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Toxicological and Histological Effects of Licorice *Glycyrrhiza glabra* L., Roots Aqueous Extract on Mediterranean Fruit Fly, *Ceratitis capitata* Wied. (Diptera: Tephritidae) under Laboratory Conditions

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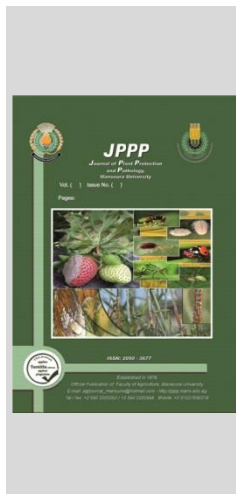

ABSTRACT

Mediterranean fruit fly, *Ceratitis capitata* (Wied.) is a serious horticultural insect pest in Egypt, Mediterranean basin and many countries of the world. Licorice, *Glycyrrhiza glabra* L. is a plant growing in Egypt and many other countries and famous for saponins groups that have insecticidal effect against broad spectrum of insect pests. Licorice roots aqueous extract (LRAE) 1150, 700 and 300 ppm/l were tested for the insecticidal effects against *C. capitata* full-grown larvae and different pupal ages (one, three, five and seven day old pupae) by dipping for 30 and 60 seconds. Dipping treatment in LRAE for 60 seconds decreased the tested stages of *C. capitata* more than 30 seconds treatment. Full-grown larvae population was suppressed by 99.33% after larval dipping in LRAE 1150 ppm for 60 seconds. Based on toxicity values, three days old pupae were the most responded pupal age to LRAE dipping treatments for 60 seconds with LC_{50} 1.5×10^{1} and LC_{90} 1.80×10^{2} ppm/l. Histological studies cleared that larval tissues were affected externally and internally after treatment with LRAE. Emerged flies were not able to either feed or move normally. The histological studies of flies showed that mouthparts, the maxillary palps, foregut neuropil areas, and tracheal tubes were severely affected. The treated flies female ovaries and male testis showed normal view of tissues. Phytochemical screening of powdered licorice roots methanolic extract proved presence of phenols, flavonoids, terpenoids, saponins, tannins and steroids compounds. Saponins compounds were the major constituent of licorice roots methanolic extract.

Keywords: Green pesticides, toxicological effect, histological disorders, phytochemical screening.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (Diptera: Tephritidae), is one of the horticultural pests in Egypt and Mediterranean basin due to its ability to infest more than 300 different host fruit and leave negatively economic impact (Papadopoulos *et al.,* 2001). Flies control depends mainly on partial spray using food attractants with chemical compounds that belong to different groups (organophosphates, especially malathion and pyrethroid) mixed with protein baits (Martinez-Ferrer *et al.,* 2012) and spinosyns in the orchards as well as larvae and pupae found in soil (Stark and Vargas, 2009). However, synthetic pesticides for crop protection in aggravating use cause a number of undesirable effects on human health and the environment (Perry *et al.,* 1998). Recently, the insecticidal characteristics of some plant extracts are subjected for studying their insecticidal and toxic effects against insect pests, as they are economically approachable, biodegradable and easy to use as alternative pest management products (Compos *et al*., 2019). Licorice, *Glycyrrhiza glabra* L. family: Leguminosae is a plant that grows in Egypt and some other countries in the world. Its roots extracts have insecticidal effect on certain pests; with keeping on natural enemies in the environment (Fenwick *et al*., 1990). The root of *Glycyrrhiza sp*. contains triterpenoid saponins (glycyrrhizin, glycyrrhizic acid), phenolics, flavonoids, tannins and steroids but saponins are the major characteristic constituents of licorice (Blumenthal *et al*. 2000 & Shah *et al*. 2018). The insecticidal and the toxicological effects of licorice roots aqueous extract (LRAE) on *C. capitata* , full-grown larvae and different pupal ages are studied in addition for the histological effect on different larvae and seem normal emerged flies' tissues and presented in this work.

MATERIALS AND METHODS

Insect flies:

Ceratitis capitata full-grown larvae and pupae used in experiments were reared in PPRI, HIRD, Giza, Egypt. Adult flies kept in a controlled environment (Temperature 25±2°C, 70± 10% R.H., 12:12 L: D photophase) in cages (80cm, 50cm, 40cm). The flies were fed on enzymatic protein hydrolysate and sugar at a ratio 1:3, respectively and supplied with a water source. Larvae reared using artificial larval rearing medium according to Tanaka *et al*., (1969). Full-grown larvae were allowed to pop in fine sieved and sterilized sand for pupation. Pupae were collected for nine days in complete darkness under laboratory conditions and placed in flies cages a day before emergence.

Preparation of the licorice roots aqueous extract (LRAE):

The Licorice roots aqueous extract (LRAE) was prepared under laboratory conditions according to Siam & El-Genaidy (2021) as the following steps: 100 gm of edible parts of Licorice roots were weighted and added with 175 ml of 6% commercial acetic acid and left for fermentation process until 6 hours under room temperature. The concentrated extract was precipitated through filter paper Wattman No. 1 with 500 ml distilled water for eight hours. Another 500 ml of distilled water were added to the filtrate and left for more four hours until the concentrated extract

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became colorless. The two filtrates were mixed to obtain one liter of aqueous extract (LRAE). A series of dilutions were done to obtain 1150, 700 and 300 ppm/L of LRAE. The three concentrations were tested for their toxicity against *C. capitata* full-grown larvae and different pupal ages (one, three, five and seven days old pupae) by dipping them for 30 and 60 seconds.

Effect of licorice roots aqueous extract (LRAE) different concentrations on *C. capitata* full-grown larvae and pupae:

Ceratitis capitata full-grown larvae were collected in fresh water immediately at popping from the artificial rearing medium and divided into five groups to obtain larvae and four pupal ages (one , three, five and seven days old pupae) for the test. A hundred individuals from each group at its determined age were dipped in LRAE 1150ppm/l for 30 seconds then sieved and transferred in a Petri dishes (9cm) lined with filter paper (Whattman No. 1) disc, covered and kept in complete darkness till flies emergence. All the treatments were replicated five times with control treatments in parallel. Dipping larvae and pupae in distilled water for 30 and 60 seconds was considered the control treatment. The same steps were repeated to test the toxic effect of LRAE 700 and 300 ppm/l on the same stages. Dead individuals, malformed ones and emerged flies were counted and classified. The effect of LRAE concentrations on the larvae and pupal ages by dipping for 60 seconds were ran as mentioned above.

Effect of licorice roots aqueous extract (LRAE) on *C. capitata* emerged flies - histological studies:

The treated larvae and the emerged flies seemed normal that could not either feed or move as normal flies were subjected to some histological studies for effect of the LRAE. The studied larval parts were the larval body wall cells, internal systems cells. The studied flies' parts were mouthparts (labellum) and maxillary parts, foregut, neuropil areas, tracheae areas and the gonads (ovaries and testis). The preparation of insect paraffin embedded sections was done according to Kucherenko *et al*., (2010). The paraffin blocks were cut with 7–10 µm. The sectioned tissues were stained with hematoxylin and eosin (Suvarna *et al*., 2013).

Preparation of licorice roots, *G. glabra* extract for phytochemical studies:

The collected roots of licorice, *G. glabra* L. were cleaned and dried in shade then grind with mechanical grinder then sieved to collect fine powder. The powdered roots were extracted with methanol (50g/250ml methanol) for 24hrs. The extract was filtered using filter paper (Whattman No.1). The extract was subjected to rotary evaporator to remove the solvent and then the extract was used for the further analysis.

Phytochemical screening (qualitative analysis):

Methanolic crude extract of licorice roots, *G. glabra* L. was screened for presence or absence of six bioactive secondary metabolites: phenolic, flavonoid, terpenoid, saponin, tannin and steroid compounds

Qualitative phytochemical tests:

The presence of various phytochemical constituents of methanol extract by following standard phytochemical tests.

Phenols:

To 1 ml of solvent extracts, 3 ml of distilled H_2O was added. To this, a few drops of neutral 5% $FeCl_3$ solution was added. Formation of a dark green colour indicated the presence of phenolics (Gorbach 1993).

Flavanoids:

The extract was treated with a few drops of $FeCl_3$ solution. Formation of a blackish red color indicates the presence of flavonoids (Raman, 2006 & Harborne, 2005).

Terpenoids:

To 1 ml of the solvent extract, 2 ml of chloroform was added. Then 3 ml of conc. H_2SO_4 was added carefully to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids (Evans, 1997).

Saponins:

About 2 ml of distilled H_2O and 1 ml of solvent extract were mixed and shaken vigorously. Formation of a stable persistent froth indicated the presence of saponins (Kumar *et al.,* 2009).

Tannins:

To 1 ml of the solvent extract, few drops of 1% $FeCl_3$ solution were added. The appearance of a blue, black, green or blue green precipitate indicated the presence of tannins (Trease & Evans 1989).

Steroids:

Two ml of acetic anhydride was added to 0.5g ethanolic extract of each sample with 2 ml H_2SO_4. Change in color from violet to blue or green indicates the presence of steroids (Whistler & Bemiller, 1993).

Spectrophotometric measurements (quantitative analysis):

UV/Vis double beam spectrophotometer (Systronix, Model 2202) and standard quartz cuvetts were used for all the absorbance measurements.

Estimation of total phenolic content (TPC) by Prussian blue method:

Spectrophotometric methods are most commonly used for the quantification of phenolic content. The total phenolic content in the methanolic extract was determined by the colorimetric assay by Prussian blue method with some modifications. Briefly, 400 µl 0.1M ferric chloride hexahydrate ($FeCl_3.6H_2O$)in 0.1 M HCl solution was added to 400 µl of sample (1mg/ml) and left to react for 2 min. Then, 400 µl of 0.0008M potassium ferricyanide K3[Fe(CN)6] were added and shaken for 20 s. The final volume was 10 ml. The absorbance was recorded at $\lambda = 700$ nm after 15 min reaction in the dark at 25°C. Different concentrations (0.01-0.2 mg/ml) of gallic acid as standard were prepared in methanol. Results are expressed as gallic acid equivalent (GAE) per gram of sample.

Determination of total flavonoid content (TFC):

The total flavonoid content (TFC) was determined according to the aluminium chloride colorimetric method of Chang *et al*., (2002). with minor modifications. The samples (0.5 ml extract; 1mg/ml stock) were mixed with 1.5 ml distilled water and 0.2 ml 5% $NaNO_2$ and the resultant solution could stand for 2min at room temperature (27± 2°C). Subsequently, 0.2 ml of 10% $AlCl_3$ in ethanol and 0.6 ml 1N sodium hydroxide were added successively with vortexing in each step. The samples were incubated in dark at room temperature for 10 min and the absorbance was measured at 510 nm using a spectrophotometer. The total flavonoid content was quantified and calculated from the calibration curve of Rutin (ranging from 50 to750 μg/ml)

and the result was expressed as mg Rutin equivalent per g dry weight of sample. A standard graph was plotted using various concentrations of Rutin and their corresponding absorbance

Determination of total tannin content (TTC):

TTC were estimated using the modified method of Price & Butler (1977). The extract (500 μl; 1mg/ml stock solution) were mixed with distilled water (8 ml), 0.5 ml of 0.1M $FeCl_3$ and 0.5 ml of 8mM potassium ferricyanide sequentially and incubated at room temperature (27± 2°C) for 10 min. The absorbance was measured at 720 nm using a spectrophotometer. Reagent blanks for each solvent were prepared similarly without adding the sample. The amount of total tannin in the samples was quantified from a calibration curve of tannic acid (ranging from 5.0 to 30 μg/ml) and expressed as mg tannic acid equivalent (TAE) per g dry weight of sample.

Determination of total terpenoids content (TTC):

Total terpenoids were estimated according to the method of Koleva *et al*., (2002) with minor modifications. About 0.2 ml of each extract prepared in ethanol (mg/ml) was evaporated by keeping it in boiling water bath and to the residue; 0.3 ml of vanilin/glacial acetic acid (W/V) was added. 1 ml of perchloric acid was added and incubated at 60°C for 45 minutes. Tubes were cooled in ice and to the mixture, 5 ml of glacial acetic acid was added and the color intensity was measured at 548 nm. The standard curve was plotted using Linalool (10–100 mg/ml). The values were expressed as mg linalool (LE)/g dry sample.

Determination of total saponin content (TSC):

The total saponin content was determined colorimetrically according to the procedures described by Makkar *et al*., (2007). A 250µl sample (1mg/mL) was mixed with 250 µl vanillin (8g/100 ml ethanol) and topped up with 2.5 ml sulfuric acid (72%). The mixture was heated for 10 min at 60ºC, and then cooled in an ice-water bath for 5 min. The absorbance of the mixture was recorded by a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) at 544 nm. Diosgenin (0.1–0.7 mg/ml) was used as the standard calibration curve. The results are expressed as mg diosgenin equivalent per g sample (mg DE/g).

Quantitative Estimation of Steroids:

The total steroids content was determined colorimetrically by Madhu *et al*., (2016). 1ml of test extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70±2^0 C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank. Cholesterol (0.1–0.5 mg/ml) was used as the standard calibration curve. The results are expressed as mg cholesterol equivalent per g sample (mg CE/g).

Statistical analysis:

Larval and pupal mortality percentages were corrected using Abbott's formula (1925) then subjected to one-way analysis of variance (ANOVA) for the differences of variance (SAS, 1985). Pearson's chi squared test was applied for describing the toxicological effects of the tested compounds against *C. capitata* larvae and pupae.

RESULTS AND DISCUSSION

Insecticidal and toxicological effects of LRAE on *C. capitata* larvae and pupae:

Ceratitis capitata full-grown larvae and pupae had responded differently to licorice roots aqueous extract (LRAE) that could decrease the populations after dipping treatment in different concentrations for 30 and 60 seconds. The insecticidal effect of LRAE concentrations on *C. capitata* larvae and pupae was expressed in dead individuals and resulted malformations i.e. the malformed emerged flies were died few hours after emergence. Full-grown larvae were affected significantly after dipping in LRAE 1150, 700 and 300 ppm/l for 30 seconds (F=870.08, P<0.0001, df=3,19, χ^2 =11.60, P=0.0109) (Table 1). LRAE 1150 ppm/l suppressed larval population by 89.76% and 99.33 after dipping for 30 and 60 seconds, respectively while 700 ppm/l decreased the larval population by 83.33% and 79.72% after treatment for 30 and 60 seconds, respectively. The 300ppm/l decreased the larval population by 73.15% and 71.90% after dipping larvae for 30 and 60 seconds, respectively. The larval-pupal intermediate individuals showed significance (F=26.95, P<0.0001, df=2,14, χ^2 = 2.08, P=0.3376). The lowest larval-pupal intermediates percentage was resulted after dipping larvae in LRAE 1150ppm/l while the highest were recorded after dipping in 700 and 300 ppm/l that differed insignificantly (F=1.49, P=0.7577). Pupal mortality appeared after larval dipping in LRAE 1150, 700 and 300 ppm/l and differed significantly (F=3.95, P=0.0430, df=2,14, χ^2 = 1.82, P=0.4019). The highest and lowest percentages of pupal mortality were recorded after dipping in 700 and 300 ppm/l, respectively. Partially emerged flies (head-only) appeared after larval dipping in LRAE 1150ppm/l only. The percentages of the emerged flies that seem normal varied significantly as compared to control treatment (F=1601.58, P<0.0001,df=3,19, χ^2 =11.14, P=0.0110) and within treatments (F= 45.67, P<0.0001, df=2,14, χ^2 =3.64, P=0.1625). There was no significance between the emerged flies percentages after larval treatment with LRAE 1150 and 700 ppm/l (t=0.47, P=0.4767) (P<0.05) but showed significance with 300 ppm/l (t=8.68, P<0.0001). Emerged flies percentages resulted from larval treatment with 700 and 300 ppm/l were significant (t=7.95, P=0.0014). The larval dipping treatment in LRAE 1150, 700 and 300 ppm/l for 60 seconds increased larval mortality percentages and differed significantly (F=300.81, P<0.0001, df=2,14, χ^2 = 0.307, P=0.8577). LRAE 1150 ppm/l caused the highest mortality percentage while the lowest was treatment with 300 ppm/l. Larval-pupal intermediate individuals appeared after larval dipping in all LRAE concentrations specially at 700 ppm/l that resulted in the highest percentage and 1150 ppm/l that showed the lowest one (F=10.52, P=0.0005). The highest percentages of dead pupae after larval treatments resulted after larval dipping in LRAE 700ppm/l and varied significantly with the other treatments (F=11.73,P=0.0015, df=2,14, χ^2 =0.601, P= 0.7405). The partially emerged flies (head-only) percentages were recorded after treatment with 700 and 300 ppm/l showing significant difference (t=2.95, P=0.0418). Dipping larvae in LRAE 1150 ppm/l for 60 seconds caused the lowest emerged flies percentages that differed significantly with the other treatments (F= 1862.33, P<0.0001, df=3,19, χ^2 = 22.097, P<0.000) (Table 1).

Table 1. Insecticidal effect of LRAE different concentration on *C. capitata* full-grown larvae

LRAE in ppm/l	Mean % of dead larvae ±SE	Mean % of larval-pupal intermediates ±SE	Mean % of dead pupae ±SE	Mean % of partially emerged flies ±SE	Mean% of seem normal flies ±SE
	Dipping of larvae for 30 seconds				
1150	68.33±1.60	10.84±0.74	9.69±0.63	0.40±0.25	10.04±0.63
700	49.01±1.28	22.49±1.63	11.25±1.21	0.00±0.00	16.47±0.93
300	42.81±0.71	22.71±1.48	7.63±0.71	0.00±0.00	26.65±1.73
0	0.00±0.00	0.00±0.00	0.20±0.09	0.00±0.00	99.80±0.54
	Dipping of larvae for 60 seconds				
1150	94.89±1.41	0.62±0.55	3.82±0.97	0.00±0.00	0.47±0.03
700	57.63±1.07	10.84±0.73	9.64±0.63	1.61±0.55	20.08±1.24
300	58.64±1.20	1.61±0.01	7.63±0.95	4.02±0.40	27.90±0.51
0	0.00±0.00	0.00±0.00	0.20±0.09	0.00±0.00	99.80±0.54

0 concentration = dipping in distilled water

C. capitata pupae aged one, three, five and seven days were affected after dipping in LRAE different concentrations. LRAE 1150ppm/l lowered the treated one-day old pupae for 30 and 60 seconds by 51.04% and 50.63%, respectively. One-day old pupae dipped in 700 ppm/l for 30 seconds resulted in the highest pupal mortality percentage (53.09%) and differed significantly as compared to 1150, 300 ppm/l and control treatment (F=2455.51, P<0.0001, df=3,19, χ^2 = 2.23, P=0.5265) and within treatments (F= 1560,22, P<0.0001, df= 2,14, χ^2 =0.424, P=0.8090). The highest pupal mortality percentage accompanied LRAE 700ppm/l while the lowest was after dipping in 300 ppm/l (11.70%). Partially emerged individuals (head-only) were recorded after dipping in LRAE 1150,700 and 300ppm/l and percentages varied significantly (F=11.23, P=0.0018, df=2,14, χ^2 = 0.936, P+0.6261). Partially emerged individuals were not observed at the control treatments. Flies emerged with wrinkled wings appeared after one-day old pupae dipping in LRAE and the highest percentage accompanied 1150 ppm/l for 30 seconds that differed significantly with 700 ppm/l (t=5.21, P=0.0065) and with 300 ppm/l (t= 8.84,P=0.0009). The seem normal emerged flies percentages of all LRAE treatments showed variation (F= 375.85, P<0.0001, df=2,14, χ^2 =7.35, P=0.0262). There was no significant difference between the percentages resulted from 1150 and 700 ppm/l (P>0.05) but high significance occurred with 300 ppm/L (P<0.001). Dipping one-day old pupae in LRAE 1150, 700 and 300 ppm /l for 60 seconds decreased flies emergence (F= 2545.22, P<0.0001, df=2,14, χ^2 =2.87, P=0.2470) (Table 2). LRAE 1150 ppm/l resulted in the highest pupal mortality percentage(50.63%) and varied significantly with 700 ppm/l (50.08%)(P<0.01) and highly significant with 300 ppm/l (38.29%) (P<0.001). Dipping one-day old pupae in LRAE for 60 seconds decreased flies emergence more than 30 seconds. Pupal mortality percentages resulted from dipping one-day old pupae in LRAE 1150 ppm/l for 60 seconds were highly significant with those treated for 30 seconds (t=34.00, P<0.0001), 700 ppm/l showed significance (t=4.47, P=0.0090) while 300 ppm/l were not significant (t=1.81, P=0.1447). The partially emerged (head-only) individuals differed insignificantly after dipping in 1150 ppm/l for 30 and 60 seconds (t=0.34, P=0.7489) but 700 and 300 ppm/l showed significant difference between the two dipping periods (t=6.00, P=0.0039) and (t=8.62, P=0.0010), respectively. Flies emerged with wrinkled wings percentages increased after pupal dipping in LRAE 700 ppm/l for 60 seconds more than 30 seconds(t=6.00, P=0.0039) and the same with 300 ppm/l (t=31.24, P<0.0001). Treating three days old pupae by dipping in LRAE 1150 ppm/l for 30 seconds showed no dead pupae but decreased the population by 97.45% (Table 2).

Table 2. Insecticidal effect of LRAE different concentration on *C. capitata* different aged pupae

LRAE in ppm/l	Dipping for 30 seconds: Mean % of dead pupae ± SE	Mean % of head-only emerged flies ± SE	Mean % of wrinkled wings flies ± SE	Mean % of seem normal flies ± SE	Dipping for 60 seconds: Mean % of dead pupae ± SE	Mean % of head-only emerges flies ± SE	Mean % of wrinkled wings flies ± SE	Mean % of seem normal flies ± SE
				One-day old pupae				
1150	34.52±0.51	1.61±0.37	15.31±2.68	48.56±2.13	44.60±0.50	1.61±0.32	4.82±0.34	48.97±0.51
700	46.45±0.44	2.01±0.24	6.02±0.40	46.51±0.81	41.25±0.37	0.80±0.21	8.43±0.40	49.52±0.49
300	10.04±0.51	1.13±0.21	0.93±0.01	87.90±0.50	9.04±0.20	3.14±0.44	26.51±0.90	61.31±0.36
0	0.40±0.25	0.00±0.00	0.00±0.00	99.60±0.11	0.40±0.25	0.00±0.00	0.00±0.00	99.60±0.11
				Three days old pupae				
1150	0.00±0.00	8.60±0.40	88.85±0.58	2.55±0.87	19.90±0.97	0.60±0.55	77.11±0.37	2.39±0.25
700	0.00±0.00	0.80±0.20	94.78±0.37	4.42±0.24	8.23±1.22	0.80±0.36	87.15±0.52	3.82±0.81
300	0.00±0.00	4.62±0.32	85.54±0.75	9.84±0.51	10.35±0.41	4.21±0.40	79.91±0.33	5.53±0.49
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
				Five days old pupae				
1150	5.22±0.41	0.20±0.21	5.62±0.34	88.96±0.88	1.04±0.25	1.81±0.37	22.92±0.81	74.23±0.71
700	3.61±0.32	0.61±0.31	5.22±0.41	90.56±0.68	0.61±0.32	1.21±0.40	19.16±0.40	79.02±0.25
300	2.01±1.03	0.20±0.24	10.84±0.58	86.95±1.08	0.40±0.37	0.21±0.26	17.87±0.33	81.52±1.53
0	0.20±0.09	0.00±0.00	0.00±0.00	99.80±0.54	0.20±0.09	0.00±0.00	0.00±0.00	99.80±0.54
				Seven days old pupae				
1150	0.68±0.20	0.20±0.01	0.20±0.23	98.92±0.34	6.23±0.42	0.21±0.20	3.01±0.44	90.55±0.61
700	0.20±0.22	0.01±0.20	0.02±0.21	99.97±0.37	10.84±0.38	1.01±0.26	5.42±0.21	82.93±0.25
300	0.00±0.00	0.00±0.00	0.00±0.00	100±0.00	0.00±0.00	0.20±0.24	2.98±0.20	96.82±0.22
0	0.20±0.09	0.00±0.00	0.00±0.00	99.80±0.54	0.20±0.09	0.00±0.00	0.00±0.00	99.80±0.54

0 concentration = dipping in distilled water

Partially emerged individuals (head-only) resulted from dipping pupae in LRAE 1150, 700 and 300ppm/l for 30 seconds differed significantly (F=166.77, P<0.0001, df=3,19, χ^2 =1.96, P=0.5817) and within treatments (F=152.40, P<0.0001, df=2,14, χ^2 =1.63, P=0.4433). Flies emerged with wrinkled wings were present at LRAE treatments with significant difference as compared to control treatment (F=7307.83, P<0.0001, df=3,19, χ^2 =4.61, P=0.2028) and within LRAE treatments (F=62.92, P<0.0001, df=2,14, χ^2 =1.69, P=0.4495). The seem normal flies resulted from dipping pupae in LRAE 1150, 700 and 300 ppm/l showed high significant difference as compared to control treatment (F= 7790.44, P<0.0001, df=3,19, χ^2 =8.06, P=0.0449). The emerged seem normal flies percentages resulted from dipping pupae in 1150 and 700 ppm/l for 30 seconds differed insignificantly (P>0.05) but showed significant difference with 300 ppm/l (P<0.001). The flies percentages showed inversely proportional relationship as the LRAE concentration decreased the emerged seem normal flies increased. Partially emerged (Head-only) individuals percentages appeared after dipping pupae in LRAE 1150, 700 and 300 ppm/l were significant (P<0.001). The highest percentage of flies emerged with wrinkled wings observed after dipping pupae in LRAE 700 ppm/l for 30 seconds that differed significantly with 1150 ppm/l (P<0.001) and with 300 ppm/l (P<0.05). Dipping three days old pupae in LRAE 1150, 700 and 300 ppm/l for 60 seconds increased dead pupae percentages by 97.61%, 96.18% and 94.47%, respectively and differed significantly as compared to control treatment (F= 128.55, P<0.0001, df=3,19, χ^2 =8.98, P=0.0307) and within LRAE treatments (F=44.81, P<0.0001, df=2,14, χ^2 =?, P=0.1859). The partially emerged (head-only) individuals percentages differed insignificantly among LRAE treatments compared to control (F=1.52, P=0.2488, df=3,19, χ^2 =2.91, P=0.5303) and within treatments (F=0.47, P=0.6380, df=2,14, χ^2 =0.63, P=0.7306). Flies emerged with wrinkled wings after LRAE 1150, 700 and 300 ppm/l dipping treatments differed highly significant as compared to control treatments (F=11635.14, P<0.0001, df=3,19, χ^2 =2.051, P=0.5618) and within treatments (F=212.24, P<0.0001, df=2,14, χ^2 =0.868, P=0.6479). Dipping three days old pupae in LRAE 1150 and 700 ppm/l for 60 seconds showed significant difference in flies emerged with wrinkled wings (P<0.001) while were not significant with 300 ppm/l (P>0.05). The seem normal flies percentages produced after LRAE, 1150, 700 and 300 ppm for 60 seconds were highly significant as compared to control treatments (F=8877.49, P<0.0001, df=3,19, χ^2 =7.248, P=0.0644) and were significant within treatments (F=50.042, P<0.0001, df=2,14, χ^2 =4.435, P=0.1089). LRAE 1150 and 700 ppm/l affected production of the seem normal flies significantly (P<0.001) and with 300 ppm/l (P<0.01). Dead pupae percentages increased as pupal dipping periods increased. Dead pupae percentages resulted from dipping three days old pupae in LRAE 1150 ppm/l for 30 and 60 seconds revealed high significance (t=20.835, P<0.0001), 700 ppm/l (7.661, P=0.0016) and 300 ppm/l (t= 41.50, P<0.0001). There was a significant difference between the partially emerged individuals percentages after dipping in 1150 ppm/l and 300 ppm/l for 30 and 60 seconds (F=10.156, P=0.0005) and (t= 6.668, P=0.0026), respectively, while dipping in 700 ppm/l differed insignificantly (t=1.00, P=0.3739). The percentages of flies emerged with wrinkled wings were significant after dipping three days old pupae in LRAE 1150 ppm/l for 30 and 60 seconds (t= 19.33, P<0.0001), 700 ppm/l (t= 18.50, P<0.0001) and 300 ppm/l (t= 9.274, P=0.0008). Five days old pupae dipped in LRAE 1150 ppm/l for 30 seconds resulted in dead pupae percentage (10.84%) and differed insignificantly with 700 ppm/l (9.24%)(P>0.05) but showed significant difference with 300 ppm/l (12.85%)(P<0.05). Partially emerged (head-only) individuals highest percentages accompanied dipping pupae in LRAE 700 ppm/l for 30 seconds. Flies emerged with wrinkled wings were recorded after dipping five days old pupae in LRAE 1150 and 700 ppm/l that differed insignificantly (P>0.05) while the highest percentage was observed after dipping in 300 ppm/l that reflected a significant difference with the other treatments (P<0.001). The highest pupal percentage was recorded after dipping pupae in 1150 ppm/l for 60 seconds (25.57%) and the lowest was after dipping in 300 ppm/l for the same period (18.28%). LRAE 1150, 700 and 300 ppm/l resulted in dead pupae percentages that differed insignificantly (P>0.05). Partially emerged (head-only) individuals appeared after dipping pupae in LRAE all concentrations. Percentages of head-only individuals resulted after dipping pupae in 1150 ppm/l differed insignificantly with 700 ppm/l (P>0.05) and significantly after dipping in 300 ppm/l (P<0.01). Percentages of flies emerged with wrinkled wings after dipping pupae in LRAE 1150 ppm/l for 60 seconds differed significantly with 700 ppm/l (P<0.01) and with 300 ppm/l (P<0.001). The least percentage of the seem normal flies were due to dipping pupae in LRAE 1150 ppm/l and the highest was related to 300 ppm/l treatment. The seem normal flies percentages due to dipping five days old pupae in LRAE concentrations for 60 seconds differed significantly (F=23.51, P<0.0001, df=2,14, χ^2 =8.663, P=0.0132). Seven days old pupae treated by dipping in LRAE 1150, 700 and 300 ppm for 30 seconds resulted in dead pupae percentages 0.88%, 0.17% and 0.00%, respectively and differed insignificantly (F=1.88, P=0.1919, df=3,19, χ^2 =2.43, P=0.9703) (P>0.05). Dead pupae percentages increased by increasing dipping period. Dipping seven days old pupae in LRAE 1150 ppm/l for 60 seconds increased dead pupae percentages (8.85%) more than dipping in 30 seconds (0.88%) (t=1184.,P=0.0003). Partially emerged (head-only) flies percentages differed insignificantly after dipping pupae for 30 and 60 seconds (t=0.535, P=0.6213). Flies emerged with wrinkled wings percentages increased after treatment for 60 seconds (t= 7.53, P=0.0017). Dead pupae percentages increased after dipping pupae in 700 ppm/l for 60 seconds (16.87%) and differed significantly with those resulted after dipping for 30 seconds (0.17%) (t=3.21, P=0.0327). Flies emerged with wrinkled wings increased after dipping pupae for 60 seconds and reflected significance on comparison with 30 seconds (t=6.532,P=0.0028). The emerged seem normal flies percentages decreased after treating pupae for 60 seconds reflecting significance with the results of 30 seconds (t=34.52, P<0.0001). Dipping pupae in LRAE 300 ppm/l for 30 and 60 seconds resulted in dead pupae percentages 0.00% and 2.98% that differed insignificantly (t=1.00, P=0.3739).

Partially emerged (head-only) individuals percentages resulted after dipping in LRAE 300 ppm/l for 30 and 60 seconds were not significant (t=1,633, P=0.1778) while flies emerged with wrinkled wings percentages showed significance between the two dipping periods (t=16.00, P<0.0001). The seem normal flies percentages decreased by dipping pupae for 60 seconds more than dipping for 30 seconds (t= 13.90, P=0.0002). Toxicity values of LRAE tested concentrations on *C. capitata* full-grown larvae and four pupal ages (one, three, five and seven days old pupae) are presented in Table (3). The obtained LC_{50} and LC_{90} values declared that the three days old pupae were the most responded stage to LRAE treatments for 30 and 60 seconds followed by the full-grown larvae and the one-day old pupae while five and seven days pupae showed more resistance. The control of fruit flies is difficult because full-grown larvae infest the fruits and drop to the soil to pupate; consequently, both larvae and pupae are protected from foliage insecticides (Heve *et al.,* 2017). Plant extracts that have insecticidal effects are used as organic pesticides. Licorice, *Glycyrrhiza glabra* L., roots contain phenolics, tannins, flavonoids but the major ingredient are saponins (glycyrrhizin). Saponins have insecticidal activities against broad range of pest insects (De Geyter *et al.* 2007). Zapata *et al*. (2006) tested the aqueous extract of ground leaves of *Cestrum parqui* L. that contains saponins on *Ceratitis capitata* larvae and reported a significant decrease of flies' emergence. Pelah *et al*. (2002) found that saponins extracted from soapbark tree, *Quillaria saponaria* have larvicidal activity against the mosquitos' species, *Aedes aegypti* and *Culex pipiens* L. (Diptera: Culicidae) 100% mortality was obtained by using and amount of 1000 mg/L (1000000ppm/L) during five days. Malformations of the emerged flies accompanied all the applied treatments. These malformations indicate that physiological disturbance occurred during *C. capitata* development. Saponins present an excellent model of insecticidal effect and multitude physiological effects (Chaieb, 2010) and (El-Genaidy *et al.,* 2021). Khater (2020) studied the effect of azadirachtin (neem leaves extract) on peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae) larvae and pupae and reported flies' morphological abnormalities represented in incomplete flies emergence from pupae and crumbled or wrinkled wings. Khater (2020) studied the insecticidal activity of Azadirachtin 15% (EC) on the lesser pumpkin fly, *Dacus ciliatus* L. (Diptera: Tephritidae) larvae and pupae and recorded malformed emerged flies with wrinkled wings and deformed female's ovipositor. These findings may explain the similar malformation recorded in the present study. The morphological abnormalities of azadirachtin insecticide may be a result of inhibition of the release of prothoracicotropic hormones and allatotropins (Mordue & Blackwell 1993, Williams & Mansingh 1996).

Table 3. Toxicological values of LRAE in ppm/l on *C.capitata* full-grown larvae and different aged pupae

C.capitata treated	Dipping in LRAE for 30 seconds					Dipping in LRAE for 60 seconds				
stage	LC_{50}	LC_{90}	χ^2	Slope±SE	P	LC_{50}	LC_{90}	χ^2	Slope±SE	P
Full-grown larvae	7.7×10^{1}	12.47×10^{2}	0.624	1.068±0.28	0.4295	2.81×10^{2}	16.15×10^{2}	127.54	1.782±0.15	0.0000
One-day old pupae	8.18×10^{2}	2.61×10^{4}	48.93	2.542±0.1473	0.0000	6.60×10^{2}	5.23×10^{5}	2.66	0.675±0.14	0.1031
Three days old pupae	1.7×10^{1}	2.81×10^{2}	0.022	1.120±0.22	0.9018	1.5×10^{1}	1.80×10^{2}	2.28	1.283±0.34	0.1307
Five days old pupae	3.36×10^{5}	3.06×10^{8}	0.101	0.122±0.18	0.7508	1.80×10^{4}	4.55×10^{8}	0.167	0.533±0.15	0.6828
Seven days old pupae	2.23×10^{5}	4.24×10^{8}	1.243	1.001±0.672	0.2649	4.24×10^{4}	2.59×10^{8}	0.139	1.654±0.22	0.7721

Histological studies on *C. capitata* larvae and emerged flies:

The larvae treated with LRAE showed variation of the external and internal body wall cells (Fig.1 a&b) and (Fig.2 c&d). The insecticidal activities of plant extract may be attributed to high content of saponins. The insecticidal effects of the saponin extracts were evaluated by El Genaidy et al., (2021) who studied the effect of *G. glabra* L extract on *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) by contact toxicity test in sandy and clay soils. The dark color of *C. capitata* treated larvae body (Fig. 1 b) is not ordinary to be seen after treatment. Cui et al (2019) studied effect of saponins on *E. obliqua* and found that saponins disrupt the waxy layer of the epidermis causing serious loss of water, and can penetrate the intestine of insect, this may prove that saponins can destroy not only the waxy layer on the surface of, but also the chitin layer both outside and inside**.** These findings may support our histological studies results (Fig.2 d and Fig. 3 e&f). The seemed normal *C. capitata* flies emerged from treated pupae with LRAE were different from those emerged from the control treatments. The flies were neither able to feed nor to move naturally. Mouth and maxillary parts of the untreated flies showed normal view of pseudotracheal structure with clear chitinacious structure and food (Fig. 4 g) while the treated flies reflected absence of pseudotracheal tubes and necrosis of the labellum cells (Fig. 4 h). The foregut of treated flies was affected showing atrophied enterocyte, degeneration necrosis of enterocytes and adjacent cells with sloughing in many parts. Foregut of treated fly stucked and plugged with coagulated substance with degenerative changes of its epithelium (fig.5 j&k). Chaeib *et al*., (2009) showed structural modifications at the fat body of *Spodoptera littoralis* (Lepidoptera: Noctuidae) as well as on the foregut and the gastric caeca of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) that were due to the cytotoxicity effect of *Cestrum parqui* saponins. Their results indicate that tea saponins can cause physiological and morphological damage to gut epithelial cells. Excessive toxicity of tea saponins leads to smaller microvilli in the gut and to cell death. The mode of action of saponins seems in relation to the property of its molecules to be interacted either with structural cholesterol (membrane) or with metabolic cholesterol (food). Saponins affect the water balance of the treated pest by destroying the waxy layer on the epidermis surface allowing penetration into the body and finally resulting in death due to water loss (Balabanidu *et al*., 2018). The histological studies declared changes in flies neuropil area showing severe lipolysis of fat body with loss of nuclei (Fig. 7. l) and vaccuolations of neuropil area and nuclei of neuronal cells (Fig.8 m). Normal flies tracheae showed Normal tracheal tubes with epithelial cells and taenidia covered by epicuticle and normal spiracles (Fig.9. n) while treated fly showing degenerated tracheal tube with loss of taenidial tubular structure and loss of chitin

layer (Fig.9. o). Female flies ovaries and males testis showed no changes in treated tissues (Fig.10. p,q,r &s).

Phytochemical screening and qualitative analysis of licorice *G. glabra* L. roots:

Phytochemical screening revealed the presence of secondary metabolites phenols, flavonoids, terpenoids, saponins, tannins and steroids in the methanolic root extract of *G. glabra* L. The qualitative analysis of licorice roots methanolic extract showed variety in amounts of detected constituents that could be arranges as follows: saponins, steroids, phenols, tannins, flavonoids while terpenoids showed the lowest amount (Table 4). Saponins in *G. glabra* L. roots methanolic extract occupied the major amount. Saponins, a group of steroidal or triterpenoidal secondary plant metabolites with insecticidal activities (Francis *et al*., 2002). The insecticidal activities of plant extract may be attributed to high content of saponins. The insecticidal effects of the saponin extracts were evaluated by El-Genaidy et al (2021) who studied the effect of *G. glabra* L extract on *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) by contact toxicity test in sandy and clay soils. The dark color of *C. capitata* treated larvae body (Fig. 1 b) is not ordinary to be seen after treatment. Cui et al (2019) studied effect of saponins on *Ectropis obliqua* P. and found that saponins disrupt the waxy layer of the epidermis causing serious loss of water, and can penetrate the inside of the intestine of insect, this proved that saponins can destroy not only the waxy layer on the surface of, but also the chitin layer both outside and inside. These findings may support our histological studies results (Fig.2 d and Fig. 3 e&f). Ecdysteroid receptors are potential targets in the development of selective insect growth regulators because they work selectively against insects, and sometimes even a particular order or stage. Sterols were also reported by Nayer and Fraenkel (1962) to inhibit the feeding of some insects. It is an evident that these steroids as constituents of the G. glabra L. may have properties cause retardation in the pupal and adult morphogenesis. Nomura and Itioka (2002) studied the efficiency of synthesized tannins and found that higher dosages of tannins, approximately from 2 mg/g, cause mortality also in the course of larval development. Furthermore, tannins possess larvicidal and repellent properties; they influence the growth, development, and fecundity of several phytophagous insects (Acheuk *et al.,* 2014). Indeed, ingestion or fumigation of tannins affect the integrity of the digestive tract of phytophagous insects (Ayres *et al.,* 1997), and cause death and malformations in offspring (Carpinella *et al.,* 2003). Phenols and flavonoids were reported to be toxic to some insects (Salama *et al.,* 1970). The presented results correlate those found in the literature (Rampadarath *et al.,* 2014), where phenolic compounds were reported to exert antimicrobial and insecticidal effects. Khan *et al.,* (2016) reported the toxic effect of terpenoid compounds of some plant extracts on *B. zonata*.

Table 4. Phytochemical analysis and quantitative estimation of licorice, *Glycyrrhiza glabra* L. aqueous extract

Secondary metabolite	Phytochemical analysis test	Results	Contents (mg/gm)
Phenolics	Ferric chloride test	Positive	86.91±3.75
Flavonoids	Lead acetate test	Positive	63.73±10.65
Terpenoids	Salkowski test	Positive	15.67±1.53
Saponins	Foam test or Froth test	Positive	416.67±5.77
Tannins	Ferric chloride test	Positive	64.53± 2.63
Steroids	Salkowski test	Positive	239.53±10.18

Fig. 1. a) Normal *C. capitata* larva; b) *C. cpitata* larva treated with LRAE.

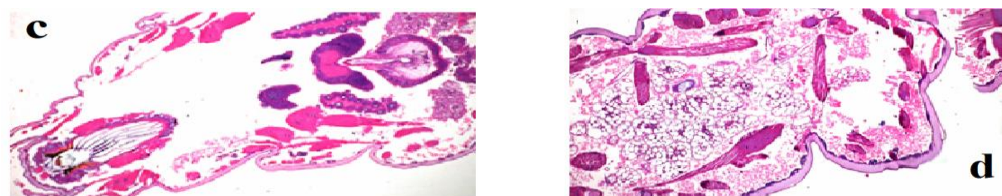

Fig. 2. c) Normal larva anterior area contains eye structure, mouth parts, brain and wing disc; d) presence of fat body vacuoles and absence of body systems (Stain H&EX 400).

Fig. 3. e & f) Treated larva showing atrophied rudimentary and compressed body of larva with atrophied and calcified internal cells in the periphery of body wall and calcification of internal structure (Stain H&EX 400).

Fig. 4. g) Control mouth part (labellum part) showing normal regular distributed pseudotracheal structure and normal food content ; h) Treated fly showing bending and twisting of mouth part and normal eye and brain structure (StainH&EX600).

Fig. 5. i) Normal maxillary palp and hypopharynx structure in head part (StainH&EX200; j) Treated fly showing modification and severe distortion of the maxillary palp (which have olfactory sensilla) labral and cibarial organs located inside the pharynx and have taste organ (StainH&EX600).

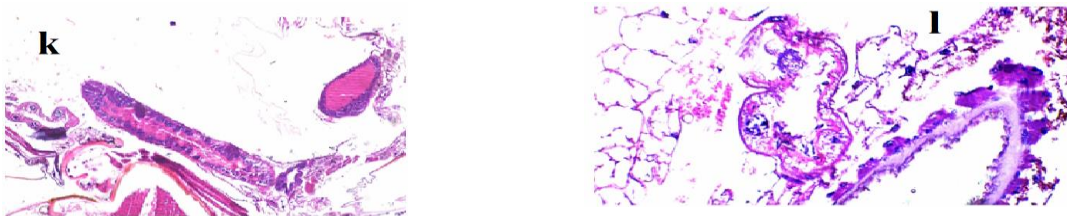

Fig. 6. k) Foregut of treated fly stucked and plugged with coagulated substance with degenerative changes of its epithelium (StainH&EX400).

Fig. 7. l) Treated fly showing severe lipolysis of fat body with loss of nuclei (StainH&EX600).

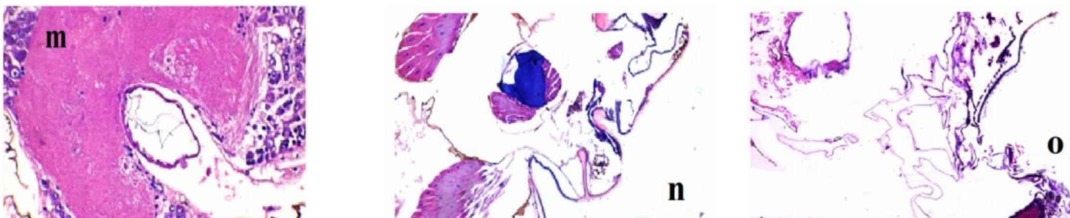

Fig. 8. m) LRAE treated fly showing vaccuolations of neuropil area and nuclei of neuronal cells (StainH&EX600).

Fig. 9. n) Normal tracheal tubes with epithelial cells and taenidia (tubular structure have chitinious structure) covered by epicuticle and normal. spiracles (Stain H&EX600); o) treated fly showing degenerated tracheal tube with loss of taenidial tubular structure and loss of chitinious layer (StainH&EX400).

Fig. 10. p & q) Control and treated fly showing ovary with primordial follicles (long thick arrow) surrounded by granulosa cells in the periphery, developing follicle and mature follicle (StainH&EX400); r&s control and LRAE treated male posterior part normal structure with normal testis (StainH&EX400).

CONCLUSION

Licorice, *Glycyrrhiza glabra* L. roots aqueous extract (LRAE) has an insecticidal potential against *Ceratitis capitata* larvae and pupae. Histological studies on larvae and emerged flies proved the deterioration of external and internal tissues of larvae and degenerative effect on foregut, neuropil areas, tracheae, while the females and males gonads were not affected. The phytochemical screening and qualitative analysis proved presence of saponins as a major

constituent of licorice roots in addition of steroids, phenols, tannins, flavonoids and terpenoids.

Licorice roots aqueous extract (LRAE) is an effective green insecticide that can control Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann).

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التأثير السُمى و النسيجى لمستخلص جذور العرقسوس المائى *Glycyrrhiza glabra* L. على ذبابة فاكهة البحر المتوسط *Ceratitis capitata* Wied. (Diptera: Tephritidae) تحت الظروف المعملية

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تعد ذبابة فاكهة البحر المتوسط من الأفات الحشرية الهامة في مصر ودول حوض المتوسط و دول العالم الأخرى؛ و يعتبر نبات العرقسوس من النباتات واسعة الإنتشار في مصر و دول العالم الأخرى و الذى بدوره يتميز بوجود مجموعة الصابونين ذات التأثير الإبادى واسع النطاق على العديد من الأفات الحشرية. تم في هذه الدراسة إختبار التأثير الإبادى للتركيزات 1150،700 و 300 جزء في المليون/ لتر على اليرقات الكاملة و العذارى عمر يوم ، ثلاثة ، خمسة وسبعة أيام عن طريق الغمس لمدة 30 و 60 ثانية. أوضحت النتائج أن مستخلص جذور العرقسوس المائى بتركيز 1150 جزء في المليون/لتر كانت له القدرة على خفض التعداد اليرقى بنسبة 99.33% بعد غمسها لمدة 60 ثانية. وبينت الدراسة أن العذارى عمر ثلاثة أيام كانت أكثر أعمار العذارى استجابة لتأثير مستخلص جذور العرقسوس المائى بعد غمسها لمدة 60 ثانية حيث كانت القيم السُمية للمستخلص فقد كانت LC_{50} تساوى 1.5×10^1 و LC_{90} تساوى 1.80×10^2 جزء في المليون/لتر. و قد بينت الدراسة النسيجية تأثير مستخلص جذور العرقسوس المائى على أنسجة اليرقات ظاهريا و داخليا و كذلك التأثير على أنسجة الذباب الناتج من معاملات اليرقات و العذارى ؛ فقد تأثرت أنسجة الأجزاء الفمية و المعى الأمامى و النسيج العصبى و خلاياه و المجارى التنفسية أما الأعضاء التناسلية للإناث متمثلة في المبايض و الأعضاء الذكرية ممثلة في الخصية فإنها لم تُظهر تأثرا بمستخلص العرقسوس المائى. و بتحليل المجموعات الكيمائية للمستخلص الميثانولى لمسحوق جذور العرقسوس فقد ظهر مجموعات الفينولات و الفلافونويدات و التيربينويدات و الصابونين و الطانين و الستيرويدات و كان لمجمعة الصابونين النصيب الأكبر في المحتوى للمستخلص الميثانولى لمسحوق جذور العرقسوس.