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Anti-Cryptosporidial Activity of Egyptian Propolis and Garlic Oil Against *Cryptosporidium* Oocysts *In vitro*

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Abstract: The present study aimed to evaluate the efficacy of ethanolic extract of propolis (EEP), water extract of propolis (WEP) and garlic oil (GO) against *Cryptosporidium* spp. *in vitro*. *Cryptosporidium* oocysts were isolated from faecal samples of naturally infected neonatal calves and propagated in goat kids then purified and stored at 4°C. For infectivity test, seventy female BALB/c mice, weighing 18-20g, were randomly assigned into 10 equal groups and numbered individually. These mice were injected subcutaneously with 0.5ml of 4mg/ml dexamethasone every 48h for 8 days. On the day of the last injection, the groups from II to X were inoculated orally with 10^6*Cryptosporidium* oocysts pre-suspended in different concentrations (6.25, 25 and 50 mg/ml) of EEP, WEP and GO. Untreated infected control group I was inoculated with 10^6*Cryptosporidium* oocysts. The oocysts' shedding was recorded daily from the 1st to the 9th day post infection. Faecal smears were stained by modified Ziehl-Neelsen and oocysts counts per 50 fields at magnification ×100 were determined. The overall oocysts count was obtained by collecting the oocysts scores of all mice at all periods. The result revealed that all extracts with different levels reduced faecal oocysts shedding but marked reduction (P<0.001) was observed in mice infected with oocysts pre-treated with WEP at concentrations 6.25 and 50 mg/ml compared to untreated control group. Also, at a concentration of 25 mg/ml, faecal oocysts count was significantly decreased (P<0.001) in mice infected with oocysts pre-treated with WEP than EEP and garlic oil. In conclusion, Egyptian WEP has a powerful anti-cryptosporidial activity than EEP and GO.

Key words: Propolis · Garlic Oil · Infectivity Test · *Cryptosporidium* · Treatment

INTRODUCTION

Cryptosporidium was first described by Tyzzer [1] which was detected in gastric gland of laboratory mice (*C. muris*). Moreover, Tyzzer [2] identified a similar small intestinal parasite of mice (*C. parvum*). In early 1970's, *Cryptosporidium* was reported as a cause associated with chronic diarrhoea in calf [3] then reported in humans suffered from diarrhoea [4]. From that time, the research on cryptosporidiosis has been done. Many outbreaks of human cryptosporidiosis have been attributed to contaminated drinking water, recreational water and food [5]. In immunodeficient individuals, such as people with AIDS, cryptosporidiosis may lead to chronic diarrhea and emerged as life-threating disease in this subpopulation [6].

Propolis (Bee glue) is a resinous hive product, produced by honey bees from various plant sources, mixed with wax and used in construction and adaptation of their nests. Propolis has many activities as antiviral [7], antibacterial [8], antifungal [9], anti-parasitic [10], anti-oxidant [11], anti-inflammatory [12], antitumor [13],

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immunomedulatory [14] and nephroprotective effects [15]. Although propolis has been extensively used in the treatment of a variety of diseases, there is no published data has been recorded for its anti-cryptosporidial activity.

Garlic *(Allium sativum* L.) has been shown to have multiple beneficial effects such as antibacterial [16], antihelmintic [17], anti-coccidiosis [18] and anti-cryptosporidiosis [19] activities. Garlic contains many active chemical constituents such as; amino acids as arginine, organosulphate compounds as aliin and allicin, enzymes as allinase, minerals and vitamins A, B1 and C. The physiological activity of dietary garlic is attributed to allicin (Diallyl thiosulphinate), which is one of the organosulphate compounds found in the bulb [20]. The major components for the essential garlic oils were sulfur compounds with diallyl trisulfide, diallyl disulfide and methyl allyl trisulfide [21].

Despite of several chemotherapeutic drugs have been tested, no consistently effective therapy for cryptosporidiosis exists. Therefore, the present study aimed to investigate the infectivity of *Cryptosporidium* oocysts pre-treated with different concentrations of ethanolic (EEP) and water (WEP) extracts of propolis and garlic oil (GO) in immunosuppressed mice (Infectivity test).

MATERIALS AND METHODS

This study was carried out according to the guidelines for animal experimentation and approved by the Institutional Animal Care and Use Committee, National Research Centre Animal Care Unit, Dokki, Giza, Egypt.

Isolation of *Cryptosporidium* spp. From Calves Faecal Samples: Fresh faecal samples were taken from 70 neonatal Holstein-Friesian calves, aged from 3 to 15 days, at governmental farm at Abu-Rawash, Giza Governorate, Egypt. All calves were removed from their dams and housed in a heifer barn after birth. Faeces were obtained by rectal sampling and placed at 4°C for transport to the laboratory, where they were processed. Faecal smears were prepared and stained with a modified Ziehl-Neelsen technique [22]. The stained slides were examined by bright-field microscopy using ×100 oil-immersion objective lens. Specimens containing oocysts were categorized into three groups as follows: (+) < 5 oocysts, (++) 5 to 10 oocysts, (+++) > 10 oocysts per High power field (HPF).

Purification of Oocysts from Faecal Samples: The pelleted faecal solids were resuspended in appropriate volume of 2.5% potassium dichromate (w/v, $K_2Cr_2O_7$) and stored at 4°C. The $K_2Cr_2O_7$-preserved stool was passed through stainless steel sieve to remove large debris. The sieved stool was applied to discontinuous sucrose gradients according to the method of Arrowood and Sterling [23].

Goat Kids for Maintenance and Amplification of *Cryptosporidium* Oocysts: Two female Baldi goat kids (2 days old) separated from their dams at birth were used. In the first 24h, they were fed goat colostrum from their dams. Kids were fed twice daily with milk replacement *ad libitum*. Each kid was inoculated orally at the 4th day of age with 10^6 oocysts in 10 ml of water just before the kids drank milk. The oral dose was determined from the mean of four hemocytometer counts of oocysts stock suspension. In order to monitor the development of the parasite, freshly faecal samples were collected from the 1st dpi until no oocysts were detected in faeces. A faecal sample was spread on microscopic slide, stained by a modified Ziehl-Neelsen technique and oocysts counts per 50 fields under oil immersion lens at ×100magnifications were scored. Following the onset of oocysts shedding, faeces were collected daily in screw-top containers, mixed with an equal volume of 2.5% potassium dichromate and stored at 4°C. Faeces were sieved sequentially through stainless steel screens. Oocysts were purified by discontinuous sucrose gradients, counted using a hemocytometer under bright field microscope (Model CX41, Olympus, Japan) and stored in 2.5% potassium dichromate at 4°C.

Dexamethasone Phosphate (DEX_P): The DEX_P (Forticortin, Merck, Germany), a synthetic glucocorticoid, was used to induce chemical immunosuppression in BALB/c mice to establish *Cryptosporidium* infection.

Propolis: Propolis sample was collected from beehives located in Dakahlia Governorate, Egypt. The sample was kept in the dark and stored at-20°C up to its processing.

Ethanolic (EEP) and water (WEP) extracts of propolis were prepared with some modification as described by Abd El-Aziz *et al.* [11].

Garlic (*Allium sativum* L.): Garlic cloves purchased from a local market were used. The peeled garlic cloves (500g) were blended with 600 ml distilled water for 5 min in a commercial blender. The mixture was heated for 2 h at

boiling temperature in the water distillation apparatus until a brilliant yellow-greenish crystal-clear liquid was obtained. The oily extract was recovered, dried over anhydrous sodium sulfate (Na_2SO_4) and stored in sealed vials at-20°C until used.

***In vitro* Activities of Propolis Extracts and Garlic Oil Against *Cryptosporidium* Oocysts (Infectivity Test):** Immunosuppressed female BALB/c mice -as animal model- were used to evaluate the infectivity of *Cryptosporidium* oocysts by quantification of excreted oocysts [24].

Incubation of Oocysts with EEP, WEP and GO: Stock solutions of EEP and GO (100 mg/ml) were emulsified by adding 1% Tween-80 and then suspended in Hank's buffered salt solution (HBSS). Stock solution of WEP (100 mg/ml) was prepared directly without adding Tween-80. In each 1.5 ml micro centrifuge tube, 100µl of 10×10^6*Cryptosporidium* oocysts suspension were added to 900µl of different concentrations of EEP,WEP and GO (6.25, 25 and 50mg/ml). Samples were vortexed and then incubated for 24h at 4°C. The control solution consisted of 100µl of *Cryptosporidium* oocysts suspension mixed with 900µl HBSS and 1%Tween-80. Following incubation, each sample was washed in HBSS and centrifuged (12,000 rpm for 30 seconds at 4°C). The supernatant was removed and the pellets were re-suspended in HBSS.

Animal Model Infection: Seventy female BALB/c mice, weighing 18-20g, were assigned into 10 groups (7mice/group), numbered individually and maintained separately in plastic cages with wire mesh tops and wood shavings for bedding in well ventilated animal room under standardized conditions (20±3°C; relative humidity 50±5% and 12 hours light/dark cycle). All nutrients including water were supplied *ad libitum* to meet the requirements of the NRC [25]. The mice were acclimatized for 15 days before the start of the experiment. These mice were injected subcutaneously with 0.5 ml of 4 mg/ml of DEX_P every 48h for 8 days. On the day of the last injection, the mice were inoculated orally by stomach tube with 10^6 *Cryptosporidium* oocysts that had been stored in different concentrations of EEP, WEP and GO.

Infectivity Assay in Immunosuppressed Mice: This experiment was performed to illustrate the infectivity of *Cryptosporidium* oocysts pre-suspended in different concentrations (6.25, 25 and 50 mg/ml) of EEP, WEP and GO in mice. The oocysts shedding were recorded from the 1st to 9th dpi. To compare the efficacy of different concentrations of extracts, the expression of overall oocysts count was used. This parameter was obtained by collecting the oocysts scores of all mice at all periods.

Quantification of Oocysts Excretion: Freshly excreted pellets were collected from individually labeled mice from the 1st to 9th dpi. A faecal pellet was homogenized in a drop of water and was spread on microscopic slide. Faecal smears were stained by modified Ziehl-Neelsen technique and oocysts counts per 50 fields of a faecal smear were scored under oil immersion at ×100 magnification.

Statistical Analysis: Differences in the oocysts shedding between untreated infected control and treated groups were analyzed by Wilcoxon Mann-Whitney U test. Statistical significance was tested at α= 0.05 and 0.1 level [26] using SPSS version 16.0 computer program.

RESULTS

Isolation of *Cryptosporidium spp.* from Calves Faecal Samples: A total of 70 faecal samples collected from calves suffering from diarrhea were examined for *Cryptosporidium spp.* oocysts; 46 (65.7%) of them were found to be positive. On the basis of oocysts counts per each HPF, 30 (65.2%) samples were assessed as mildly infected, 11 (23.9%) as moderately infected and 5 (10.9%) as heavily infected.

The *in vitro* Activities of EEP, WEP and GO Against *Cryptosporidium* Oocysts (Infectivity Test): Although the different concentrations of all extracts significantly reduced faecal shedding of *Cryptosporidium* oocysts in comparison with the untreated infected control group, they failed to prevent the establishment of infection. Moreover, mice infected with oocysts pre-treated with WEP at concentrations 6.25 and 50 mg/ml showed very highly significant decrease (P<0.001) in faecal oocysts shedding compared to the untreated infected control group. No significant difference in the median of overall oocysts counts was observed between EEP, WEP and GO at each concentration. Only at concentration of 25 mg/ml, the median of overall oocysts counts was highly significantly decreased (P < 0.01) in mice infected with oocysts pre-treated with WEP than that of EEP at the same concentration. The present experiment showed that the efficacy of EEP against *Cryptosporidium* oocysts *in vitro* was approximately equal to that in GO (Table 1).

Table 1: Median of overall oocysts counts in mice infected with *Cryptosporidium spp*. oocysts pre-suspended overnight in different concentrations of ethanol extract of propolis (EEP), water extract of propolis (WEP) and garlic oil (GO)

		Oocyst counts	
Groups	Concentration (mg/ml)	Median	Range
Untreated control	-	92	0-138
Ethanol extract of propolis	6.25	9**	2-99
	25	18**	0-87
	50	12.5**	0-83
Water extract of propolis	6.25	6.5***	0-38
	25	6**, a	2-59
	50	8***	0-38
Garlic oil	6.25	12**	0-92
	25	12**	0-63
	50	14**	0-61

** = Significant at P <0.01 and *** = Significant at P <0.001 compared to untreated control group.

a = Significant at P <0.01 compared to ethanol extract of propolis group at the same level of concentration (25mg/ml)

DISCUSSION

This study aimed to evaluate the efficacy of propolis (EEP and WEP) and garlic oil (GO) against *Cryptosporidium* spp. *in vitro*. The infectivity of *Cryptosporidium* oocysts pre-treated with different concentrations of EEP, WEP and GO was evaluated in immunosuppressed mice.

The chemical composition of propolis is highly variable and depends upon time, vegetation and area of collection [27]. Sixty-five compounds such as flavonoids, aromatic acids and their esters, aliphatic acids and sugar were identified in Egyptian propolis collected from Dakahlia Governorate by GC/MS [28]. Moreover, The investigators reported that Egyptian propolis contained two new caffeate esters, tetradecenylcaffeate (Isomer) and tetradecanylcaffeate and triterpenoids including lupeol and alpha-amyrin. They stated that Dakahlia propolis sample was a typical popular propolis. The anti-cryptosporidial activities of EEP and WEP may be due to their highly antioxidant capacity [1] and rich contents of phenolic compounds such as organic acids [28] that were confirmed by the result of Al-Mathal and Alsalem [29]. Furthermore, propolis can stimulate the immune system of rabbits [14] and rats [7, 30] by the way of increasing the antibody titers which is positively correlated with the reduction of oocysts shedding [31].

The present experiment showed that GO at different doses was moderately effective against *Cryptosporidium* oocysts *in vitro* compared to that of WEP. This effect may be due to sulfur compounds with diallyl trisulfide, diallyl disulfide and methyl allyl trisulfide contained in GO [21]. Similarly, garlic extract caused mild reduction of oocysts output in chickens infected with *C. baileyi* [32] and in mice infected with *C. parvum* [33, 34]. On the other hand, allicin-based product at doses 40 and 80 mg/calve dissolved in milk replacer did not significantly reduce faecal oocysts score in neonatal calves infected with *C. Parvum* [35].

CONCLUSION

Egyptian water extract of propolis has a powerful anti-cryptosporidial activity than the ethanolic extract of propolis and garlic oil. The infectivity test revealed that at the concentration of 25 mg/ml of both propolis extracts, the median of overall oocysts count was significantly decreased in mice infected with oocysts pre-treated with WEP than that of EEP at the same concentration.

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