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Phytochemical and anthelmintic investigation of Citrus aurantifolia Swingle seeds

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Abstract

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 results revealed variation in the lethal effect with different organisms. Petroleum ether (40-60) extract tested at 100% concentration showed 100% mortality effect against all parasites except *T. colubriformis*. larvae (39.6% mortality) .The larvicidal effect of acetone extract vary from *T. colubriformis* (adult worms) , *F. gicantica* (mature fluks) and *T.spiralis* larvae (LC_{50} = 415,522,630 ppm) respectively, but no effect was observed against ascaris (adult worms) or *T. colubriformis* (larvae). Acetone extract was effective against the eggs of *T.vitulorum, F. gicantica and T. colubriformis* (LC_{50} 275, 155, 309 ppm) respectively. An oocidal effect was observed against Eimeria (LC_{50} = 631 ppm) Phytochemical study of the bioactive extracts lead to identification of limonin and limonexic acid beside nine coumarins from acetone extract, the isolated compounds were identified by chemical and spectral analysis. The petroleum ether extract was saponified and analyzed by GC/Mass.

Key Words: Anthelmintic , *Citrus aurantifolia* , coumarins and limonoids.

Introduction

The aim of work is to investigate the larvicidal and oocidal effect of *Citrus aurantifolia* seeds extracts on some common parasites. And define the chemical composition of each extract.Family Rutaceae is characterized by the presence of trinorterpenoids related derivatives C26 limonoids. Most of Citrus limonoids and their glycosides were reported in seeds and juice. (1,2). Citrus plants are very rich in several types of coumarins: simple and prenylated coumarins, furanocoumarin, pyrano-coumarin and coumarin dimers were reported in different Citrus species (3-6) The fatty acid profiles of citrus juice and seeds were different containing iso and anteiso simple branched fatty acids (7,8) Egypt is considered as one of the semitropical countries. The climatic conditions in particular the temperature favors the parasitic infection in human and animals ; Beside the bad hygienic conditions encourages the spread of parasitic infection. *C.aurantifolia,* Swingle (Rutaceae), was reported to show anthelmintic activity (9-11) but further studies are recommended to evaluate their effects against different types of worms, and to determine the nature of their bioactive constituents to provide a useful source of effective simple and relatively nontoxic remedy.

Materials and Methods

General experimental

Melting points were uncorrected, measured on Buchi electrothermal digital melting point apparatus. Ultraviolet–visible Spectronic 2000 spectrophotometer system UV- Visible recording spectrophotometer 240. Mass spectrophotometer

MAT 112, electron impact ionization 70 ev. The infrared spectra were recorded in potassium bromide disks on a Pye Unicam SP- 3-300 and Shimadzu FT IR 8101PC infrared spectrophotometers .The NMR spectra were recorded on a Varian Mercury VX–300 NMR spectrometer ^{1}H spectra were run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deuterated chloroform (CDCl3) or Dimethyl Sulphoxide (DMSO –d 6) chemical shifts are quoted in δ and were related to that of the solvents. X-single crystallography data collection on Kappa CCD, Mo Kappa radiation (0.71073).

GC/MS analysis of Fatty acids methyl ester

Capillary column DB-5 Fused silica [(5% phenyl) methyl polysiloxane], 30 m length ,0.25 mm ID and 0.25µm thickness was used, temperature progr-amming;150^{o}C for 3min ,increase temperature from 50–280^{o}C at a rate of 5^{o}C/min ,maintained at 280^{o}C for 5min. injector mode splitless, detector MS flame ionization EI, EV 70, injector temperature 250^{o}C, flow rate 1ml/min, duration 34min.

GLC analysis of unsaponnifiable matter

The instrument used was HP 6890 GC provided with FID detector capillary column, B.P. Polysilphenylen–siloxane length 60 m, diameter 320 µ m and film thickness 0.254µm. programmed temperature 70^{o}C for 2 min., increased at rate 8^{o}C / min for 30min and final temp. 280^{o}C. Injector temperature was 240^{o}C and detector temperature at 300^{o}C. Hydrogen and nitrogen were used as carrier gas at flow rate 30ml/min., and air flow rate 300ml/min.

Materials for biological study

Parasites

Different types of parasites were used in the in vitro studies to evaluate the efficacy of the different *Citrus aurantifolia* extracts as anthelmintics. These parasites are belonging to different phylia; trematoda and nematode phylum and protozoa, as follow: Eggs and adults of *Toxocara vitulorum* (Ascaris). (parasite of cattle and buffaloes). Eggs, larvae and adults of *Trichostrongylus colubriformis*. (Parasite of man and animals).Larvae of *Trichinella spiralis* (Parasite of man and animals). *Fasciola gigantica* mature flukes and eggs (parasite of man and animals Eimeria Sp oocysts (poultry-coccidiosis) Worms and eggs for in vitro anthelmintic studies were isolated from fresh animal organs obtained from Basatien - Cairo abbatoir. The parasites were incubated in phosphate buffer solution, PH =7.2 at 28^{o}C. Also, the control non treated group was applied using PH=7.2 .and blank solution .

Plant materials

Citrus aurantifolia seeds (1kg) were purchased from local market, comprared with authentic sample and identified by Prof. Dr. Ahmed El Ezaby department of Horticulture, faculty of Agriculture, Cairo University.

Preparation of the plant extracts for biological study

i: The powdered seeds of *Citrus aurantifolia* were defatted with petroleum ether (40-60) , evaporated under vacuum , to obtain the petroleum ether fraction which was tested as anthelmintics without dilution. Results in (Table 1).

ii: The defatted seeds were extracted with acetone, evaporated to dryness, 1 gram was dissolved in 10 ml methanol and diluted to 1000 ml by phosphate buffer solution to prepare different concentrations (mg/L) to be tested against parasites. Results in (Table 2, Figs1-17). Each concentration was tested in triplicates.

Extraction and isolation

The petroleum ether extract

The ground seeds of *C. aurantifolia* (1kg) were extracted with petroleum ether, evaporated, to gave (500g) oil, (10ml) was saponified, according to Tsuda (12) extracted with ether to separate the unsaponifiable matter (0.5%), the aqueous liquor was acidified and extracted with ether to isolate the fatty acids (95%), followed by methylation of fatty acid (13).

The unsaponifiable matter and the methyl esters of isolated fatty acids were subjected to GLC and GC/Mass analysis respectively adopting the previously mentioned conditions. Peak assignments were carried out by comparing the mass fragmentation patterns and relative retention times with those of reference compounds, literature sources, and the Wiley MS database of the equipment.

The acetone extract

The defatted seeds were extracted with acetone, evaporated to give residue (25) g, ١٢g of the acetone extract was applied on silica gel flash column, eluted with petroleum ether with gradient ethyl acetate then ethylacetate /methanol. Fractions were monitored by TLC pre-coated silica gel GF254 (Merck), developed using solvent systems: (A) toluene /ethylacetate (8:2) and (B) chloroform: methanol (37:3) examined under UV. At 365 nm and sprayed with Iodine / Potassium Iodide reagent

or vanillin/sulfuric acid. The matching fractions were added together to give finally eight fractions .Fraction I (0.005g): eluted with pet.ether showed to contain mainly hydrocarbons Fraction II (1.2g) from 40%.ethyl acetate / petroleum ether allow isolation of three fluorescents compounds CI,CII,CIII with Rf (0.76 , o.65 and 0.63) respectively by preparative TLC using solvent system A. Fraction III. (0.4g) eluted with 60 % EtOAc / pet ether offered compounds CIV& CV Fraction IV(0.3g) eluted with 80 % EtOAc / pet .ether was applied on a silica gel subcolumn , eluted with n-hexane / dichloromethane which led to isolation of four coumarins CVI,CVII,CVIII,CIX. Fraction V (4g): from 100 % ethyl acetate the compound LI was isolated Fraction VI (3g): from 4% methanol / ethyl acetate the compound LII was isolated. The isolated compounds were identified by physical, chemical methods and by comparison with authentic.

Results and Discussion

Anthelmintic effect of petroleum ether extract

The promising larvicidal and oocidal effect of the petroleum ether extract of *C. aurantifolia* seeds against the tested parasites, with 100% mortality except *Tricostrogylus colubriformis* larvae, (39.6%) mortality were observed (Table 1 Figs 15-17).

Anthelmintic Effect of acetone extract

The larvicidal and oocidal effects of the acetone extract expressed in terms of percent mortality and the LC_{50} were reported in (Table 2 Figs1-14) which reflects the difference in sensitivity of several types of parasites to the acetone extract .

The results revealed that *Fasciola gicantica* (Trematode worms) was the most susceptible parasite to both acetone and petroleum ether extract while *T. colubriformis* larvae was the most resistant parasite to acetone extract with low sensitivity to the petroleum ether extract. Both ascaris adult worm and *Trichinella spiralis* larvae (Nematodes) show great resistance to the acetone extract; whereas the petroleum ether extract was very effective against *Trichinella spiralis* larvae. Eimeria (protozoa) showed no resistance to both extracts. Also, it is well noticed that both petroleum ether and acetone extracts were effective against all the eggs of the tested parasites that reflect the promising anthelmintic effects of *C. aurantifolia*; thus enhance the investigation of the bioactive petroleum ether and acetone extract for their chemical constitution.

Isolation and identification of the constituents of the extracts

Compounds identification in petroleum ether extract :

GLC of unsaponifiable matter allow identification of the folowing hydrocarbons: Nonane, Tridecane, Tetradecane, Pentadecane, Heptadecane, Nonadecane, Cosane , Unacosane , Docosane. GC/Mass of the methylated fatty acids allow identification of 28 fatty acids the most dominant were palmitic , linoleic , oleic (46.4 , 36.9 , 9 .8 %) respectively .Also several branched chain fatty acids were detected (Table 3).

Compounds isolated from acetone extract

Column chromatography of the bioactive acetone extract revealed the isolation of nine coumarins and two limonoids.

Compound CI: Rf = 0.76 crystallized from methanol (2mg) m.p. = 68°C, EIMS: m/z [M^+] 298(100%), $C_9H_6O_4$, 255(33) [M+-CH_3CO], 199(16) [M^+-CH_3CO+C_4H_8], 143(33).

The compound was identified as aurapten.

Compound CII: R_f = 0.65 crystallized from petroleum ether / ethyl acetate (1: 2) ,(5mg) m.p. 86-87 °C. Mass: EIMS m/z=[M+H]+330, $C_{20}H_{24}O_4$; UV: λ max nm (213 , 271, 320). 1H -NMR ($CDCl_3$). δ 7.9-7.8 (dd J = 9.5 , 0.5 Hz) H4, δ 6.4 (d J= 2 , 0.5Hz) H 8, δ 6.2 (d J= 2.0 Hz) H6, δ 6.16 (d J= 9.5 Hz) H3, δ 5.35(tq J= 6.5, 1.0 Hz) H2`, δ 5.09 m H7`, H1` δ 4.2 d ,OCH3 δ 3.8 s, δ `2.2 H5m, δ 2.3 H6`m , δ 1.6 br s H4`, δ 1.2 s H9`, δ 1.3 H10` the chemical shifts and assignments agreed with the previously reported data [14] The compound was identified as 5- geranoxy 7-methoxycoumarin (Fig.19)

Compound CIII: R_f = 0.63 The compound was obtained as colorless oil. EIMS, m/z [M+] = 313, $C_{19}H_{22}O_4$ .296.29(30%) [M^+-OH], 264(100) Epoxy aurapten.

Compound IV: R_f = 0.61the compound was obtained as colorless needles (4mg) and as analyzed by X-ray single crystallography. $C_{11}H_{10}O_4$, Mr = 206.19Orthorhombic, All diagrams and calculations were performed using maXus [15, 16] (Bruker Nonius, Delft & MacScience, Japan). Fig.1 Pna21 , a = 10.5430 (6)Å, b = 13.7067 (10)Å ,c = 6.7311 (4)Å , γ = 90.00°,β = 90.00° , γ = 90.00° ,V = 972.71 (11)Å3 , Z = 4 , Refinement on F2fullmatrix least squares refinement , R(all) = 0.083, R(gt) = 0.039 ,wR(ref) = 0.078,wR(all) = 0.090.the molecular structure determined by this method is illustrated in (Fig 18).the data was deposited at the Cambridge Crystallographic Data Center (CCDC).

The compound was identified as 5, 7 dimethoxy coumarin.(limettin(Compound CV: R_f = 0.58 The compound crystallized from methanol as yellow needle (50mg), m.p. = 148- 151^oC .Mass : EIMS, m / z $[M^+]$ at 246(100%), $C_{13}H_{10}O_5$. 230.9 (55) $[M^+-CH_3]$, 203.4(25) $[M^+-CH_3+CO]$ This was confirmed by ^{1}H-NMR ($CDCl_3$) represented in showed signals at δ 8.13 and 6.30 ppm (d J= 9.6 Hz) corresponding to H-4and H-3 respectively, at δ 7.63 (d J= 2.1 Hz) H-7, at δ 7.00 (d J= 2.1 Hz) H-6 and two singlet at δ 3.60 and 3.65 for two methoxy groups. Thus the compound was identified as Isopimpinellin. (Fig.19).

Compound CVI: R_f= 0.42 It was obtained from fraction eluted with 2 % methanol in dichloromethane, crystallized from methanol (4mg) m.p.128-129^oC MS: EIMS $[M^+]$ m/z 297(20%), $C_{15}H_{18}O_5$, 256 (45%) $[M^+-CH_3+CO]$, 213(15)[$M^+-C_4H_9O$].the compound was identified as Meranzin hydrate.

Compound CVII: R_f = 0.35 it was obtained from fractions eluted with 3% methanol in dichloromethane crystallized from methanol as white needles (2mg) m.p. (204-206^oC). Co chromatography with authentics the compound was identical to Scopoletin

Compound CVIII: R_f = 0.26. Obtained from 3 % methanol in dichloromethane, crystallized from methanol as colorless needles (1mg), m.p. 224-226^oC, Co chromatography with authentics the compound was typical Umbelliferone.

Compound CIX: R_f = 0.20 Obtained from 4% methanol in dichloromethane afford The compound was crystallized from methanol as colorless needle (2 mg) m.p. (268-270^oC). Co chromatography with authentics the compound was deduced to be aesculetin.

Compound LI: Obtained by crystallization from methanol white shiny plates were obtained (3.5g) mp. 276 – 278 ^oC. The compound was examined on silica gel TLC plates, eluted with either system chloroform: methanol (37: 3). Showed one reddish spot when sprayed with vanillin sulfuric acid R_f = 0. 32. ETMS: $[M^+]$ 471 m/z $C_{26}H_{30}O_8$. ^{1}H-NMR: spectrum of compound LI in $CDCl_3$ the chemical shifts and assignement are listed in (Table 4) which agree with the reported data for limonin (2) further confirmation was obtained by ^{13}C-NMR analysis showing signals for 26 carbon atoms represented in (Table 4,Fig 19) the compound was identified as Limonin.

Compound LII: an amorphous yellowish powder (2.3g) with mp = 299-300oC. Mass: +ve ESI, TOFMS M/e: $[M^+ + Na^+]$ = 525, $[M^+]$ =503 m/z $C_{26}H_{30}O_{10}$. IR cm-1: 1700 for C=O and additional bands 1752 for C =O in five member ring and 3400 for OH ^{1}H-NMR, DMSO showed difference in oxidation of furan ring from limonin, verified by ^{13}C-NMR which revealed absence of two signals at ∂H 143 and 141 characteristic for C21and C23 respectively in the furan ring also the spectrum showed resonance corresponding to three carbonyl carbons [δ 206 .6, 169.4, 169.2] Table 5 . Comparing the obtained data with the previously published data (17) and the signals in the ^{13}C-NMR suggested the compound to be a mixture of limonexic acid and its isomers isolimonexic acid (Fig 19)which were identified for the first time in *C. aurantifolia*.

Table (1): percent mortality of 5 ml 100 % petroleum ether extract of Citrus aurantifolia seeds on the studied parasites:

Parasites	Mortality percentage
Toxocara vitulorum eggs	100
Trichostrongylus colubriformis eggs	100
Trichostrongylus colubriformis larvae	39.6 ± o.69
Trichostrongyluscolubriformis adult worms	100
Trichinella spriralis larvae	100
Fasciola gicantica eggs	100
Fasciola. gicantica mature flukes	100
Eimeria sp. Oocysts (coccidia)	100

N.B. Toxocara vitulorum adults worms was not included in the trials

Table (2): Lethal concentration (LC_{50})of acetone extract of *C aurantifolia* seeds on the studied parasites

Parasites	LC_{50} mg/L
Fasciola.gigantica mature flukes	522
Trichinella spiralis	630
Trichostrongylus colubriformis adult worms	415
Trichostrongylus. colubriformis egg	309
Toxocara vitulorum egg	275
Fasciola.gicantica egg	155
Eimeria sp. Oocysts	631

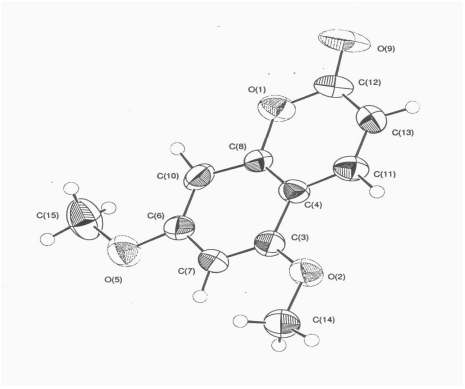
N.B: no effect was observed on *Toxocara vitulorum* adult worms, *Trichostrongylus colubriformi* larvae up to 1g/L

Table (3): Results of GC/MS analysis of methylated fatty acids from *C.aurantifolia* seeds

Relative Retention time	Percentag %	Compounds	Molecular formula	M^+	Fragmentation pattern
0.32	0.03	Octanoic acid methyl ester	$C_9H_{18}O_2$	158	143,126,99,75
0.47	0.04	Hexanoic acid -2-propenyl ester	$C_9H_{16}O_2$	156	141,111,87,74
0.53	0.02	nonanoic acid methyl ester	$C_{10}H_{20}O_2$	172	157,129,73
0.54	0.03	Octanoic acid 6- methyl – methyl ester	$C_{10}H_{20}O_2$	172	157,115,57
0.58	0.02	Octanoic acid 8,8 –dimethoxy methyl ester	$C_{11}H_{22}O_4$	218	202,165,57
0.62	0.29	Nonanoic acid 9-oxo methyl ester	$C_{10}H_{18}O_3$	168	153,139,115,53
0.72	0.19	nonanedioic acid , dimethylester	$C_{11}H_{20}O_4$	216	201,186
0.75	0.02	12,12-dimethoxydodecanoic acid methyl ester	$C_{15}H_{30}O_4$	274	201, 137,75
0.79	0.01	dodecanoic acid 12-methyl methyl ester	$C_{14}H_{30}O_2$	232	217,202,189
0.83	0.06	tetradecanoic acid methylester(methyl myristate)	$C_{15}H_{30}O_2$	242	199,143,74
0.92	0.04	Pentadecanoic a methyl ester	$C_{16}H_{32}O_2$	256	241,213, 57
0.93	0.04	tetradecanoic acid 13 methyl methyl ester	$C_{17}H_{32}O_2$	268	236,194,152,74
0.95	0.01	pentadecanoic acid 13- methyl methyl ester	$C_{17}H_{34}O_2$	270	255,213 143,74
0.96	0.08	9-hexadecenoic acid methyl ester (methyl palmitoleate)	$C_{17}H_{32}O_2$	268	236,194,152,74
1	46.4	hexadecanoic acid methyl ester (methyl palmitate)	$C_{17}H_{34}O_2$	270	227,143,74
1.07	0.02	hexadecanoic acid 15-methyl methyl ester	$C_{18}H_{36}O_2$	284	269,251,57
1.08	0.01	heptadecanoic acid methyl ester	$C_{18}H_{36}O_2$	284	269,227,74
1.14	36.9	octadecadienoic acid methyl ester(linoleic acid)	$C_{19}H_{34}O_2$	294	264,222,96
1.19	9.82	octadecenoic acid methyl ester(methyl oleate)	$C_{19}H_{36}O_2$	296	264,222,81
1.20	0.27	9-octadecenoic acid ethyl ester(ethyl oleate)	$C_{20}H_{38}O_2$	310	264,222, 180
1.20	0.05	octadecadienoic acid methyl ester	$C_{19}H_{34}O_2$	294	279,251,83
1.21	0.17	heptadecanoic acid 15-methyl methyl ester	$C_{19}H_{34}O_2$	294	237,223,57
1.22	0.17	11- eicosasenoic acid metyl ester(arachidinic acid methyl eater)	$C_{21}H_{40}O_2$	324	292,250,71
1.25	0.78	eicosanoic acid methyl ester(arachidic acid methyl eater)	$C_{21}H_{42}O_2$	326	294,283,143,74

Table (4): NMR of compound LI

H	δ ppm , J(Hz)	C	δ ppm
ά - furan	7. 41 s	C1	80.3
	7.25 s	C2	35.6
β – furan	6.34 s	C3	168.9
17	5.46 s	C4	79.2
15	4.02 s	C5	53.9
11	1.77 m	C6	36.4
12	1.84 m	C7	205.9
9	2.64 dd (10, 2.0)	C8	51.4
6 α	2..85dd (15 , 2.8)	C9	46.0
β	3.09 t (15)	C10	48.1
5	2..45 dd (15 , 2.8)	C11	17.6
1	4.73 s	C12	30.2
2 α	2..24 (14 5 , 3.1)	C13	38.0
β	2.64 (14.5 , 3.1)	C14	65.7
19 α	4.43 d (13)	C15	60.6
β	4.78 d (13)	C16	166.4
CH_3	1.50s	C17	77.8
	1.21s	C19	65.3
	1.17s	C20	120.0
	1.07s	C21	143.2
		C22	109.6
		C23	141.1
		C-methyls	30.8
			21.4
			20.7
			19.0



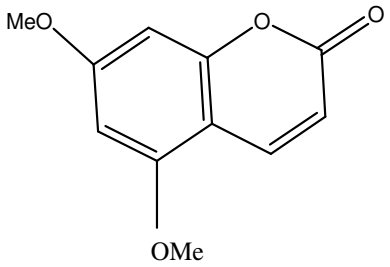
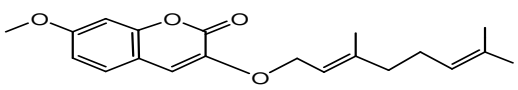
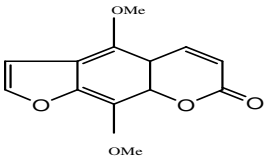


Figure 18

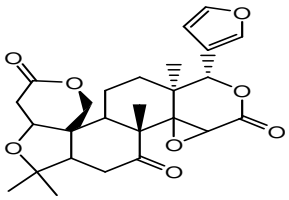
X-ray of compound IIIa (Mackay,1999(15), Altomar, 1999(16) (5,7 dimethoxy coumarin)



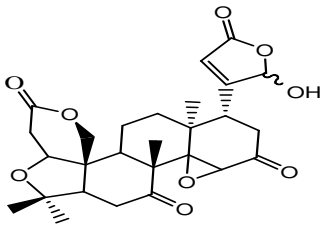
5-geranoxy 7-methoxy coumarin



isopimpenelline



limonin



isolimonexic acid

Figure 19

Discuscion

Control and treatment of parasites in human and animal are important strategies towards the improvement of human public health and also improvement of animal resources and increasing the national income .The traditional treatment against parasites is known by anthelmintic . Inspite of their wide use, anthelmintics have their drawbacks. Many drugs are ineffective at recommended dose, beside the incomplete elimination of the parasites and development of resistant strain of parasite against drug. Moreover, the therapeutic and toxic doses of some anthelmintics are very close and the health hazards of the drug residues upon both human and animal. Recently herbal treatment of parasites is now taken in consideration either by researchers and or pharmaceutical companies due to its safe use and inducing clean environment.

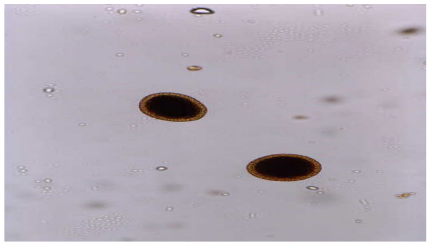
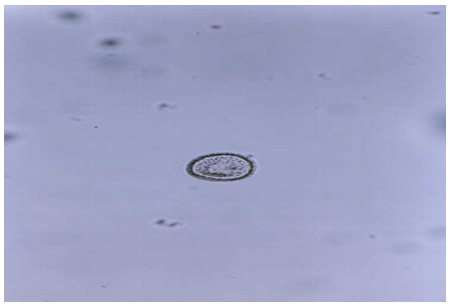
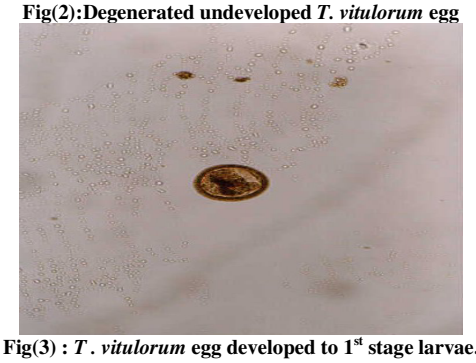


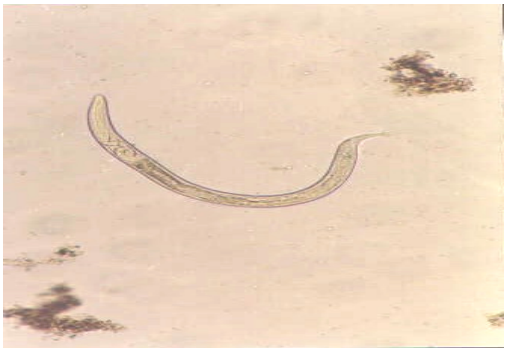
Fig (1):Normal *Toxocara vitulorum* egg



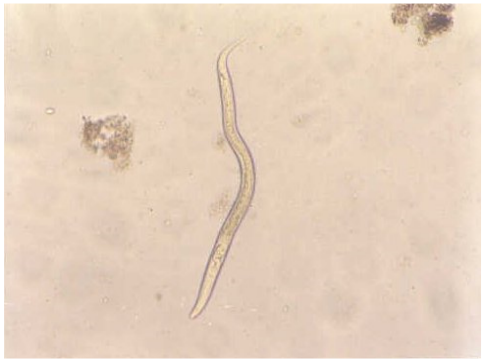
Fig(2):Degenerated undeveloped *T. vitulorum* egg



Fig(3) : *T . vitulorum* egg developed to 1st stage larvae. Lethal effects of the botanical acetone extract of *Citrus aurantifolia* seeds on eggs of Ascaris (*Toxocara vitulorum*) .



Fig(4): Normal *Trichostrongylus colubriformis* larvae



Fig(5):Treated unchanged *Trichostrongylus colubriformis* larvae

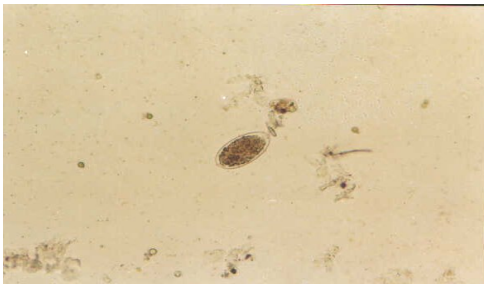


Fig (6): Normal *Trichostrongylus colubriformis* egg

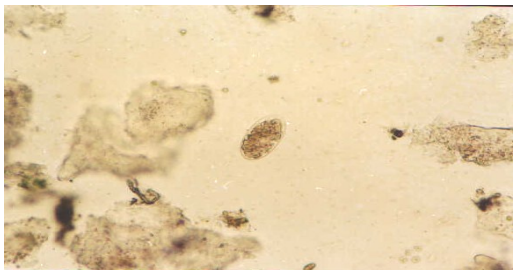
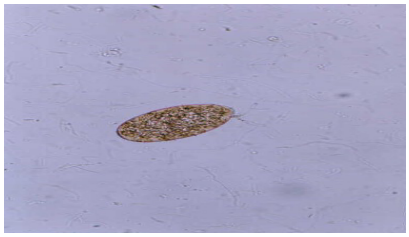
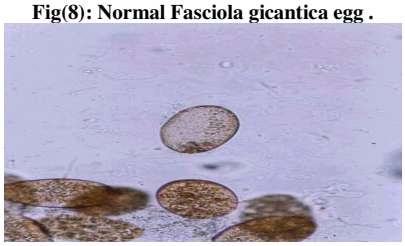


Fig (7) : Degenerated *Trichostrongylus colubriformis* egg

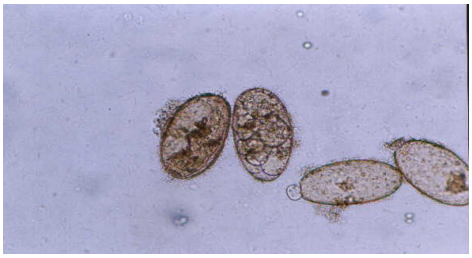
Lethal effect of the botanical acetone extract of *Citrus aurantifolia* seeds on the eggs of *Trichostrongylus Colubriformis*



Fig(8): Normal Fasciola gicantica egg .

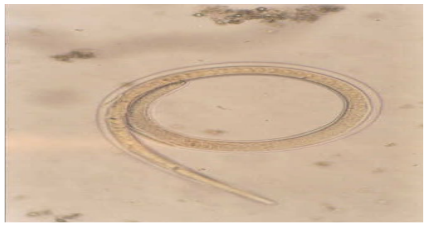


Fig(9) : Degenerated Fasciola egg.



Fig(10) : Developed miracidia &libration of miracidium .(emptyegg)

Lethal effect of the botanical acetone extract of *Citrus aurantifolia* seeds on *Fasciola gigantica* eggs fig9



Fig(11): *Trichinella spiralis* normal larvae

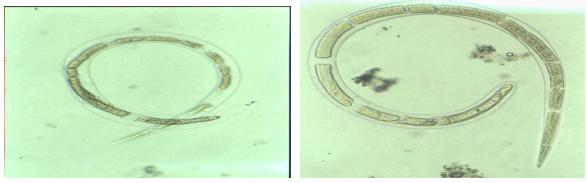
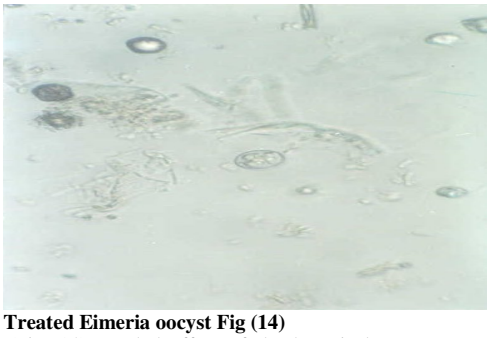


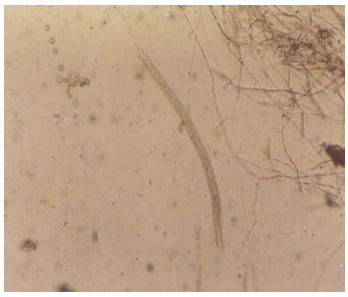
Fig (12): Treated and degenerated *T. spiralis* larvae.

Fig 12 Lethal effect of the botanical acetone extract of *Citrus aurantifolia* seeds on *Trichinella spiralis larvae*

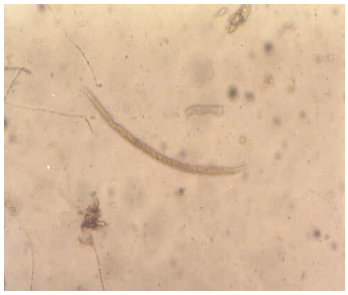


Treated Eimeria oocyst Fig (14)

(Fig. 14) Lethal effect of the botanical acetone extract *of Citrus aurantifolia* seeds on *Eimeria sp*. Oocysts , coccidian parasites of poultry morbid oocyst showed corrugated or pitted wall with degeneration of the developed sporoblasts and the sporozoites



Fig(15) :Untreated *T.colubriformis* larvae



Fig(16) : Treated *T. colubriformis* larvae pitted irregular cuticle

Fig (17) :Treated *T. colubriformis* larvae with detached & separated cuticle.

Phytochemical investigation of the acetone extract revealed it consist of coumarins (20%), isopimpinellin the most dominant whose antibalharzial effect was reported by Haggag (10) meanwhile the limonoids (60%) represented by limonin the most common limonoids in Citrus aurantifolia and limonexic acid. The antifeedant effect of limonoids was reported (18) it was found that three limonoids : limonin , nomilin and obacunone isolated from seeds of C. reticulate inhibit the emergence of fourth instar larvae of mosquito Culex with EC50:59.5,26.6 and 6.3 respectively. Another study (19) reported the in vitro antimalarial activity of limonoids from Khaya grandifoliala .The crude extracts from bark and seeds were active in vitro against plasmodium falciparum. The extract was purified to obtain seven limonoids; the most active was genuine, exhibiting an additive effect when combined with chloroquineThe antifeedant properties of natural limonoids obtained from Citrus limon seeds and their semisynthetic derivatives were evaluated against a commercially important pest Spodoptera frugiperda a highly significant antifeedant activity ($p<0.01$) for limonin, obacunone and three semisynthetic derivatives was observed (20).

Conclusion

The previous data concluded the anthelmintic effect with variation in sensitivity of several types of parasites to the acetone and petroleum ether extract of C. aurantifolia seeds. Fasciola gicantica (Trematodes worms) was the most susceptible parasite to both acetone and petroleum ether extract while T . colubriformis larvae was the most resistant parasite to acetone extract with low sensitivity to the petroleum ether extract. Althougth both Ascaris adult worms and Trichinella larvae (Nematodes) show great resistance to the acetone extract, it was found that the oil was very effective against Trichinella larvae. Concerning Eimeria (protozoa) no serious resistance was observed. Also, it is well noticed that the tested extracts were effective against all the eggs of the tested parasites that reflect the promosing chemoprophylactic effects of C. aurantifolia.

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