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STUDY THE EFFECT OF AQUEOUS EXTRACT OF PROPOLIS ON *TRICHOMONAS GALLINAE*, IN VITRO

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ABSTRACT

This study was designed to investigate the in vitro inhibitory activity of aqueous extract of propolis (AEP) on the growth of *Trichomonas gallinae* in comparison to metronidazole. Aqueous extract of propolis inhibited the growth of *T. gallinae* trophozoites and the level of inhibition varied according to the extract concentration and incubation times. The highest reduction of parasitic growth (100%) was observed in cultures treated with 100 and 75 mg/ ml of propolis aqueous extract after 24 h. The same result was detected in cultures treated with 50 mg/ ml of AEP but after 48h. While Growth reduction by 92.5 and 80% was observed in 25 and 12.5 mg/ml propolis-treated cultures respectively after 96 h. Minimal lethal concentration of aqueous extract of propolis was 50mg /ml after 48 hours.In comparison complete inhibition of parasite growth was obtained by metronidazole (50 µg/ml) after 24hours. Light microscope observations revealed changes of the pear-shaped aspect of the cell as a result of presence of large vacuolations in the cytoplasm of the trophozoites. Our results hold the perspective for the utilization of propolis as an antitrichomonal agent after the complementary in vivo studies.

Key words: *Trichomonas gallinae; Propolis; Trophozoites; Growth rate.*

INTRODUCTION

Pigeons are worldwide free living species which found of ancient time, (Sari *et al.*, 2008) and are most widely distributed among hoppy in the world, in some countries pigeons are used for human food as well as ornamental purposes, also feral pigeon used as a bioindicator of chemical pollution (Nam *et al.*, 2004 and klein *et al.*, 2008). On other hands pigeons act as reservoir or carrier and an important source of infection for other avian host, which share the common parasitic fauna (Kumar, 1998).

Trichomonas gallinae causes avian trichomoniasis and affects upper digestive and respiratory tracts of different avian species, especially the crop and esophagus (Levine, 1985). However, other organs, such as liver, bones, sinuses of the skull, lungs, air sacs, peritoneum and pancreas of birds can also be parasitized partly depending on the virulence of the *T. gallinae* strains (Narcisi *et al*., 1991).

The life cycle of *T. gallinae* is direct and the pathogen is transmitted by water, food and orally (Levine, 1985).

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The clinical signs of birds infected by *T. gallinae* vary ranging from asymptomatic to anemia, loss of body weight, anorexia, diarrhea, dehydration and finally death are the probably results of infection with these parasites (Burton and Doblar, 2004).

Avian trichomoniasis is mostly a disease of young birds which may result in a high mortality in young pigeons within 10 days. A high incidence of latent infection (up to 90%) has also been reported (Soulsby, 1986). They can cause granulomatous lesions that occlude the esophageal lumen, leading to the death of birds as a result of severe starvation (Narcisi *et al.*, 1991). Because it causes high mortalities and great losses in birds, especially in columbiformes, *T. gallinae* is considered one of most serious pathogen in pigeons all over the world (Villanúa *et al.*, 2006).

Propolis is a complex resinous material produced by honeybees from plant exudates, beeswax, and bee secretions (Kusumoto *et al.*, 2001) and has protective function on honeycombs, especially against microrganisms (Bosio *et al.*, 2000). Propolis is a sticky dark-colored material that honey bees collect from plants. The chemical composition of propolis is very complex and is dependent upon the plant source.

But exudates of different poplar species are the main sources of propolis in the temperate zone, including Europe, Asia and North America. Samples originating from these regions are characterized by similar chemical composition; the most important constituents appeared to be phenolics: flavonoids, aromatic acids, caffeic acid and its esters, cinnamic acids (Garcia-Viguera 1992 and Marcucci *et al.,* 2001). In addition, propolis extracts shows in vitro anti-microbial activity against protozoa, inhibiting proliferation o *Toxoplasma gondii* and *Trichomonas vaginalis* (Starzyk *et al.,* 1977), *Trypanosoma cruzi* (Higashi and Castro 1994, Prytzyk *et al.,* 2003) and *Giardia duodenalis* (Freitas *et al.,* 2006).

The present study was carried out aiming to evaluate the in vitro effects of different concentration of aqueous extract of propolis (AEP) on the growth and morphological change of *Trichomonas gallinae* trophozoites in comparison to metronidazole.

MATERIALS AND METHODS

A total number of 40 oropharyngeal samples were taken randomly from the mouth and crop of apparently healthy squabs using moistened microbiology swabs, according to Samour and Naldo (2003). Samples were examined by high power for detection of *T. gallinae*.

The positive samples were cultured in vitro by dipping of swabs in test tubes containing glucose-serum broth medium (GSB) at pH 7 (El-Sayed, 2005) and incubated at 37 °C for 7 days. One drop, from the bottom of each tube, was microscopically examined daily for the presence of trichomonads according to Abd El-Motelib and Galal (1993). A hemocytometer was used to count individual flagellates in a wet-mount preparation with a drop of glycerin added to reduce flagellate movement. (Swinnerton *et al.,* 2005).

Preparation of aqueous extract of propolis (AEP):
Ten grams of crude propolis was added to 90mL of distilled water. The mixture was gradually heated, allowed to boil for 3 minutes, and then shaken for 1/2 hour. After that it was left at room temperature for 24 hours. This procedure was repeated daily for 5 successive days. The extraction was filtered, stored in a screw-capped tube, and refrigerated until use (Molan, 1992).

In vitro studies
In order to evaluate the propolis effect on the growth of *T. gallinae*, 10^4 trophozoites were incubated in glucose-serum broth medium containing propolis in different concentrations (12.5, 25, 50, 75 and 100 mg/ml) and examined at 24, 48, 72, 96 h and 120h at 37 °C. In addition, controls were included cultures containing only the parasites; cultures treated with metronidazole (50µg/ml). All drugs were tested in duplicates. Effect of propolis extract and metronidazole on the growth inhibition of *T. gallinae* was evaluated by comparing the number of organisms in treated cultures with the number in non-treated cultures.

Evaluation of the drug efficacy was done by:
Calculation of the percent of inhibition of multiplication according to the equation:

$$\text{Percent inhibition of growth} = \frac{a - b}{a} \times 100$$

Where: a=Mean number of trophozoites in control tubes and b= Mean number of trophozoites in tested tubes (Palmas *et al.,* 1984).

The minimal lethal concentration (MLC) was calculated as the lowest concentration of the tested of propolis aqueous extract at which no organism was observed (Meingasser and Thurner 1979).

A drop of freshly prepared samples was obtained on a clean slide, let to dry in air, fixed in absolute ethyl alcohol for few minutes, and stained with 10% buffered Giemsa stain for 30 minutes (Soulsby, 1986). It was examined under oil immersion lens of microscope for the detection any morphological changes in treated samples.

RESULTS

In the present study *T. gallinae* was observed in 13 out of 40 squabs microscopically in freshly prepared wet mount, thus the overall prevalence was 32.5 %. Table 1.

Table 1: Prevalence of *T. gallinae* in examined squabs.

No. Examined squabs	No. Infected squabs	%
40	13	32.5

In the present study, *Trichomonas gallinae* (in vitro) showed sensitivity to metronidazole, so that with high concentrations of aqueous propolis extract (100 and 75 mg/ml) and the minimum time (24hours) caused 100% death of the parasites. While 50mg/ml AEP caused 100% inhibition of growth of the *T. gallinae* trophozoite after 48 hours (Minimal lethal concentration).

However, lower doses of AEP (25 &12.5 mg /ml) failed to completely inhibit the parasite growth, they only showed growth reduction by 92.5 - 80% respectively after 96 hours' incubation periods (Table 1). Culture treated with of metronidazole (50 µg/ ml) showed a complete reduction the parasite growth after 24 h (Table 2).

Table 2: Mean count and percentage of growth inhibition n of *T. gallinae* per culture after exposure to various concentrations of AEP in comparison to normal control.

Dosage of Treatment	24 h		48 h		72 h		96 h		120 h
	Mean	%	Mean	%	Mean	%	Mean	%	Mean
NTC	90	0	110	0	40	0	40	0	No organism
AEP 12.5mg/ml	30	66.7	20	81.8	17	57.5	8	80	No organism
AEP -25mg/ml	20	77.7	15	86.4	3	92.5	3	92.5	No organism
AEP -50mg/ml	10	88.9	0	100	0	100	0	100	No organism
AEP -75mg/ml	0	100	0	100	0	100	0	100	No organism
AEP100mg/ml	0	100	0	100	0	100	0	100	No organism
MTZ 50 mg/ml	0	100	0	100	0	100	0	100	No organism

NTC = Non Treated Culture Control MTZ = Metronidazole AEP= Aqueous extract of propolis

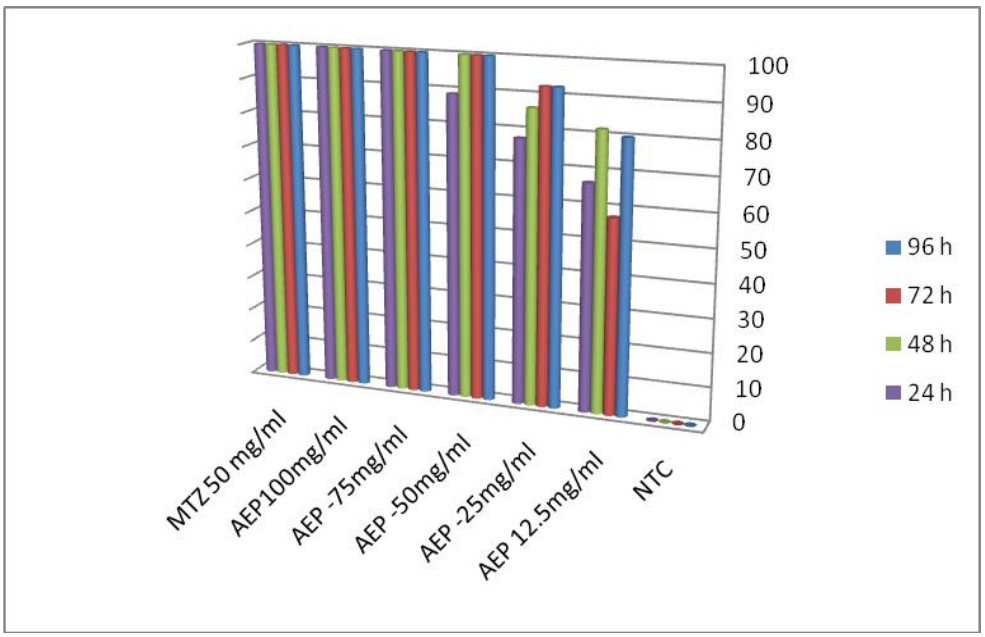
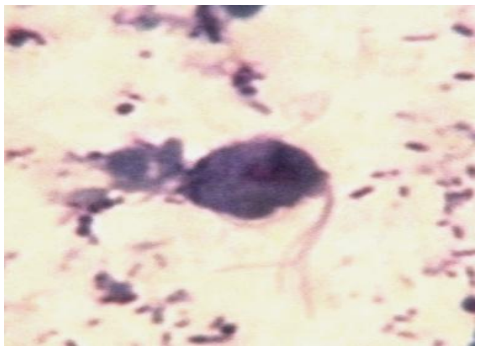


Fig. 1: Percentage of growth inhibition of *T. gallinae* per culture after exposure to various treatments in comparison to normal control.

Morphologically: *T. gallinae* trophozoites which collected from squabs were appear varying in shape (ovoidal or pyriform) provided with four flagellae anteriorly. While at the opposite pole the axostyle proceeds into a short peak (Fig 2 A). They have sluggish movements and their mean size about 7–9x 5-7µm.

Besides propolis effect on growth *T. gallinae*, light microscope observations, revealed presence morphological changes for trophozoites in cultures treated with propolis at 12.5, 25 and 50 mg /ml, at24–96-h incubation. These changes characterized by large vacuolations in cytoplasm masked all morphological details and giving the parasites abnormal shape, in addition to areduction of flagellar beating frequency in the great part of trophozoites (Fig 2 B). While there is no changes were detected in morphological characters of trophozoites in the untreated culture.



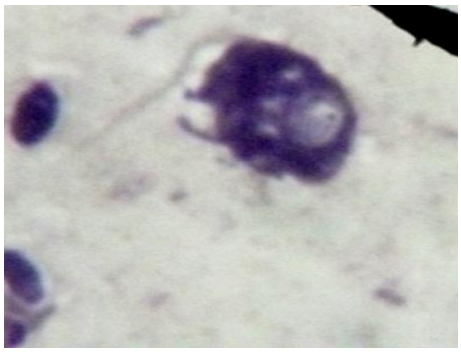


Fig. 2: **A-** Untreated *T. gallinae* trophozoite x1000

B- Treated *T. gallinae* trophozoite (Large vacuolations) x1000

DISCUSSION

Trichomoniasis is a common disease of pigeons, causing high losses among pigeon squabs. It is not only a world-wide, but also occurs all over the seasons of the year (McDougald *et al.,* 2003).

It was not until the 1990 that the first therapeutic failures have been described for treatment of avian trichomonosis and that the existence of resistant *T. gallinae* strains has been reported. However, only a few studies report the existence of resistant strains of *T. gallinae* to the commercial drugs (Lumeij and Zwijnenberg, 1990).

In the present work the infection rate of *T. gallinae* in squabs was 32.5%. these result was relatively similar with Abd El-Motelib and Galal (1993). Whereas, high prevalence of infection with *T. gallinae* was detected previously by Helmy (1995) who stated that the infections with *T. gallinae* was, 68.50% in squabs also Eman (2005) who inferred that the infections with *T. gallinae* was 61.60% in squabs at Al Sharkia province. These differences may be due to different localities.

Propolis is a resinous hive product collected by bees, it is a natural remedy, and may have many antibiotic, antifungal, antiviral and antitumour properties, although reports of allergic reactions are not uncommon, and is relatively non-toxic (Burdock, 1998).

The anti-parasitic properties of ethanolics propolis were studied previously against *Trypanosoma cruzi* (Saloma˜o *et al.,* 2004; Cunha *et al.,* 2004), *Trichomonas vaginalis* (Starzyk *et al.,* 1977) coccidian (Hollands *et al.,* 1984) and *Giardia* (Miyares *et al.,* 1988).

The present work was carried out to evaluate the in vitro activity of aqueous propolis extract (AEP) on growth and morphological characters of *T. gallinae* trophozoites.

The obtained results cleared that the highest concentration of AEP (100 and 75mg/ml) were more than the others and were close to the effect of metronidazole on *T. gallinae* trophozoite where mortality rate was100% at 24h post inoculation. While the culture treated with 50µg ml of AEP give the same effect at 48 h post inoculation (minimal lethal concentration). However, at this time, results for 25 and 12.5 mg /ml were 92.5 % and 80% at 96 h post inoculation, respectively. According to the available literatures, the present work is considered the first study on the effect of the aqueous extract of propolis on the protozoal parasites specially *T. gallinae*.

In fact, propolis containing several constituents that act on the enzymes involved in controlling airway responsiveness, like quercetin that inhibits the lipoxygenase, protein kinase C, cyclic AMP phosphodiesterase and apigenin that inhibit the MAP kinase (Marcucci *et al.,* 2001).

On other hand, Castaldo and Capassob (2002) mentioned that the pharmacologically active molecules in the propolis are flavonoids, phenolic acids and their esters they suggested that it's therapeutic activity depends mainly on the presence of flavonoids, volatile oils and aromatic acids, waxes, resins, balms, pollen grains which are a rich source of essential elements such as magnesium, nickel, calcium, iron and zinc.

Also, Lianet *et al.* (2011) they mentioned that some Cuban propolis extracts exhibited activity against both intracellular and extracellular protozoal parasites (*Plasmodium falciparum, Leishmania spp., Trypanosoma cruzi and Giardia intestinalis*). They return it to four flavonoids which are among the major components of propolis extract (biochanin A, 3, 8-dihydro methoxypterocarpan, formononetin, and liquiritigenin).

The commercial drugs used in the treatment and prevention of *Trichomonas gallinae* are nitroimidazole compounds such as metronidazole‹ dimetridazole etc. (Munaz *et al.*, 1998).

The important point of this study, comparison between the effect of extract concentrations to each other, and groups treated with metronidazole and the control group. The obtained results shown that the effect of highest concentration of AEP (100 and 75mg/ml) and metronidazole, on *Trichomonas gallinae* in vitro are similar.

Abdulkader (2009) mentioned several possible mechanisms for propolis compounds could inhibit *trichomonias* growth: these could be cytotoxic to the parasite or interferes with the function of surface glycoproteins or act on the enzymes involved in controlling airway responsiveness.

Microscopical examination of treated culture with AEP (12.5, 25 and 50 mg /ml) revealed presence certain morphological alterations in the parasite these changes were not detected in untreated cultures. These alterations represented in large vacuolations in cytoplasm and reduction of flagellar beating frequency.

Naksheen *et al.* (2011) showed marked changes in the cell wall of trophozoites of *Entamoeba histolytica*, treated with ethanolic extract of propolis and them adding that this change may lead to alternative for the chemotherapy.

It could be concluded that aqueous extract of propolis (AEP) is an efficient as metronidazole on *T.gallinae* in vitro with the added advantage of being a natural product. Even so, the present results hold the perspective for the finding of new therapeutic alternative to trichomoniasis treatment. Further experimental and clinical investigations are needed to evaluate and standardize the doses of these natural products.

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دراسة تأثير مستخلص البروبوليس المائي على طفيل التريكوموناس جالينى معمليا

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أجريت هذه الدراسة بهدف التعرف على تأثير مادة البروبوليس (صمغ النحل) على نمو طفيل التريكوموناس جالينى. ويعد البروبوليس مادة طبيعية تتكون من مصادر نباتية وتجمع بواسطة النحل كما تعد مصدراعلاجيا طبيعيا. وقد تم دراسة تأثير مستخلص البروبوليس المائي على طفيل التريكوموناس جالينى معمليا كمضاد طبيعي للبروتوزوا وذلك بالمقارنة بعقار ميترونيدازول. وقد اظهرت الدراسة ان للمستخلص المائي للبروبوليس تأثيراً مثبطاً على نمو طفيل التريكوموناس جالينى. حيث ظهرت أعلى نسبة تثبيط (١٠٠%) لنموالطفيل عند التركيزين ١٠٠،٧٥ ملغم /مليلتير من المستخلص خلال ٢٤ ساعة. وبنفس النسبة كان تأثير المستخلص ولكن بعد ٤٨ ساعة وذلك عند تركيز ٥٠ ملغم /مليلتير وهى تعتبر اقل جرعة مثبطة لنمو الطفيل. بينما بلغت فعالية المعاملة عند التركيزين ٢٥ ،١٢.٥ملغم/مليلتير ٨٠ و٩٢.٥% على التوالى بعد مرور ٩٦ساعة. فضلاً عن ذلك فقد لوحظ تغيرات شكلية لطفيل التريكوموناس جالينى نتيجة تكون فجوات كبيرة داخل السيتوبلازم مما ادى الى تضخم حجم الطفيل. وفي المقابل توقف نمو طفيل التريكوموناس جالينى تماما (١٠٠%) عند استخدام لعقار ميترونيدازول (٥٠ ميكروجرام / مليلتير) وذلك بعد ٢٤ ساعة.

وقد يرجع هذا التاثير لمستخلص البروبوليس لاحتوائه على العديد من المركبات الكيميائية حيث انه يحتوي على مواد فلافونية وبيتولين وبيتيولين ايزوفانيلين ومواد صمغية واحماض عطرية غير مشبعة مثل حمض الكافئين وحمض الفيرولين وزيوت طيارة بالاضافة إلى بعض حبوب اللقاح. لذلك توصي الدراسة بعمل دراسات مستقبلية للوصول إلي الجرعة الكافية من المستخلص المائى لصمغ النحل حتى يمكن استخدامه في العلاج للتخلص من الطفيل دون إحداث أي تأثير ضار موضعي أو عام.