

Synergistic Anthelmintic Effect of *Citrus aurantifolia* Swingle Seeds and Mebendazole in Egyptian Dogs Infected with *Ancylostoma caninum* and *Toxocara canis*: Trial to Solve Drug Resistance Problem

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Abstract: Synergistic Combination has been described in human and veterinary medicine as to solve the anthelmintic resistance and overcome it. The objective of the present study is to evaluate the anthelmintic efficacy of combination of the herbal plants; *Citrus aurantifolia*; Egyptian lemon seeds extract and mebendazole against mixed infection with *Ancylostoma caninum* and *Toxocara canis*. A total number of 25 local Egyptian dogs naturally infected with *A. caninum* and *T. canis* were used in the experiment. The animals were randomly divided into 5 groups, five dogs each. Group A; animals were non infected non treated control one, group B; animals were naturally infected with both *A. caninum* and *T. canis* and not treated. The infected C, D and E groups; animals were treated in serials with mebendazole alone, *Citrus aurantifolia* seeds extract only and the latter were treated with combined therapy mebendazole and *Citrus aurantifolia* seeds extract. The treatment extended 2 weeks; 3 times per week; day after day. The results showed that treatment with the different anthelmintic gives a variable efficacy, but the high efficacy was produced by the combination treatment of mebendazole and *Citrus aurantifolia* seeds extract which produce significant decrease in fecal egg count (FEC) and the percent of FEC reduction of *A. caninum* and *T. canis* reached to 100% and 98.2%, respectively. The hematological assay during different period of treatment and after recovery were done and the infected treated dogs with combined therapy of *C. aurantifolia* extract with mebendazoles, showed blood parameters (Hb, RBCs, PCV, WBCs, total protein and albumin concentration) reaching to normal values of non-infected untreated control group.

Keywords: Dogs, Anthelmintic resistance, Synergistic effect, *Citrus aurantifolia*, Mebendazole, *Ancylostoma caninum*, *Toxocara canis*.

1. INTRODUCTION

Hook worms, *Ancylostoma caninum* and ascarids round worm, *Toxocara canis* were the major and harmful gastrointestinal parasites in dogs producing severe enteritis, dehydration, un-thriftiness, dullness, in-appetence, diarrhea, emaciation, loss of weight and finally death if didn't treated [1], [2], besides its zoonotic significance and public health risks [3]. The anthelmintic resistance in parasitic infection of pet animals is common and developed due to different factors as due to gene or genes being present in the parasite population [4], infrequent usage of the same group of anthelmintic [5], improper doses of drugs [6] and antibody-mediated response in infected host [7].

Different strategies were adapted for preventing anthelmintic resistance and one of them is the herbal treatment and the synergistic combination of two drugs, particularly if one of them is herbal in origin. [8] compared the efficacy of percentage of *Nigella sativa* (Kalongi), *Saussurea lappa* (Qust-e-Shireen) and Pyrantel pamoate against *Ancylostoma* spp. infected dogs which it was 89.6, 79.9 and 100 %, respectively, they stated that no side effects were produced by any of the drugs of plant origin. The aqueous crude extracts of *Euphorbia hirta* Linn were showed broad spectrum anthelmintic activity against *Ancylostoma caninum*, *Toxocara canis*, *Echinococcus granulosus* and *Dipylidium caninum* in dogs than Mebendazole [9]. Also, the efficacy of aqueous crude extracts of *Vernonia amygdalina* and Mebendazole for the treatment of *A. caninum*, *T. canis* infected dogs was assessed by

[10] and the results modified that aqueous extracts of *V. amygdalina* produced significance improvement of blood parameter and remarkable and significant reduction in fecal eggs counts and identified helminthes and they concluded that the plant extracts were broad spectrum in action. Drug leaf infusion (DLI) of Paico (*Chenopodium ambrosioides*) extracts was used by [11] to determine its efficacy for control of nematode infection in canines and its anthelmintic effect reached 99.01 %. The synergistic effect of chemical drugs was done by different authors as, the effect of pyrantel and the febantel metabolite febendazole on adult *T. canis* were done by [12] and [13], they concluded that combination of pyrantel and febendazole produce lethal damage faster and lower doses as compared to incubation in pyrantel or febendazole alone in high doses.

The *Citrus aurantifolia*, swingle (*Rutaceae*), showed different anthelmintic activity against human *Ascaris lumbricoides*, anti bilharzial activity and against intestinal flagellates of poultry as *Tetratrichomonas gallinarum* and *Histomonas meleagridis* [14], [15] and [16]. In vitro and in vivo anthelmintic activity of ethanolic extract of citrus peels against *Ascaridia galli* infection in chicken was investigated and concluded that citrus peels extracts have potentials anthelmintic properties against *A. galli* [17]. In vitro studies, petroleum ether and acetone extracts of *Citrus aurantifolia* swingle seeds were tested against *Toxocara vitulorum* (Ascaris), and *Fasciola gicantica* (larvae and eggs), *Trihconstronylus colubriformis* (adult worms , larvae and eggs), larvae of *Trichinella spiralis* , in addition to *Eimeria* oocyst. The different two extracts of *Citrus aurantifolia* showed variable results with 100% mortality effect against all parasites except *T. colubriformis* [18].The objective of the current study is to evaluate the anthelmintic efficacy of the combination of herbal plant, *Citrus aurantifolia* swingle seeds ethanolic crude extract and mebendazol against *Ancylostoma caninum* and *Toxocara canis* parasitic infection in dogs as a trial to solve the problem of anthelmintic drug resistance in dogs infested with internal parasites.

2. MATERIALS AND METHODS

2.1. Citrus Aurantifolia Swingle Seeds Ethanolic Extraction

Citrus aurantifolia (Egyptian lemon) seeds (200 g) were purchased from local market; the seeds were identified and authenticated at the herbarium by Prof. Dr. Ahmed El Ezaby department of Horticulture, faculty of Agriculture, Cairo University [18].The seeds were dried at room temperature (26 ± 2 °C) for one week [19].The dried seed were ground into powdered using electric blender and soaked in absolute ethanol for 48 hours with continuous stirring. The mixture was then filtered through #1 Whatman filter paper. Ethanol was removed under reduced pressure at 40 °C by a rotary evaporator [17]. Five grams of ethanolic extract was dissolved in 50 ml distilled water to get tock solution of 0.1 g / ml. the stock solution was stored in the refrigerator.

2.2. Anthelmintics

Mebendazole (MBZ), *Citrus aurantifolia* seeds herbal extract and combination therapy of both mebendazole and *Citrus aurantifolia* seeds extract were used in the experiment. The mentioned compounds were administrated orally in a daily dosage 50mg/kg bodyweight and 40mg/kg bodyweight for both mebendazole and *Citrus aurantifolia* seeds extract, respectively. The dose of the herbal ethanolic extract was determined according to [18] based on the LC50 in vitro study against *Trihconstronylus colubriformis* adult nematode.

2.3. Animals

A total number of 25 local dogs ; puppies (5 uninfected and 20 infected with *A. caninum* and *T. canis*) with average weight 5-8 kg were procured from local market, near Giza. The experimental dogs were selected from 45 dogs some of them were be naturally uninfected and others were be naturally infected with *A. caninum* and *T. canis* nematodes. The dogs were maintained in clean, separate cages in hygienic and fumigated kennels in animal care center at the faculty of Veterinary Medicine, Cairo University, Egypt where they supplied with balanced food and adequate clean water.

2.4. Experimental Design

Each animal from the twenty five dogs was examined coprologically 3 times during a week before the experiment to be sure of the persistence of the non-infection in the five uninfected dogs and persistence of infection in the other twenty *A. caninum* and *T. canis* infected dogs. The dogs were randomly divided into 5 groups (A, B, C, D & E), five dogs of each. Group A animals were that previously sure as non-infected with any parasites and not subjected to any treatment, so they were

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considered as negative control group. The group B animals were naturally infected with both *A. caninum* and *T. canis* and not treated (positive control group). The infected C, D and E group animals were treated in serials with mebendazole alone, *Citrus aurantifolia* seeds extract only and with combined therapy of both mebendazole and *Citrus aurantifolia* seeds extract respectively, where the treatment course extended for 2 weeks; as oral administration 3 times per week; day after day. At the end of treatment course, dogs received 2 doses of lactulose laxative, once/ week during treatment.

All the experimental dogs were subjected to the parasitological and hematological examination before the start of the experiment and at one week after stoppage of the treatment.

2.5. Parasitological Examination

Fecal egg counts (FEC) was calculated as the number of egg per gram of feces using modified McMaster egg counting technique [20]. Decrease in the percentage of the FEC describes the percent of reduced FEC between non treated control and treated groups, by the following formula:

$$\% \text{FEC reduction} = \frac{\text{FEC infected untreated (+ve control)} - \text{FEC infected treated}}{\text{FEC infected untreated (+ve control)}} \times 100$$

2.6. Hematological Assay

Blood samples were collected from cephalic vein of each animal mixed with EDTA. Estimation of hemoglobin (Hb) concentration was performed using Sahli's methods [21]. Total Erythrocytic and Leucocytic counts was done manually using Neubauer haemocytometer counting chamber; Packed cell volume (PCV) was determined by the conventional methods; the microhematocrit, the differential leucocytic and platelets count also were detected according the methods described by [22]. The blood sera separated to determine the total protein (TP) and albumin concentration (ALB) using spectrophotometer method [23].

2.7. Statistical Analysis

Data were statistically analyzed using the M-STAT and STATISTICA (6.0) computer programs. The Average and Standard Deviation, T-test, ANOVA test were determined to compare group results among the different parameters. Results were considered significant at $P < 0.05$.

2.8. Ethical Approval

The experiments were progress according to the guide for the Care and animal rights and the work is approved ethically by the Medical Research Ethics Committee-National Research Centre, Egypt-under registration number 1-2 /0-2-1.2012.

3. RESULTS AND DISCUSSION

3.1. Results

The in-vivo current study used animals in group B (+ve control) were found to be heavily infested with *A. caninum*, 1620 and *T. canis* 1800 egg per gram. Also, there is a gradual decrease in FEC from the end of the first week of treatment to the end of one week post treatment with percentage of reduction reached to 70.80, 74.10, 87.10, 91.08, 100.00 and 98.20 for both *A. caninum* and *T. canis* nematodes corresponding to the three infected anthelmintic treated groups C, D and E, respectively. The usage of combined therapy of *C. aurantifolia* extract with mebendazoles, showed significant effect of treatment ($P < 0.05$) and significant reduction ($P < 0.05$) in fecal egg count with high efficacy against infestation with mentioned nematode parasites (Table 1).

The parasitological examination of fecal samples obtained from the dogs used in the experiment from the different groups revealed normal *Ancylostoma caninum* and *Toxocara canis* eggs mainly from infected non treated group, B (+ve control) animals (Fig.1), and the abnormal degenerated *A. caninum* and *T. canis* eggs mainly obtained from infected treated groups C, D and E (Fig.2).

Concerning the hematological parameters of *Ancylostoma caninum* and *Toxocara canis* infected and infected treated dogs; the hemo-gram of infected treated dogs with combined therapy of *C. aurantifolia* extract with mebendazoles, showed significant ($P < 0.05$) in Hb concentration (12.1 ± 0.3), total RBC count (5 ± 0.4), PCV (41.7 ± 0.1), and reaching to normal values of non-infected untreated control group. Also lymphocytosis produced in infected untreated dogs (36 ± 0.2) was

significant decrease to normal values in dogs treated with *C. aurantifolia* alone (30±0.02) and with combined therapy of *C. aurantifolia* extract with mebendazoles (30±0.01). On the other hand, the blood parameters showed no significant changes in blood protein and in albumin concentration in any of the infected treated animal groups (Table 2).

Table1. Fecal egg counts (FEC) of *A. caninum* and *T. canis* infected dogs and its percentage of reduction after treatment

	Non infected untreated (Group A)	Infected untreated (Group B)		Infected Mebendazole treated (Group C)		Infected <i>C. aurantifolia</i> extract treated (Group D)		Infected combined treated Mebendazole & <i>C. aurantifolia</i> (Group E)	
		A. <i>caninum</i>	<i>T. canis</i>	A. <i>caninum</i>	<i>T. canis</i>	A. <i>caninum</i>	<i>T. canis</i>	A. <i>caninum</i>	<i>T. canis</i>
Start of the experiment	0	1620 ±19.3	1800 ±13.5	1680 ±20.1	1200 ±20.6	1870 ±20.5	1010 ±15.3	1050 ±15.8	990 ±10.6
End of first week of treatment	0	1800 ± 11.5	2000 ±15.3	750 ±13.6	1600 ±15.3	995 ±14.6	970 ±10.2	640 ±15.5	320 ±10.3
End of second week of treatment	0	1650 ±20.1	1660 ± 17.2	650 ±20.1	405 ±10.5	500 ±10.3	565 ±10.5	60 ±20.7	100 ±10.5
one week post treatment	0	1640 ± 19.3	1680 ±20.4	510 ±9.5	310 ±9.1	240 ±10.5	290 ±11.2	--	45 ±9.6
% of reduction	--	--	--	70.80	74.10	87.10	91.08	100	98.20

Table2. Blood profile of *A. caninum* and *T. canis* non-infected, infected untreated and infected anthelmintics treated dogs

	Non infected untreated (Group A) (-ve control)	Infected Untreated (Group B) (+ve control)	Infected Mebendazole treated (Group C)	Infected <i>C. aurantifolia</i> extract treated (Group D)	Infected combined treated Mebendazole & <i>C. aurantifolia</i> (Group E)
Hb conc (mg/dl)	13.9 ±0.11	7.9 ±0.4	8.1 ±0.4	8.8 ±0.5	12.1 ±0.3 *
RBCs (1x10 ⁶) ul	5.3 ±0.01	3.7 ±0.07	4.4 ±0.15	4.8 ±0.20	5.0 ±0.40 *
MCV (F1)	77.5 ±0.81	64.0 ±0.77	58.6 ±0.44	70.2 ±0.30	83.4 ±0.10
MCH (pg)	24.3 ±0.37	21.3 ±0.66	18.7 ±0.21	18.3 ±0.61	24.2 ±0.30
MCHC (g/dl)	33.01 ±0.70	33.1 ±0.91	31.5 ±0.35	26.1 ±0.81	29.01 ±0.71
PCV %	42.1 ±0.33	23.8 ±1.7	25.7 ±1.5	33.7 ±1.90	41.7 ±0.10 *
WBCs (1x10 ³) ul	9.4 ±0.41	14.2 ±2.90	11.7 ±0.50	10.7 ±0.66	10.5 ±0.31
Platelets	195.000 ±70	192.000 ±50	196.000 ±50	195.000 ±60	196.000 ±60
Diff WBCs					
Neutrophils %	57 ±0.01	42 ±0.04	52 ±0.03	57 ±0.03	57 ±0.01
Lymphocytes %	32 ±0.01	36 ±0.02	33 ±0.02	30 ±0.02*	30 ±0.01*
Monocytes %	7 ±0.45	11 ±0.44	9 ±0.45	5 ±0.51	5 ±0.51
Eosinophils %	3 ±0.10	9 ±0.21	5 ±0.44	7 ±0.55	7 ±0.55
Basophiles %	1 ±0.001	2 ±0.001	1 ±0.001	1 ±0.001	1 ±0.001
Total Protein conc (TP)	6.5 ±0.2	6.0 ±0.4	6.1 ±0.3	6.1 ±0.1	6.2 ±0.1
Albumin conc (ALC)	4.9 ±0.1	4.1 ±0.7	4.5 ±0.3	4.9 ±0.2	5.1 ±0.4

* = means with superscripts are significantly different at P<0.05

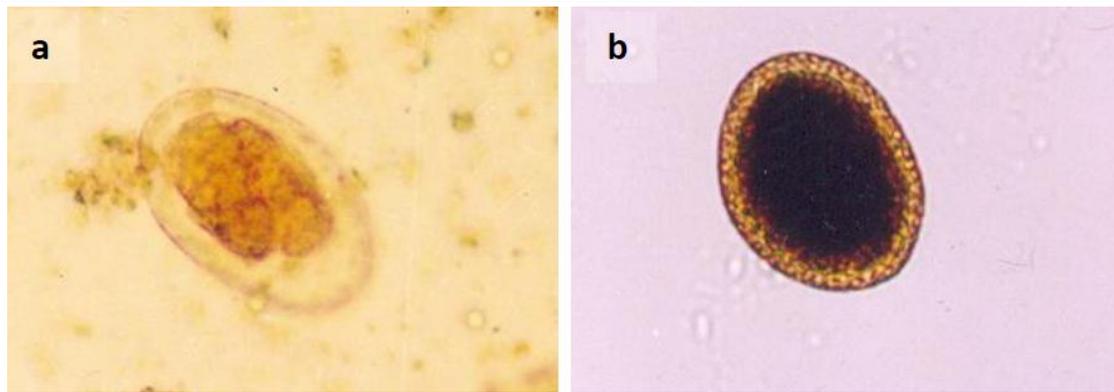


Fig1. Normal *Ancylostoma caninum* (a) and *Toxocara canis* (b) eggs obtained from infected non treated group's dogs

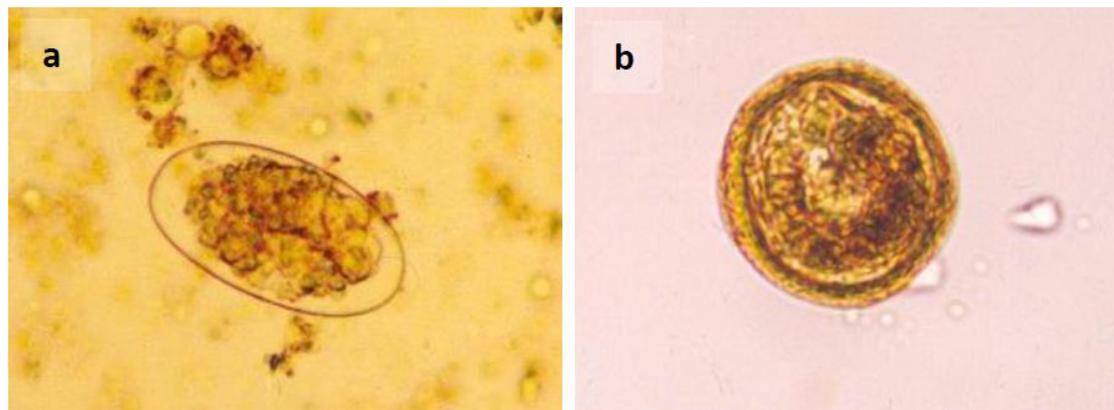


Fig2. Abnormal degenerated *Ancylostoma caninum* (a) and *Toxocara canis* (b) eggs obtained from infected treated group's dogs.

3.2. Discussion

The current study discussed the efficacy of different anthelmintics; mebendazol, *Citrus aurantifolia* seeds herbal plants extract and combined mixture of mebendazol and *Citrus aurantifolia* extract against mixed infection with *Ancylostoma caninum* and *Toxocara canis* in dogs in trial to reach high potency and overcome the problem of drug resistance. The used anthelmintics in the present study give variable degrees of anthelmintic efficacy and reached to high percentage; 100% and 98.2% in animals treated with combined mixture of mebendazol and *Citrus aurantifolia* extract. The results were in agreement with that proved by many previous studies assess the anthelmintic efficacy of aqueous crude extract of some herbal plants against nematode infection in dogs [8], [9] and [10].

3.2.1. Synergistic Anthelmintic Affect

Recently, combination therapies have been used to combat challenges with drug resistance. This strategy could also allow for drugs to target separate pathways, which will increase its efficacy and help prevent recrudescence [24]. Thus, at the same time, [25], [26], [12] and [27] concluded that a good synergistic effect of pyrantel and febantel in high doses can produced against *A. caninum* and *T. canis* dog nematodes. Also, [28] reached to that high level of pyrantel can play a role in resistance in hook worm, *A. caninum* and need for greater vigilance and more judicious use of anthelmintics in small animals. Moreover, [29] showed the modelling of the benefits of anthelmintic combination serving the synergistic action for the nematodes of small ruminants.

3.2.2. Parasitological Examination

The use of herbal plants was of the most accepted trial to escape from producing anthelmintics resistance particularly in dogs and give high efficacy reached to 100%. The use of *Citrus aurantifolia* swingle seeds extract alone in the present experiment gave high efficacy against both *A. caninum* and *T. canis*, dog nematodes infection reached to 87.1% and

91.08%, respectively with improvement of the blood parameters and health status of the infected dogs. Also, in the same way, many studies proved that, citrus plant plays as important and successful anthelmintics therapy with high efficacy reached to 100% against human, poultry and animal parasitic infections [14],[15], [16] [17] and [18].

3.2.3. Hematological Assay

Concerning the blood parameter in the present study, the infected untreated (+ve control) group B, dogs revealed apparent decrease in hemoglobin (Hb) concentration and RBCS count, (7.9 ± 0.4) and (3.7 ± 0.07) than normal values (13.9 ± 0.11) and ($5.3 \pm .01$) in non-infected untreated (-ve control) group dogs, these results compatible with that declared by Lee et al., (2010), who proved that *T. canis* and *A. caninum* can result in acute anemia and significant lower of plasma proteins in infected dogs. While, infected dogs with nematode parasites and subjected to the three anthelmintic treatments showed, an improvement in the hemoglobin and total RBCS reaching to normal values in the non-infected non treated, control group dogs. Also, PCV % in infected animals is decreased reached to 23.8 ± 1.7 and by treatment it improved reached to its normal value 41.7 ± 0.1 in dogs treated with combined therapy of mebendazole and *C. aurantifolia* extract. Also there was a significant change in lymphocytosis produced by parasites in infected dogs (36 ± 0.02) shifting to normal values, 30 ± 0.02 and 30 ± 0.01 in dogs treated only with *C. aurantifolia* and combined therapy of mebendazole and *C. aurantifolia* extract, respectively. Similar results, to assess the anthelmintic efficacy of the aqueous crude extracts of *V. amygdalina* after its administration into local Nigerian dogs infected with ascarids (*Toxocara canis*) and hookworm (*Ancylostoma caninum*), also, produced significant changes ($p < 0.05$) in PCV, RBC, Hb concentration, and TWBC (Adedapo et al., 2007). The administration of the anthelmintic drug and extract brought about a remarkable improvement in the hematology of dogs in groups C, D and E because the worms that were responsible for reduction in the levels of these hematologic parameters have been removed to some extent. It is expected that through hemopoiesis, the parameters will begin to appreciate with time (Oyerinde 1980; Onamegbe and Uche, 1985).

4. CONCLUSION

Although some parasitic helminthes, particularly nematodes have variable genetic features that favor development of anthelmintic resistance, different researches have been developed in order to solve or reduce the occurrence of this major problem. One of the most acceptable and faster method to solve or avoid this problem, is the combination of two anthelmintics together and in particular one of them is herbal with their property in having synergistic action. Results of the present study revealed successful way in using two anthelmintics; one of them is chemical drug, mebendazole and the other is herbal, in origin, *Citrus aurantifolia* swingle seeds extract against dog nematodes, *A. caninum* and *T. canis*, in a synergistic mechanism.

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