IN VIVO EVALUATION OF TOXIC EFFECTS OF AVERMECTIN, *CITRUS SINENSIS* VAR. BALADY AND *C. LIMON* ON FEMALE *HYALOMMA DROMEDARII* (ACARI: IXODIDAE)

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AVERMECTIN
ESSENTIAL OIL
CITRUS SINENSIS
CITRUS LIMON
HYALOMMA DROMEDARII
TICK
SDS PAGE
GC-MS

SUMMARY: The chemical composition of the essential oil obtained from fresh fruit peel of *Citrus limon*, was elucidated by GC-MS analysis. Three main compounds, Limonene (45. 99), Myrcenol (21. 85%), Cis Ocimene (15. 49%) were found. In this study, we evaluated two essential oils obtained from two plant species, *Citrus sinensis* var. balady, *Citrus limon* and avermectin as control agents against the engorged female of *Hyalomma dromedarii*. In vitro study, *Citrus limon* oil had a stronger effect than that of *Citrus sinensis* oil. LC_{50} and LC_{90} were 0.0151; 0.0316 and 0.022; 0.0527 for *C. limon* and *C. sinensis* respectively. In vivo study, Avermectin together with each essential oil have a stronger effect than that of each essential oil alone and avermectin alone respectively, the mortality rate was 36, 36, 20, 30 and 13% respectively. Histological examination (HE) and sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were done for midgut; ovary and hemolymph of the engorged female *H. dromedarii* within 7 days after injection with avermectin and essential oils. HE revealed that the basement membrane and digest cells of midgut were completely damaged. The vacuoles have disappeared from the main cell of the gut and there was no hematin in the digest cells. Gut lumen was filled with hemolysis blood after injection with *C. sinensis*, *C. limon* and avermectin together with each essential oil respectively. SDS-PAGE exhibited 5, 6, 11, 5, 5, 6, 8 bands for ovary and 6, 3, 6, 9, 5, 4 for hemolymph of engorged female *H. dromedarii* injected with *C. limon, C. sinensis*, avermectin and avermectin together with each essential oils, negative and positive control respectively. Our results indicated that the essential oils of *C. limon* and *C. sinensis* have strong toxic effect on female *H. dromedarii* than avermectin alone.

INTRODUCTION

Worldwide, about 800 species of ticks are known and some can carry diseases-causing agents, e. g. bacteria, viruses or other organisms giving rise to health problems (THORSELL *et al.* 2006).

Hyalomma dromedarii is one of those ticks that attack camels as a main host in Egypt, ABDEL-SHAFY,

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(2000). The tick *Hyalomma dromedarii* is an obligate parasite, principally on camels. This species is known as the most important obstacle to camel production in several areas of the Middle East. In an integrated tick control program covering different methods, the control of this parasitic is performed mainly through chemical acaricides. However, continuous use of acaricides has led to the problem of resistance in these arthropods, Klafke *et al.* (2006). The most widely used methods for controlling tick populations are based on the application of synthetic acaricides both in the environment and to animals. However, the indiscriminate use of these substances inevitably leads to resistance and potentially can harm the environment. There is a need to reduce the use of synthetic acaricides and to introduce alternative and/or supplementary methods for the tick population control.

A study of the literature shows that promising results have been achieved using bacteria and fungi that are pathogenic for arthropods (Hassanain *et al.* 1997, Zhioua *et al.* 1999, Habib & Swiefy, 2002), and the extracts of certain plants. The essential oils of *Azadirachta indica* (Neem Tree oil), *Ocimum suave*, *Ageratum houstonianum*, *Melaleuca alternifolia* (Tea Tree oil TTO) and *Citrus sinensis* var. balady have shown acaricidal and repellent properties against the larvae of *Amblyomma variegatum*, all stages of *Hyalomma anatolicum excavatum*, *Rhipicephalus appendiculatus, Rhipicephalus lunulatus*, *Ixodes ricinus* and the egg of *Hyalomma dromedarii*. (Mwangi *et al.* 1995, Ndumu *et al.* 1999, Lwande *et al*. 1999, Kaaya, 2000, Abdel-Shafy & Zayed, 2002, Tedonkeng Pamo *et al.* 2005, Lori *et al.* 2005, Habeeb *et al.* 2007).

Macro–cyclic lactones (MLS) are represented by two groups, avermectins (ivermectin, abamectin and doramectin) and milbemycins (moxidectin and milbemycine oxime). They are largely used in Egypt for the control of internal (gastrointestinal nematodes and microfilariae) and external parasites (ticks and mites [mange]). The mode of action of these compounds in arthropods is attributed to their high affinity to glutamate-gated chloride channels (Glu-Cl) that are present in muscle and nerves. The opening of these channels causes a slow and irreversible membrane conductance increase, resulting in somatic muscular paralysis and the consequent death of the parasite (Cully *et al.* 1994, Davey & George, 2002 & Davey *et al*. 2005).

In this study, the effects of systemic avermectin and essential oil of *Citrus sinensis* var. balady and *Citrus limon* were evaluated on the protein content of hemolymph and ovary and on the pathological changes in mid gut of the engorged females of *Hyalomma dromedarii*.

Material and Methods

1- Collection of ticks — About 400 fully engorged females of *Hyalomma dromedarii* (Koch 1818) were collected from the ground of camel pens, Burkash village, Giza governorate, Egypt and identified according to Estradà–Peña *et al*. (2004). Females were incubated at 26°C and 75 RH in plastic cups till further use. About 250 females were used for vitro studies; about 150 females were used for vivo studies.

2- Extraction of the essential oil — *Citrus sinensis* var. balady from Rutacea Family and *Citrus limon* were collected at the ripening stage from trees cultivated in the experimental station of Horticulture Department, Ministry of Agriculture at El-Marg, Kalubyia Egypt. Fresh fruits peal (flavedo and albedo) of orange and lemon samples were subjected to hydro-distillation until there was no significant increase in the volume of the collected oils. The oils were dried over anhydrous sodium sulphate and kept in a dark bottle at refrigerator till performing chemical analysis and biological activity of each essential oil, Salido *et al.* (2004).

3- Chemical analysis of the essential oil — The essential oil was determined; 20μl of the respective essential oil was diluted with 1000μl diethyl ether and then 2μl of diluted oil was injected in Perkin Elmer Gas Chromatograph model XL with a split ratio of 1:10.The oil constituents were separated on 60m D 13. 5 capillary column having 0.32mm internal diameter, Soliman *et al.* (2003) & Habeeb *et al.* (2007).

4- In vitro study- toxicity test of essential oils — Toxicity test was carried out on engorged females. Concentrations of the essential oils, *C. sinensis* var. balady or *Citrus limon* were prepared by using

ethanol 80% as solvent. The test included four concentrations; 1:40, 1:30, 1:25 and 1:10, (oil: ethanol 80%), 80% ethanol was used alone as a control treatment. Each concentration or control treatment was replicated 5 times and the replicats included five females. The treatment was applied by dipping females for 30 second in each concentration or alcohol in case of control treatments, transmitted to filter paper. The females were separated in plastic cups cone (female/cup). The females incubated at room temperature, the mortality rate was noted daily for 7 days. Calculated mortality percentages of females were based on females with brown–black color. LC_{50}, LC_{90} values for each concentration from *Citrus sinensis* var. balady or *Citrus limon* were calculated according to Finnney (1971).

5- In vivo study — 5-1- Injection of avermectin — The avermectin used in this study is a registered product of Merial with claims for use against a variety of internal and external parasites. A stock solution of avermectin (1mg/mL) in 1. 2% saline was stored at -20°C until needed, Friesen *et al.* (2003). From stock solution, 15 μg/mL in 1. 2% NaCl, (isomotic to tick hemolymph), were injected at 1μL/100mg body weight (bw). Avermectin was injected into the hemocoel through the camerostomal fold (articulation between the scutum and capitulum). Ticks were isolated in covered plastic cups after injection. Mortality was recorded at 7 day. The survived ticks were allowed to desiccate, hemolymph and tissue samples were collected. Control ticks were injected with 1.2% saline.

5-2- Injection of essential oils — From stock oils, *C. limon* and *C. sinensis* oils, 15 μl/ml in 1. 2 NaCL were injected at 1μL/100 mg body weight (bw). The injection was carried out as previously described. The engorged ticks were divided into four groups: ticks injected with *C. sinensis* oil, ticks injected with *C. limon* oil, ticks injected with *C. sinensis* oil and avermectin, and ticks injected with *C. limon* oil and avermectin in ratio 1:1. (30 ticks per group).

5-3- Collection of hemolymph, ovary and midgut samples — Ticks were secured ventrally to a Petridish with glue and refrigerated for 15 min. Cooling ticks prior to hemolymph collection inhibits gut contraction, thus reducing the chance of breaking the gut and contaminating the hemolymph, Kaufman (1991). Incisions (1-2mm long) were made in the integument with a fine scalpel blade. The exuding hemolymph was collected with capillary tubes and diluted 1:1 in phosphate buffer saline, pH 7. 2, Friesen *et al*. (2003) hemolymph samples were stored at -70°C until used.

5-4- Histopathological study — Gut samples were fixed in 10% formalin for 24 – 48 hr, dehydrated in the serial dilution of ethanol alcohol and embedded in paraplast. Serial sections of each gut sample were prepared and stained with Hematoxylin and Eosin stain (H &E stain) according to Agyei *et al.* (1991).

5-5- Characterization of ovary and hemolymph protein content of engorged H. dromedarii *by SDS — polyacrylamide gel electrophoresis* — The ovaries samples were individually taken in 0.01M phosphate buffer saline, pH 7.2 (PBS), homogenized in an equal amount of PBS then sonicated for 5 minutes under 150 watt interrupted pulse output at 50% power cycle using a sonifier cell disrupter. The sonicated ovaries were subjected to high speed centrifuge (10000 rpm) for one hr at 4°C. The resulting supernatant was collected. The protein content of ovaries and hemolymph were determined by the Lowery method, Lowery *et al.* (1951). 10% SDS Slab-polyacrylamide gel electrophoresis (Slab-PAGE) and running buffer consisting of 0.5M tris, 1,92M glycine and 10% SDS (pH, 8. 3) were used as descried by Hames (1987).

Results

1- GC/MS analysis of the Citrus limon — The GC/MS analysis of *C. limon* oil (Table 1) revealed the presence of 23 compounds. It was found that, Cis Ocimene 15.49%, dL-Limonene 45.99% and Myrcenol 21. 85% are the major identified compounds. It was found that the volatile oil of limon which is rich in monoterpenoid hydrocarbon compounds represented 90.18% while the sesquiterpenoid hydrocarbon compounds represent 0.98%. On the other hand, the limon oil contains non-oxygenated monoterpenoid hydrocarbon compounds 61.67%. Oxygenated hydrocarbon compounds represent 28.51%, non-oxygenated

Peak No.	Identified compounds	M.wt.	R_t (min)	Relative Area %
1	Cis-Ocimene	136	10.65	15.49
2	Sablinene	136	11.18	5.68
3	Geraniol formate	182	12.17	0.94
4	1,3,6-Octa triene	108	12.94	4.51
5	dl- Limonene	136	15.24	45.99
6	4,5 – Dimethyl hex-1-ene	112	18.37	0.71
7	5,10 – Dioxotricyclo [7.10. 0E4, 6] Decane	140	18.86	0.72
8	Naphlhalene 1,2,3,4-tetra-hydro 5,8-dimethyl-octyl	272	19.19	0.62
9	1,1′:3′,1″- tetracyclopentane, 2′-dodecyl	374	19.53	0.39
10	1-pentanol, 5-cyclo propyliden	126	21.24	0.25
11	Myrcenol hydrocarbon	154	22.07	21.85
12	3-Hexen 2- one, 5-methyl	112	23.70	0.33
13	Cis-3-Hexene-DL	100	23.87	0.06
14	3-penten-1-OL-2-methyl	100	24.49	0.22
15	1,7-Nonadien-4-OL, 4,8-Dimethyl	168	26.60	0.11
16	Geranyl acetate	196	27.24	0.28
17	1-Methyl-1- VinyL-2-isopropenyl cyclobutane	136	27.48	0.03
18	Trans - Caryophyllene	204	28.44	0.32
19	Cis – α - Bergamotene	204	28.79	0.55
20	Nerol	154	29.24	0.02
21	α - Humulene	204	30.30	0.05
22	Farnesol	222	35.28	0.08
23	L-Farnesene	204	35.68	0.03

TABLE 1: GC/MS analysis of *C. limon* oil

sesquiterpenoid hydrocarbon compounds represent 0.58% and oxygenated sesquiterpenoid hydrocarbon compounds represent 0.40%.

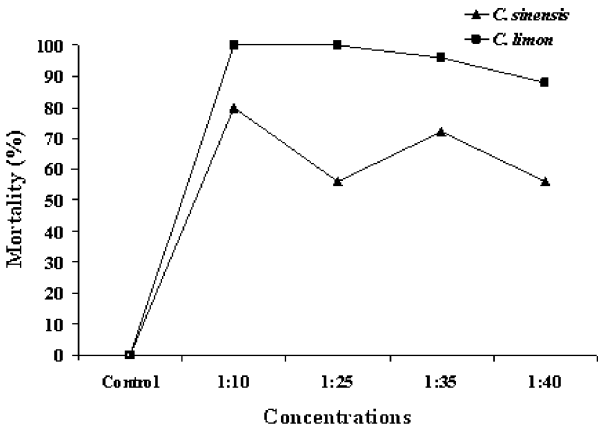

FIG 1.— Mortality rates of engorged females *H.dromedarii* exposed to different concentration of the essential oil *Citrus sinensis* var. balady and *Citrus limon.*

2- In vitro study — As shown from FIG. 1, the mortality increased with increasing oil concentrations. There was a significant difference between mortalities of all concentrations and the control of the two oils *C. sinensis* var. balady and *C. limon.* The mortality rates of engorged female of *H. dromedarii* at dilution 1/10, 1/25, 1/35, 1/40 were 80, 56, 72, 56% and 100, 100, 96 and 88% in case of *C. sinensis* and *C. limon* respectively.

The toxicity of *C. limon* was higher than that of *C. sinensis*. LC_{50} and LC_{90} values in *C. sinensis* and *C. limon* were 0.0151; 0.0316 and 0.0227; 0.0527 respectively, (FIG. 2).

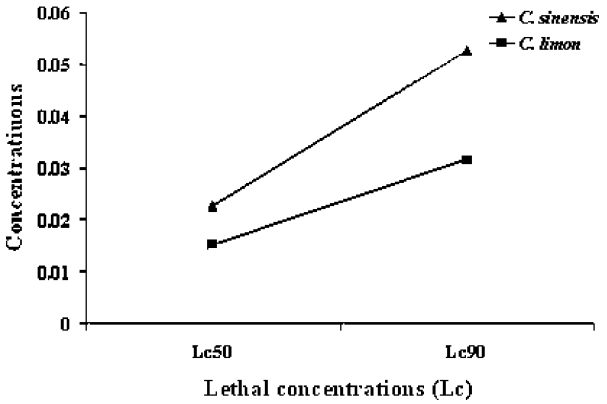

FIG 2.— Toxicity lines for the essential oil *Citrus sinensis* var. balady and *Citrus limon* against the adult females of *H. dromedarii.*

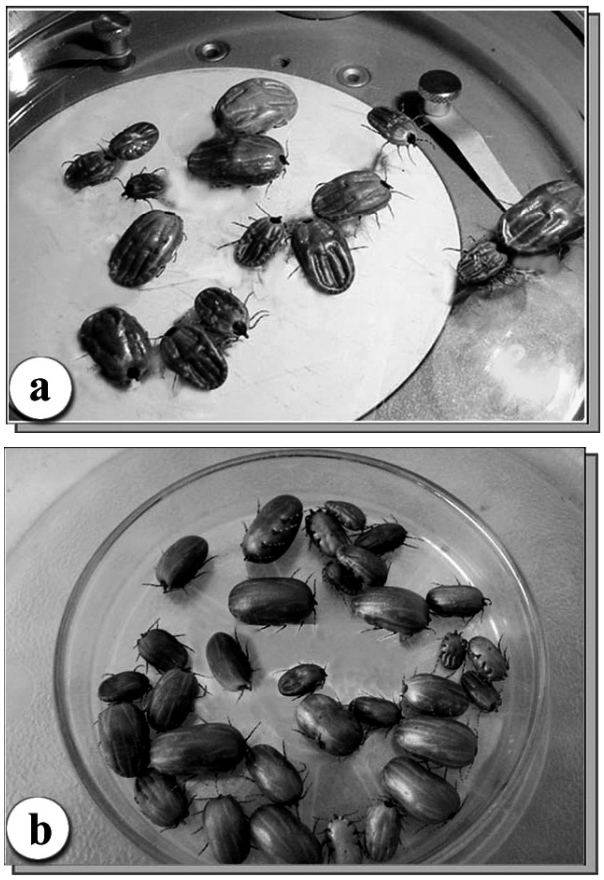

Fig 3.— a) Appearance of healthy female *Hyalomma dromedarii* ticks. b) After injection of Avermectin and essential oils.

3- In vivo study: Toxicity effect of avermectin and essential oils — The dose of avermectin used in this study killed 13% only of engorged females at the 7th day after injection. However, ticks mortality increased significantly after injection of. essential oils, *C. sinensis* and *C. limon*. The mortality was 20% and 30% for *C. sinensis* and *C limon* respectively. In addition, there was a significant increase in mortality after injection of avermectin together with each essential oil. The mortality was 36% and 36% for each oil together with avermectin. Ticks treated with avermectin and essential oils appeared bloated, had splayed legs, did not move, and displayed much shallower dorsal didges than normal healthy ticks (Fig. 3), suggesting that the major dorso-ventral muscles and leg muscles were paralyzed.

4- Histopathological study on the midgut — The classification of the cells of the midgut follows the nomenclature of other researchers (Agyei *et al.* 1991, Agyei & Runham 1995) (Fig. 4 a & b).

4-1- Gut cell after injection of avermectin and essential oils — A slight damage occurred in the basement membrane after injection with avermectin. There are in fact, all stages of cells intermediate between stem cells and digest cells. Some of these cells were small and have empty cytoplasm, other were large and have started ingesting the blood meal, and contain small food vacuoles. Digest cells were either attached to the basal lumina or were free in the lumen and contained numerous hematin granules (Fig. 4c).

A damage of the midgut epithelial cells appears after injection of *C. limon* oil. Basement membrane was completely damaged. The digest cell was the main cell type present and the midgut lumen was filled with hemolysis blood (Fig. 4d). However, after injection of *C. sinesis* oil, the midgut revealed a severe damage in the basement membrane and digest cells. The food vacuoles have disappeared from the main cell and the digest cell. In addition, the midgut lumen was filled with hemolysis blood (Fig. 4e). In response, maximal damage occurred after injection of avermectin together with each essential oil. The basement membrane was completely damaged. Indeed, digest and residual sessile digest cells were not formed. Moreover, no hematin in the digest were and gut lumen were filled with hemolysis blood (Fig. 4, f & g).

5- Characterization of ovary and hemolymph protein content of engorged female H. dromedarii *by SDS-polyacrylamide gel electrophoresis* — Electrophoretic profiles of ovary and hemolymph in the two controls; treatment with avermectin alone; each essential oil and avermectin with each essential oil are presented in (Figs. 5 a, b & Tables 2, 3). The ovary homogenates of treated and non-treated ticks reflected an appearance and disappearance of protein bands at 7th day of injection. Six and eight molecular entities were detected in the negative control ovary and positive control. The molecular weight ranged from 380.27-106.43 kDa and 414.51-106 kDa for both negative control and positive control of ovary respectively. However, after treatment with

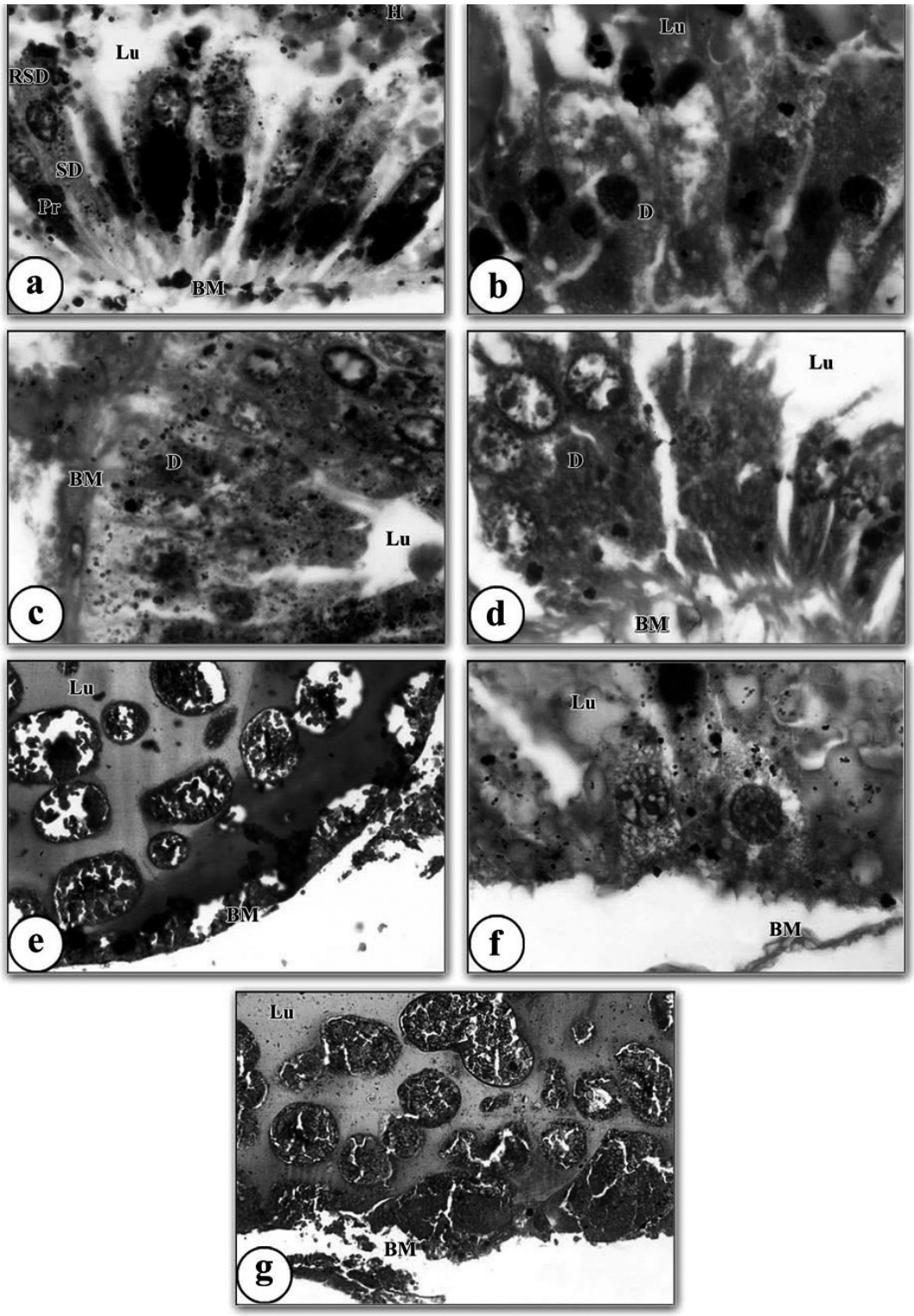

FIG.4.— Transverse sections of the midgut of *H. dromedarii* showing lumen (Lu) basement membrane (BM), digest cell (D)° with release of hematin (H) by the midgut epithelial cells, prodigest cell (Pr), sessil digest cell (SD), and residual sessil digest cell (RSD*)*.

a) control negative; b) control positive (1000X); c) midgut injected with limon oil; e) midgut injected with *C. sinensis* oil; f) injected with avermectin and *C. sinensis* oil.

C. limon	*C. sinensis*	Avermectin	Control -ve	Control +ve	Avermectin + *C. Limon*	Avermectin + *C. sinensis*
109.23	187.61	108.68	380.27	414.51	92.1	226.32
92.57	160.71	99.8	335.47	371.44*	53.8	191.16
30.76	106*	97.82	267.28	343.45	31.25	159.21
27.70	71.79	93.50	224.95	312.63	25.37	106.75*
19.51	42.57	66.1	176.44*	271.50	19.34	43.29
	26.33	33.1	106.43*	230.31		
		30.0		176.06*		
		28.42		106		
		27.7				
		23.00				
		19.4				

TABLE 2: Electrophoretic ovary protein profile of female *H. dromedarii* injected with avermectin and essential oils (at 7th day).

C. limon	*C. sinensis*	Avermectin	Control -ve	Control +ve	Avermectin + *C. Limon*	Avermectin + *C. sinensis*
210.82	89.82	199.77	118.48*	43.6*	208.00	200.11
89.51	42.25	167.71	98.34		167.71	82.00
55.81	28.04	118.80*	81.34		101.87	45.09*
43.63*		65.29	45.05*		77.36	37.47
42.40		45.05*			68.23	29.67
30.38		31.18			61.32	
					45.00*	
					43.23*	
					32.00	

TABLE 3: Electrophoretic hemolymph protein profile of female *H. dromedarii* injected with avermectin and essential oils (at 7th day).

C. limon; *C. sinensis* and avermectin alone, the ovary SDS dissociated proteins were separated into 5, 6 and 11 protein bands with molecular weights ranging from 109.23-19.51kDa; 187.6-26.33kDa and 108.68-19.4kDa respectively. A common band with molecular weight 176 was shared with control negative and control positive. In case of being treated with *C. sinensis*, the shared protein band with control negative was 106kDa. In addition, new protein bands were stimulated after injection of avermectin with *C. limon* oil and avermectin with *C. sinensis* oil. There was one common protein band shared with negative and positive control with molecular weights 106kDa (TABLE 2 & FIG. 5a).

The electrophoretic hemolymph protein profile reflected appearance and disappearance of protein bands (TABLE 3 & FIG. 5b). Four bands were detected in the non-treated hemolymph with molecular weight ranging from 118.48-45.05kDa, and the positive control showed one protein band. In case of treatment with *limon*, *sinensis* oils and avermectin, the hemolymph dissociated proteins were separated into 6, 3 and 6 bands with molecular weights ranging from 210.82-30.38; 89.82-28.04; 199.77-31.18kDa respectively. Three bands were shared with negative and positive controls with molecular weights 43.63, 45.05 and 118 kDa. Nine and five protein bands appeared after the injection of avermectin with *C. limon* and avermectin with *C. sinensis*. Molecular weights ranged from 208-32 kDa and 200.11-29.67kDa respectively. The dissociated protein bands in case of avermectin and *C. limon* showed that two bands were shared with control with molecular weight 43 and 45kDa (TABLE 3 & FIG. 5b).

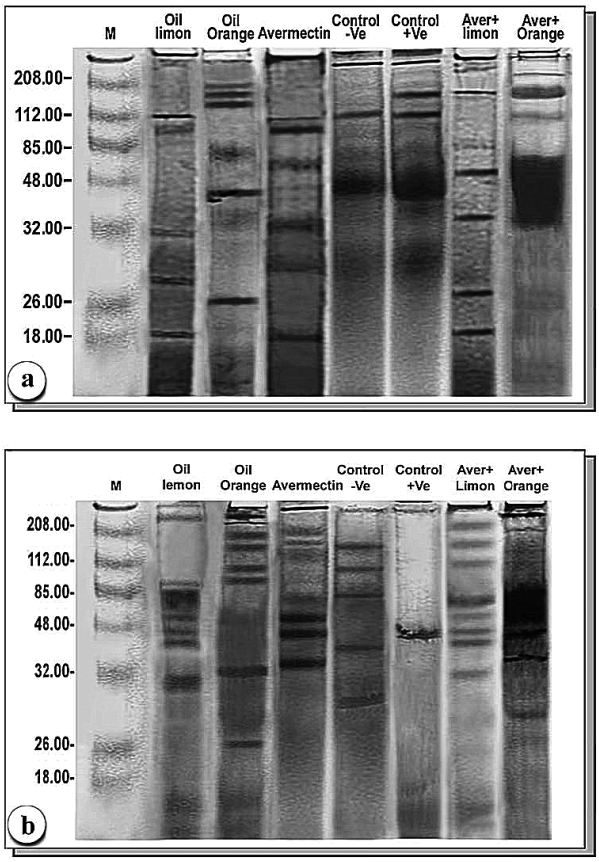

FIG. 5.— 10% SDS-PAGE electrophoretic profile for total protein of female *H. dromedarii* injected with Avermactin and essential oils (at 7th day). (a) ovary profile; (b) hemolymph protein profile.

DISCUSSION

FRIESEN *et al.* (2003) stated that Avermectin MK-243 inhibited egg development primarily at the level of vitelogen uptake by the oocytes. It reduced hemolymph ecdysteroid titer by approximately 90%. Avermectin MK-243 did not significantly reduce hemolymph vg-concentration. Ovary weight and vitellin content of the ovary were the most affected by Avermectin MK-243.

HABEEB *et al.* (2007) used the essential oil from *C. sinensis against* egg stage of the common camel tick, *Hyalomma dromedarii* for the first time in Egypt. So in our study we compared between the effects of two essential oils *Citrus sinensis*; *Citrus limon* and avermectin on engorged female of *Hyalomma dromedarii*. The main compounds of the essential oil *C. sinensis* were studied by HABEEB *et al.* (2007) who found two main compounds, limonene (83. 28%) as a hydrocarbon compound and linalool (3.97) as an oxygenated compound.

In this study, the main compounds of the essential oil of *C. limon* were dL-Limonene (45.99%) and Myrcenol 21.85%. The sum of non-oxygenated monoterpenoid hydrocarbon compounds was (61. 67%) and the sum of oxygenated hydrocarbon compounds were (28. 51%). TABLE (1). The toxicity of the oil was attributed to these two main groups of compounds. OMER *et al.* (1997) detected only 78. 36 limonene in fruit peels of balady orange *C. sinensis*. TROZZI *et al,* (1999) reported that Limonene of *C. sinensis* (L.); Osbrck cv. Maltese was 92. 6%. The toxicity of the oil is attributed to these two main compounds. CHUNGSAMARNYART & JANSAWAN (1996) found that the peel oils of *Citrus reticulate* and *C. maxima* showed higher acaricidal activity two times higher than that of (+) – limonene against the engorged females of *B. microplus*. They added that *C. sinensis* and *C. maxima* oils exhibited a higher larvicidal activity 1. 5 times stronger than that of (+) – limonene. HABEEB *et al.* (2007) found that *C. sinensis* oil showed higher acaricidal activity against all ages of the egg of *Hyalomma dromedarii* except at the 20-day age of egg, which is more resistant than other ages. In vitro, the toxicity effect of *C. sinensis* and *C. limon* oils showed higher acaricidal activity against the engorged female of *Hyalomma dramedarii* in higher concentration than in lower concentration. There was a significant difference between mortalities of all concentrations and the control through the two oils. On the other hand, *C. limon* was more effective than *C. sinensis* in all concentration. This result agrees with CHUNGSAMARNYART & JANSAWAN (1996) and HABEEB *et al.* (2007). However, FACEY *et al.* (2005) found that the oil of *Hyptis verticilla* Jacq disrupted the oviposition and hatching of *B. microplus* egg; but, it was not very lethal to the adult ticks.

In vivo, the results revealed that the mortality of engorged females of *H. dromedarii* was significantly higher after injection with *C. limon* only than with *C. sinensis* only and avermectin alone respectively. On the other hand, there were significant increases in mortality after injection of avermectin together with each essential oil. This could be due to probable

synergic effect of oils, increasing the toxic effect of avermectin. Avermectin was found less effective on tick female. The female cuticle color changed from shiny grey to dark brown with swollen body after death. Our results agree with Friesen *et al.* (2003) in case of avermectin.

The basic histology investigations of the midgut epithelial cells during the digestion of blood meal in ticks have been previously reviewed (Balashov, 1972, Coonset al. 1986, Raikhel, 1983, Rosell-Davis & Coons, 1989). The present study showed that a severe damage in the basement membrane and epithelial cells after injection with *C. sinensis* occurred. However, epithelial cells showed more severe damage after injection with avermectin together with each essential oil than injection with avermectin alone. A clear difference was obviously noticed in the extent of the damage to epithelial cells between injection with essential oils and avermectin. These results indicated that the main compounds of essential oils had marked effect in midgut epithelial cells. This could explain the rise in mortality in females injected with essential oils compared to female injected with avermectin only.

SDS-PAGE revealed that there were differences in bands numbers and molecular weights in the two controls and the treated ovary and heamolymph proteins of females. Avermectin affected the proteins of ovary. It destructs the protein structure thus inducing the formation of new protein bands. Avermectin together with *C. limon* induces more differences in the molecular weights of proteins of hemolymph. Our results agree with Friesen *et al*. (2003).

Conclusion

It was concluded that the female of *H. dromedarii* was more sensitive to the essential oils of *C. sinensis* and *C. limon* and avermectin together with each essential oil than to avermectin only.

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