolites, in addition to the elucidated inflammatory response against these stages (Schito *et al*., 1996; Dkhil *et al*., 2013). In addition, the depletion in goblet cell number may be due to the inhibitory action of parasite products on goblet cell producing cells and the down-regulation of genes related to mucin production (Dkhil *et al*., 2013).

Palm pollen extract (PPE) showed marked enhancement in the induced histopathological alterations within jejunum tissue as represented by the significant reduction of histological injury score of infected jejunum tissue and restore of goblet cell number to its control values. Such ameliorative effects on the induced histopathological alterations may be due to the previously proved anticoccidial effects of PPE and the reduced number of intracellular parasitic stages and hence inhibition of parasitic load and damage to infected jejunal tissue (Metwaly *et al*., 2014). In addition, palm pollen was found to strengthen and increase the resistance of different tissues to different harmful pathogens and toxicants due to their high phenolic and flavenoid contents (Campos *et al*., 1997; Lee *et al*., 2009). Also, the restore in goblet cell number is considered to be one of the protective mechanisms of PPE to

the intestine as these cells act as a defensive barrier against chemical or mechanical damage, and to trapping invading pathogens (Deplancke and Gaskins, 2001; MacDonald and Monteleone, 2005). Murine intestinal coccidial infection with *E. papillata* induced a potential oxidative and cytotoxic damage within infected jejunal tissue as revealed by the increased levels of mediators of nitric oxide pathway of inflammation (No, TNF-α and iNOs) and hydrogen peroxide, enhanced lipid peroxidation and protein oxidation processes, and the mutual depletion of the intracellular antioxidant defense system.

The primary mucosal response towards *E. papillata* infection is mediated through both natural killer cell and neutrophil responses through TNF-α and IFN-γ mediated processes (Schito and Barta, 1997; Schito *et al*., 1996). Eimerian infections also, lead to activation of different inflammatory cells as neutrophils, macrophages and natural killer cells which in turn produces reactive oxygen and nitrogen species including nitric oxide, hydrogen peroxides and many other free radicals (Rosen *et al*., 1995). Excessive production of these free radicals leads to destruction of cellular bio-molecules as nucleic acids, lipids, proteins and carbohydrates (Trenam *et al*., 1992; Koinarski *et al*., 2006).

Nitric oxide is also generated during host's cellular immune response to invasion by eimeria parasites and is involved as a complex part during pathogenesis of intestinal coccidiosis (Kheirabadi *et al*., 2011; Moncada *et al*., 1997). The elevated levels of the inflammatory cytokines, TNF-α and IFN-γ leads to increased production of a family of inducible nitric oxide synthase enzymes within epithelial cells, macropahges and enterocytes which in turn increases the production of nitric oxide radical (Moncada *et al*., 1997; Allen, 1997). No reach its maximum level during the time of disorganization of intestinal mucosa, and is associated with oocyst production and shedding (Allen and Teasdale, 1994; Allen, 1997).

Also, intestinal eimeriosis is usually associated with depletion of antioxidants as GSH, GPx, SOD and catalse; which leads to irreversible cell damage due to increased accumulation of reactive oxygen species leading to enhanced lipid peroxidation, protein oxidation, altered intracellular stability, damaged DNA and cellular membranes, and the induction of cell death (Stohs *et al*., 2001; El-Shahat *et al*., 2009; Georgieva *et al*., 2006).

PPE showed strong protective activities against *E. papillata*-induced cytotoxic and oxidative damage in mice jejunum. Lipid peroxidation and protein oxidation processed were significantly reduced with concurrent decrease in hydrogen peroxide levels. Also, nitric oxide pathway of inflammation was significantly decreased after treatment. In addition, the jejunal antioxidant system was strongly activated upon treatment of infected mice with the PPE.

Phytochemical studies on date palm pollen proved the presence of high concentrations of flavenoid compounds such as quercerin and rutin (Mahran *et al*., 1985). In addition, pollen is a rich source of bioactive lipids that on contact with aqueous phase releases oxylipins and phytoprostanes that modulate cells of innate immune response and inhibits antigen-induced T-cell proliferation (Gutermuth *et al*., 2007). Also, quercerin has strong free radical scavengering properties and has a synergetic action offering protection against cytotoxicity and oxidative damage, in addition to blocking action of intracellular peroxides (El-Ridi *et al*., 1952; Kara *et al*., 2005; Morales *et al*., 2006; Chow *et al*., 2005).

Lee *et al*. (2009) showed that pine pollen extract is an efficient inhibitor of lipid peroxidation and protein oxidation processes, in addition to its ability to inhibit production of NO and TNF-α. It exerts its action via down regulation of JNK and MMPs inflammatory pathways. Moreover, its high content of reductone causes termination of free radical chain reactions and hence stabilizing free radicals.

Compared to other investigations carried out using natural products against *E. papillata* infection like pomegranate (Dkhil, 2013), garlic (Al-Quraishy *et al.,* 2011) and date palm (Metwaly *et al.,* 2012), we say that palm pollen could be used as anticoccidial agent.

Collectively, our results prove that date palm pollen aqueous extract exhibits a strong protective activity against *E. papillata*-induced intestinal damage. PPE could effectively reduce jejunal histological injury score, inhibited nitric oxide

pathway of inflammation and enhance the antioxidant system within infected jejunum tissue with mutual decrease of both lipid peroxidation and protein oxidation processes. So, date palm pollens can be used in development of new herbal medicine or mixed with traditional anticoccidial drugs to control coccidiosis and its associated intestinal damage.

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