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Evaluation of molluscicidal, miracicidal and cercaricidal activities of crude aqueous extracts of *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* on *Schistosoma mansoni* and *Schistosoma haematobium*

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ABSTRACT

Schistosomiasis has been classified as a category II disease after malaria in importance as a targeted tropical disease. Praziquantel (PZQ) which is the chemotherapeutic agent of choice against adult worms, already faces drawback of drug resistance in some *Schistosoma* isolates. Therefore, searching for new alternative drugs has been the intention of many researchers. In the current study, the effect of different doses 6.25, 12.5, 25. 50. 100 and 200 mg/ml of *Origanum majorana*, *Ziziphus spina-christi*, *and Salvia fruticosa* extracts on the snails *Bulinus truncatus* of the *S. haematobium* and *Biomphalaria alexandrina* of the *Schistosoma mansoni* as well as on the free-living stages miracidia, in addition, the cercariae for both Egyptian species of schistosomes were studied. The results declared that *Origanum majorana*, *Ziziphus spina-christi* showed efficacy against snails (molluscicidal), miracidia (miracicidal), and cercariae (cercaricidal) for both Egyptian species of schistosomes in the *in vitro*, while *Salvia fruticosa* was less effective than the previous ones.

INTRODUCTION

Infectious diseases are the infection of a living organism with one of these five organisms: viruses, bacteria, fungi, protozoa, and helminthes. Worldwide, annually due to infectious diseases more than 13 million deaths occur, the largest proportion of which are in the developing world (Sachs, 2001). About 218 million people suffer from schistosomiasis worldwide, it's a weaken and sometimes fatal disease, nearly, on the African continent no country is safe from this infection, currently, 90% of the cases present in Africa alone especially in sub-Saharan, and two species only out of five species (*Schistosoma mansoni* and *Schistosoma haematobium*) infect human causing 80% of schistosomiasis in Africa (Anyan, *et al*., 2019; Ndassi *et al*., 2019), therefore according to the WHO Special Program for Research and Preparing in Tropical Diseases, schistosomiasis has been classified as a category II disease after malaria in importance as a targeted tropical disease. Possible results of schistosomiasis infection in children include nutritional deficiency, dysuria, hematuria, watery hypertrophy, dwarfism, and urinary bladder lesions (Saathoff *et al*., 2004), while if the infection persists for a long time to adulthood, it can cause cancer, increase in exposure to HIV and infertility (Kjetland *et al*., 2006; King and Dangerfield-Cha, 2008).

Blood flukes of the genus *Schistosoma* are a parasite that causes an acute or chronic disease called schistosomiasis or snail fever (Obare *et al*., 2016). It is widespread in poor communities with inaccessible safe drinking water and adequate sanitation services in the tropical and subtropical regions (WHO, 2016). Schistosomiasis in Egypt is responsible for about 35% of chronic liver diseases in children, as well as 70% of liver diseases in adults, therefore it is classified as an endemic disease (Abd El-Ghany *et al*., 2018). Also in Egypt, most people do agricultural work on irrigation farms, recreational activities, and household chores. For these reasons, they are always exposed to the water that contains the free-swimming larvae infective stage (cercariae), which comes out of the freshwater snail (Guo *et al*., 2016). In Egypt, *Biomphalaria alexandrina* freshwater snail is the intermediate host of *Schistosoma mansoni* (Ibrahim and Abdalla, 2017), also, *Bulinus truncatus* snail which is widely distributed all over Egypt is the intermediate host of *Schistosoma haematobium* (Youssef, 2010; Bakry *et al*., 2004; 2015). There are four distinct points by which we can stop transmission of schistosomiasis: first is preventing human fecal materials (sanitation) from reaching freshwater, second is controlling broad intermediate host snails to prevent the interaction of larval stages of schistosomes (miracidia, cercariae), third is preventing human exposure to the infective stage free-swimming cercariae (reducing contact with water), and fourth is the chemotherapy of PZQ on the parasite that lives inside the human host.

At present, there is no vaccine available, and Praziquantel (PZQ) which is the chemotherapeutic agent of choice against adult worms, already faces drawback of drug resistance in some *Schistosoma* isolates (WHO, 2011). Reducing the incidence and spread of schistosomiasis by using molluscicides to control snails has been led to a significant impact on a devastating decrease of the fresh-water snails' population which are the intermediate host. Environmental and economic considerations are increasing for the use of molluscicides that are biodegradable, selectively active, readily available in the affected areas and inexpensive. Niclosamide is a pesticide used as a positive control and consists of 5 chloro-N- (2 chloro-4-nitrophenyl)-2-hydroxy-benzamide (Guo *et al*., 2016). Nevertheless, any chemical compound such as niclosamide does have some drawbacks that involve a risk of toxicity for aquatic plants, fish, birds, domestic animals, and humans, lower solubility, and expensive cost. Also, to achieve snail control niclosamide should be used continuously over a longer time period, and this can lead to drug resistance (Kenawy and Rizk, 2004; Guo *et al*., 2008; Li *et al*., 2013; Guo *et al*., 2016). There are also other compounds that have a fatal effect on adult snails and embryos, such as copper, but they are not used regularly in common practices because they are absorbed by soil and organic materials (Ribeiro *et al*., 2009; Martins *et al*., 2014). Consequently, it's imperative to develop alternative substances for their control of mollusca, miracidia and cercariae, as they are effective, prove safe for aquatic organisms, cheap, easily available, and simply applicable agents.

The utilization of some medical plants with molluscicidal properties seems to be an inexpensive and simple alternative to chemical molluscicides (Perrett and Whitfield, 1996). To reveal the activity of some plants against snails, more than 1000 plant species were examined (El-Bolkiny *et al*., 1997). In Egypt, with great interest, some local plants have been examined for molluscicidal activity (Sakran, 2004; Sakran and Bakry, 2005; El-Sayed *et al*., 2006; Bakry and Hamdi, 2007; Bakry, 2009; Bakry *et al*., 2016). Several plants have already been identified as useful to control the intermediate hosts of trematodes such as *Solanum xanthocarpum*, *Phytolacca dodecandra* (Endod), *Thuja orientalis*, *Annona squamosa*, *Adenium arabicum*, and *Calotropis procera* (Al-Sarar *et al*., 2012). *Phytolacca dodecandra* in Ethiopia was the most

active plant against snail intermediate hosts of schistosomes and its aquatic larvae (molluscicidal, cercaricidal, and miracicidal activities) (Lemma, 1970). However, experiments have been proven toxic to non-target organisms such as fish (Lemma, 1970). Matos *et al*. (2020) found a fatal effect of curcumin on snails (*Biomphalaria glabrata*) and cercariae in *Schistosoma mansoni*.

Origanum majorana is also known as sweet marjoram. It belongs to the family of Lamiaceae (syn. Majorana hortensis Moench). *Origanum* herb, reported the presence of a large number of constituents in different parts of the plant, especially terpenoids (Raina and Negi, 2012), phenols (Nakatani, 2000) and flavonoids as major constituents due to its aromatic nature and other chemical constituents like steroids (Leung and Foster, 1996), fatty acids and vitamins (Janicsák *et al*. 1999) as a minor component. *Origanum majorana* leaves are also a vital medicinal herb in modern medicine primarily for extraction of *Origanum*, one of its main phytochemicals. The extracted of *Origanum majorana* have been used effectively against protozoans e.g., *Pentatrichomonas hominis* (Kozlowska *et al*., 2010); anti-bacterial (Mohamed *et al*., 2011); anti-fungal (Sharma *et al*., 2011); insecticidal (Sharma *et al*., 2011); anticonvulsant (Deshmane *et al*., 2007); anti-diabetic (Martha and Gutierrez, 2012); anti-gout (Vasudeva *et al*., 2014); anti-mutagenic activity (Al-Harbi, 2011); anti-ulcer (Vagi *et al*., 2005).

Ziziphus spina-christi (L.) belongs to the Rhamnaceae family. These trees produce small orange-yellow fruits and are also evergreen. Its common name in the countries of the Middle East is Nabka or Sidr. This plant is native to western and southern Asia and north and tropical Africa. In Egypt, wild plants are found mainly in the Sinai (Michel *et al*. 2011). *Ziziphus spina-christi* contains many essential oils for example, methyl stearate, methyl palmitate, and geranyl acetate, quercetin derivative from the flavonoids groups and triterpenoid sapogenins from the saponins groups and betulinic acid, phytosterols such as beta-sitosterol, alkaloids such as cyclopeptide, spinanine-A, tannins, phytosterols, triterpenoid sapogenins, and saponins (Kadioglu *et al*. 2016). *Ziziphus spina-christi* is used in traditional folk medicine for antibacterial, antiviral, hypoglycemic, diuretic, anticathartic, tonic activities, hepatoprotector and immunostimulant (Amin and Mahmoud-Ghoneim 2009; Michel *et al*. 2011; Mubaraki *et al*. 2017).

Salvia fruticosa (common name is Greek sage) belongs to the family Lamiaceae), it is an herb that is completely covered with hair and with the increased growth of leaves of different sizes that gives the plant an intense and silver appearance. It is a perennial plant or sub-shrub native to the eastern Mediterranean, including North Africa, the Canary Islands and southern Italy. It is especially abundant in Palestine (Koutsoulas *et al*., 2019). Numerous previous studies have identified many biologically active compounds and search their pharmacological properties, many of these species are also used in traditional medicine. These studies revealed the anti-inflammatory, antioxidant, anti-diabetic, as well as anti-tumor activities of isolates, and plant extracts. These effects are mainly attributed to terpenes, coumarins, flavonoids, plant phenols and carotenoids such as carnosic acid (California, terpenes) and rosmarinic acid (phenol) (Duletić-Laušević *et al*., 2018 and Fraihat *et al*., 2018).

The present work was planned to study the effect of lethal concentrations of the extracts of *Origanum majorana, Ziziphus spina-christi and Salvia fruticosa* on *Bulinus truncatus* with *S. haematobium* and *Biomphalaria alexandrina* with *Schistosoma mansoni* (molluscicidal), as well as on the free-living stages miracidia (miracicidal), in addition cercariae (cercaricidal) for both Egyptian schistosomes species.

MATERIALS AND METHODS

Plant materials

Origanum majorana, *Ziziphus spina-christi* and *Salvia fruticosa* leaves were obtained and identified in the Horticulture department, Faculty of Agriculture, Ain Shams University. The part of the collected plant was cleaned, washed, and dried in the shade; and was not exposed to sunlight for avoid the losing the active ingredients.

Aqueous extract preparation

By electric grinder, the dried materials were ground into a fine powder. A quantity of crushed leaves weighing 200 g was dissolved with two liters of distilled water at a ratio (1:10w/t) by the method of cool extraction and evaporated of the extract *in vacuo*. The extracts were concentrated *in vacuo* by using a rotary evaporator at 40°C. Finally, the extracts were placed in porcelain dishes in a temperature-controlled oven to remove the remaining water in the extracts to give a residue of 8.5 g. The residues were stored at 4^{o}C for further use (Ekpo and Etim, 2009).

Toxicological study

The maximum non-toxic concentration (MNTC) (the maximum concentration without toxic effect and expressed in µl/ml) of the plant extract on Vero cells had been carried out by serial dilutions of (10-300 µl/ml). Briefly, $2x10^5$ cells/ml of Vero cells were treated with the serial dilutions of the extract in microtiter plates and had been incubated at 37°C in a carbon dioxide (5% humidity) for 72 hours. Furthermore, the microscopic plates were examined in order to determine the toxic concentration of the extract through its ability to induce cell death. Cytotoxicity of the extract concentration at 50% was not exceeded to maintain the cell viability and their ability to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, Chem, St. Louis, MO), producing formazan blue product (Mosmann, 1983). Briefly, supernatants were removed from the wells, and 25 µl of an MTT (Sigma, St. Louis, MO) solution (2 mg/ml in PBS) had been added to each well, and the plates were incubated at 37°C for 90 minutes. Then, DMSO (25 µl) was added to each well to get rid of crystallized formazan. The plates were kept for 15 min on a shaker to be ready for the determination of the optical density at 492 nm (OD492). The MNTC value is the extract dilution which maintains normal cells morphologically and density in comparison with the untreated control cells with at least 95% of the optical density.

Identification of most potent plant extract

Identification of antischistosomal activity of three plants: *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* assessed at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University Egypt by extraction method, and absorbance of oil solutions in methanol measured with UV-240 spectrophotometer (Schimmadzu-Corporation, Kyoto, Japan). The preparation of the extract was done by maceration method. Maceration was done using the appropriate solvent with several times shaking or stirring at room temperature (Rader *et al*., 2007).

(GC-MS) Gas Chromatography-Mass Spectrometry analysis

Using a DELSI 121 C device equipped with a flame ionization detector and silica column contained a CP WAX 51 (25 m × 0.25 mm i.d., 0.25 µm film thicknesses), analytical gas chromatography (GC) was performed to identify the main components that present in the extracts. The temperature was maintained at 50 °C for five minutes and programmed to reach 220

°C at 3 °C per minute. ACPWAX 51merged silica WCOT column (60 m × 0.25 mm i.d., 0.25 μm) GC/MS with carrier gas helium is used. For GC/MS, a CPWAX 52 merged silica CB column (50 m × 0.3 mm; 0.25 μm film thickness) was used with HP mass spectrometer and couple to helium as a carrier gas: the energy of ionization 70 V. Temperature was Programmed to rise at a rate of 3 °C/min to reach from 50°C to 240°C. At a temperature of 240 °C samples were injected into an injector. By comparing Kovats indices (KI) linear with the components were identified, mass spectra and their time of retention (RT) with those acquired from the original samples and/or the MS library.

Experimental design

Snails

Biomphalaria alexandrina freshwater snails are the intermediate host of *Schistosoma mansoni*, and *Bulinus truncatus* freshwater snails are the intermediate host of *Schistosoma haematobium*, they were obtained from Medical Malacology Laboratory at Theodore Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt, their sizes were ranging from 13 to 18 mm for *Biomphalaria alexandrina* and from 8 to 12 mm for *Bulinus truncatus* and, the snails were placed in plastic aquaria with a size of 9 x 23 x 16 cm, ten snails per liter of water were placed in aquaria to avoid congestion between snails, with dechlorinated aerated tap water the aquaria were supplied, and covered with glass plate. The snails were feeding on dried lettuce leaves in the oven and were kept under ordinary room temperature (25±2) and the water used to change for the snails in aquaria twice weekly.

Molluscicidal activity

According to the WHO guidelines, a molluscicidal evaluation of medicinal plant extracts were performed (WHO, 2019). Four groups (equal to the number of concentrations), each group consists of ten uninfected snails (for each concentration) placed in glass beakers at room temperature.

Snails were exposed to concentrations of 6.25, 12.5, 25. 50. 100 and 200 mg/ml for 24 hr. at 26 ^{o}C. After the 24-hour period, the snails were removed from the solution containing the extracts and washed thoroughly with dechlorinated tap water, the snails were transferred to containers with deionized and dechlorinated water for another 24 hrs. of recovery. Dead snails were counted and confirmed by eliciting typical pull movements of the foot by using a wooden blunt nail and the absence of a heartbeat. This step was done in three replications. The negative control was dechlorinated water, in addition to the deionized water, in contrast, the positive control was water with niclosamide. The snails were not disturbed or fed during the exposure and recovery interval. LC_{50} and LC_{90} were calculated according to Leitchfield and Wilcoxon (1949).

Hatching of miracidia from infected hamster

Schistosoma mansoni and *Schistosoma haematobium* eggs were obtained from golden hamster (*Mesocricetus auratus*) with chronic infection maintained at the Schistosome Biological Supply Center (SBSC) at Theodor Bilharz Research Institute (TBRI), Embaba, Giza, Egypt. In a one-liter plastic container containing saline solution the hamster faeces was mixed. The mixture was passed through two sieves, the sieve size is 600 μm and the second size is 250 μm and the filter was collected in a metal plate. Then the collected filtrates were placed in a jar and left for 30 minutes in the dark. A clear supernatant was removed, a new saline was added and leave for three minutes, this step was repeated three times. Finally, the sediment was placed under artificial light for 30 minutes at a temperature from 20–25 ^{o}C until the exit of the miracidia. The later was used for miracicidal assay and snail's infection.

Infection of snails with miracidia

Biomphalaria alexandrina snails were infected with *Schistosoma mansoni* and *Bulinus truncatus* snails were infected with *Schistosoma haematobium*. This was done by using a long glass pipette to pull five miracidia from a Petri dish containing hatched miracidia. In a 24 well culture plate the miracidia were dispatched into each well. Then, individually, the snails were transferred into these wells and left for 30 minutes to allow miracidia to penetrate the soft body of snails; the wells were covered with plates to prevent the snails from crawling out. The snails were moved to a place where the lighting was 12 hr and darkness 12 hours for three weeks. In the fourth week, which is prepatent period of snails (before the infected snails pass five weeks), they were placed in the dark, to prevent trickle shedding cercariae, the container was covered with dark clothes.

Miracicidal activity

A series of concentrations (6.25, 12.5, 25. 50. 100, and 200 mg/ml) of aqueous extracts were prepared for the three tested plants. One ml of dechlorinated tap water that contains twenty-five miracidia mixed with one ml of double concentration (this means when testing the concentration of 200 mg/ml, 400 mg/ml is used actually because adding the same volume of water containing miracidia dilutes the concentration to half in each concentrations) in a 5 cm Petri dish. Three replications were done with the same preceding controls. The positive group was a 2 ml of one gram per liter of niclosamide and the negative group was a 2 ml of distilled water. Dead or immovable miracidia were recorded at intervals of 15, 30, 60, 90, 120 and 180 min because their movement indicates that the organism is alive (WHO, 2019).

Shedding of cercariae

Five weeks after the infection, the snails were transferred to beakers containing dechlorinated water. To release the cercariae, the beaker was moved to under a 100-watt lamp shaded with glass. The cercariae were collected in a beaker and mixed well.

Cercaricidal activity

A series of concentrations (6.25, 12.5, 25. 50. 100 and 200 mg/ml) of aqueous extracts were prepared for the three tested plants. One ml of dechlorinated tap water that contains twenty-five cercariae mixed with one ml of double concentration in a 5 cm Petri dish. Three replications were done with the same preceding controls. The positive group was 2 ml of one gram per liter of niclosamide and the negative group was 2 ml of distilled water. Dead or immovable cercariae were recorded at intervals of 15, 30, 60, 90, 120 and 180 minutes because their movement have been used to indicate that the organism is alive (WHO, 2019).

Infection of hamster with *Schistosoma* cercariae

At the start of bioassay (week 0), general anesthesia was administered to the hamster to produce a loss of consciousness and suppression of reflex activity and muscle relaxation. A ratio based on the volume of 3:1 Ketamine and Rompun and (Agar, Holland) was used to provide a combined effect of anesthesia. An aesthesia dose of 0.02 ml/30g hamster body weight was injected intraperitoneally. The anesthetized hamsters were shaved on the stomach area and on a wooden rack they were arranged. A piece of cotton wool had been dipped in water for use in moisturizing the shaven area to allow easy penetration of cercariae. A 1cm ring of metal was placed on the shaven area of each hamster, then, a suspension constituting approximately 250 live cercariae were dispensed in the metal ring using a micropipette and kept for a period of 30 minutes to allow cercariae to penetrate the hamster (Smithers and Terry, 1965).

LC_{50} Calculator

Sublethal (LC_{50}) values for different NLE concentrations were calculated by AAT Bioquest® calculator (Bioquest Inc. 2018).

RESULTS

Cytotoxicity

The cytotoxicity assay of tested plant extracts (*Origanum majorana*, *Ziziphus spina-christi*, and *Salvia fruticosa*) indicated that at Maximum Non-Toxic Dose (MNTD) of each extract-treated Vero cells did not show any morphological differences in comparison with control, at the value of 250, 350, and 300 µl/ml, respectively.

Phytochemistry

Origanum majorana (Sweet marjoram) is characterized by a strong, spicy and pleasant odor and flavor. The chemical compositions of the important oils acquired from *O. majorana* are recorded in Table (1) (Fig. 1).

Table (1): Identified compounds in aqueous extract of *Origanum majorana*

No.	RT	M. wt	M. formula	Prediction	Area	Area %
1	20.52	204	$C_{15}H_{24}$	Zingiberene	54727666.55	0.36
2	20.80	222	$C_{15}H_{26}O$	Nerolidol	79653206.07	0.52
3	26.89	222	$C_{15}H_{26}O$	Cubenol	90321047.45	0.59
4	26.89	222	$C_{15}H_{26}O$	Eudesmenol	118361109.56	0.78
5	28.42	220	$C_{15}H_{24}O$	Cedren	176248647.53	1.16
6	30.66	30.66	$C_{15}H_{22}O$	Phenol	98240348.64	0.65
7	30.81	30.81	$C_{11}H_{14}O_3$	Butanone	163365217.48	1.08
8	33.26	222	$C_{15}H_{26}O$	Eudesmol	106173887.45	0.70
9	36.84	188	$C_9H_{16}O_4$	Propanol	164765320.50	1.08
10	45.71	276	$C_{17}H_{24}O_3$	Gingerol	6205744296.17	40.86
11	47.93	200	$C_6H_9FN_{6O}$	Capsaicin	274275241.47	1.81
12	48.83	292	$C_{21}H_40$	Nonivamide	151523642.98	1.00
13	60.25	412	$C_{29}H_{48}O$	Stigmasterol	92898328.92	0.61
14	61.17	414	$C_{29}H_{50}O$	á-Sitosterol	351889991.52	2.32

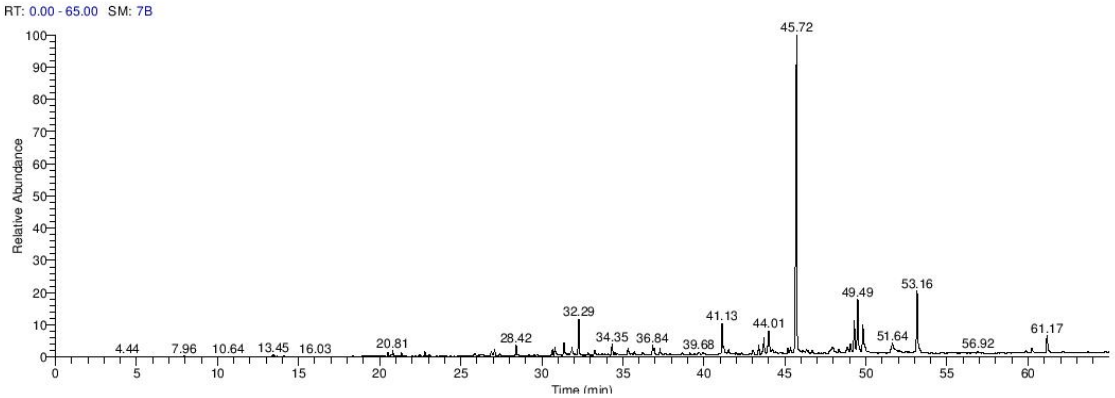

Fig. (1): GC-MS chromatogram of aqueous plant extract of *Origanum majorana*

On the other hand, the chemical compositions of the important oils acquired from *Ziziphus spina-christi* (L) are listed in Table (2) (Fig. 2).

Table (2): Identified compounds in aqueous extract of *Ziziphus spina-christi* (L.)

No.	RT	M. wt	M. formula	Prediction	Area	Area %
1	13.23	615	C23H45N5O14	D-Streptamine	8421115.74	0.48
2	17.73	592	C16H48O8Si8	Cyclooctasiloxane	36726047.85	2.11
3	19.82	110	C6H6O2	Hydrochinon	54912618.86	3.15
4	20.47	145	C8H7N3	Benzene	70108424.28	4.02
5	20.89	458	C28H30N2O4	Morphinan	28332599.99	1.62
6	24.58	220	C15H24O	Tricycloundecan	16114395.18	0.92
7	24.58	220	C15H24O,	Spathulenol	16114395.18	0.92
8	24.76	153	C6H7N3O2	Imidazole	19324582.43	1.11
9	26.32	190	C13H18O	Megastigmatrienone	17929010.41	1.01
10	26.93	283	C10H13N5O5	Guanosine	22821026.68	1.31
11	32.76	279	C10H17NO6S	Desulphosinigrin	20218112.12	1.16
12	41.28	519	C30H53NO4Si	Glycine	28017325.31	1.61
13	46.99	286	C20H30O	Phenanthrenemethanol,	33473432.07	1.92
14	53.07	439	C31H21NS	Thienopyridine	14058118.79	0.81
15	61.17	414	C29H50O	á-Sitosterol	98265259.33	5.63

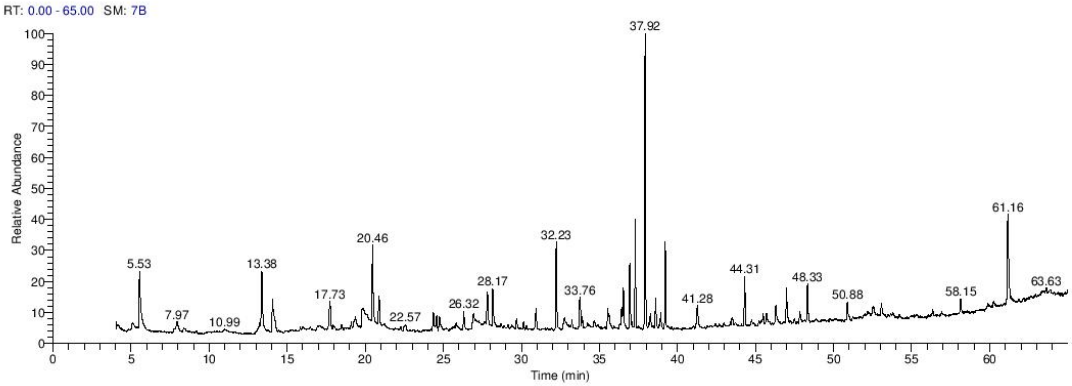

Fig. (2): GC-MS chromatogram of aqueous plant extract of *Ziziphus spina-christi*

The chemical compositions of the important oils acquired from *Salvia fruticosa* are listed in Table (3) (Fig. 3).

Table (3): Identified compounds in aqueous extract of *Salvia fruticose*

No.	RT	M. wt	M. formula	Prediction	Area	Area %
1	10.43	341	C20H23NO4	Quinolinol	3053550.06	3.26
2	14.16	576	C18H52O7Si7	Tetrasiloxane	3831389.13	4.08
3	20.88	428	C27H44O2Si	Androstadienol	2861964.11	3.05
4	22.53	283	C10H13N5O5	Purinol	1584222.84	1.69
5	30.94	428	C27H40O4	Spirosten	1522237.77	1.62
6	32.36	256	C16H32O2	Tetradecanoic acid	12521180.76	13.35
7	35.11	195	C11H17NO2	Benzenemethanol	4860449.32	5.18
8	36.42	366	C13H30N2O4SSi2	Cystathionine	1306854.22	1.39
9	49.30	514	C27H31BrO5	Terphenyl	2374908.89	2.53

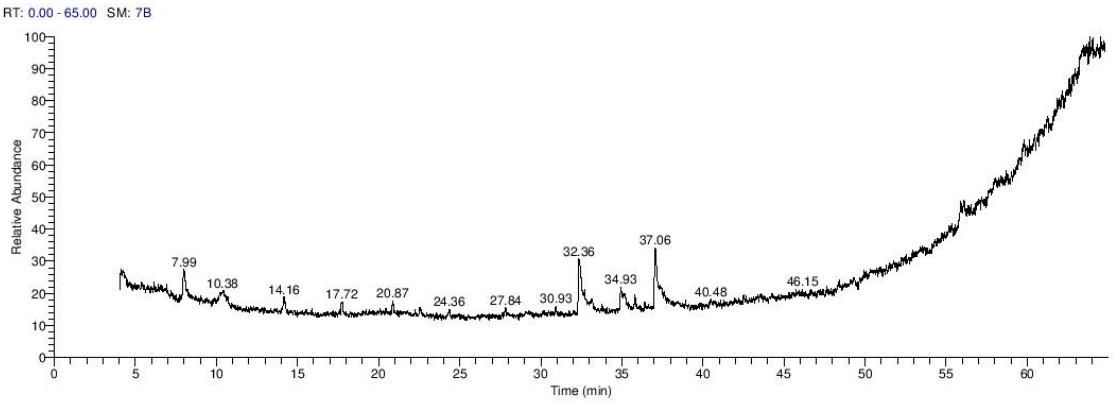

Fig. (3): GC-MS chromatogram of aqueous plant extract of *Salvia fruticose*

***In vitro* study**

The efficacy of *in vitro* treatment of *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* aqueous extracts was studied on adult snails of *Biomphalaria alexandrina* and *Bulinus truncatus* as well as on miracidia and cercariae in both Egyptian species of *Schistosoma mansoni* and *Schistosoma haematobium* at different concentrations. The effect of all aqueous extracts on adult snails, miracidia and cercariae were effective, and this mainly depends on incubation time and concentration.

Molluscicide activity

Untreated snails were moved inside the container with their feet extended outside the shell. When mechanical stimulation was applied with a sharp tool to the foot sole, they immediately retracted to their shell. The toxic effects of active plant extracts are shown on tested snails as follows. In the partially dead snails, partially dead snails there was a partial regression (withdrawal response), but in dead snails, there was no retraction at all to the mechanical stimulation of the foot sole with a sharp needle. Also, the high doses of the active extracts caused a clear swelling of a cephalopodal mass and the emergence of hemorrhagic "blisters" on the ventral surface of the foot sole and its failure to respond to mechanical stimulation with a sharp needle. Mucous secretion was also observed in the foot.

From Table (4) LC_{50} and LC_{90} of the leaf extracts of *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* were calculated. The current data revealed that the aqueous extract of *Origanum majorana* leaves have been demonstrated the highest molluscicidal activity in the two tested snails as (LC_{50}= 42 mg/ml and LC_{90}= 172 mg/ml on *B. alexandrina*, and LC_{50}= 35 mg/ml and LC_{90}= 140 mg/ml on *B. truncates*) followed by *Ziziphus spina-christi* as (LC_{50}= 43 mg/ml and LC_{90}= 374 mg/ml on *B. alexandrina*, LC_{50}= 35 mg/ml and LC_{90}= 299 mg/ml on *B. truncates*), while *Salvia fruticosa* showed molluscicide activity, but it was less than previous as (LC_{50}= 69 mg/ml and LC_{90}= 315 mg/ml on *B. alexandrina*, LC_{50}= 56 mg/ml and LC_{90}= 219 mg/ml on *B. truncates*). The results showed that the *Bulinus truncatus* snail was more affected than the *Biomphalaria alexandrina*, possibly due to the size of the snail. On the other hand, the positive group showed complete death of snails (100%) after 24 hr. of incubation. Conversely, the untreated group was still living until the experiment was ended.

Table (4): Molluscicide activity of *Origanum majorana, Ziziphus spina-christi* and *Salvia fruticosa* aqueous extracts against *Biomphalaria alexandrina* and *Bulinus truncates* after 24 hours of exposure *in vitro*

Snails	Plant extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
B. alexandrina	*Origanum majorana*	14	42	88	172
	Ziziphus spina-christi	17	43	111	374
	Salvia fruticosa	28	69	150	315
B. truncatus	*Origanum majorana*	11	35	68	140
	Ziziphus spina-christi	16	35	87	299
	Salvia fruticosa	24	56	112	219

Miracicidal activity

The results showed that the mortality rate of miracidia was high in the two Egyptian species of *Schistosoma* exposed to plant extracts with different concentrations and at time intervals from 15 to 180 min. *Origanum majorana* was the most active in killing 100% of miracidia after 30 min at (LC_{50}= 24 mg/ml and LC_{90}= 74 mg/ml on *S. mansoni* miracidia, at LC_{50}= 23 mg/ml and LC_{90}= 65 mg/ml on *S. haematobium* miracidia). The effect started at a low concentration of 6.25 mg/ml at 15 min, and the mortality rate began to gradually rise until reached 100% at a concentration of 50 mg/ml at 90 min. The results were the same on miracidia for the two species of schistosomes. As for *Ziziphus spina-christi*, it also had a fatal effect on miracidia at (LC_{50}= 31 mg/ml and LC_{90}= 96 mg/ml on *S. mansoni* miracidia, at LC_{50}= 29 mg/ml and LC_{90}= 84 mg/ml on *S. haematobium* miracidia), the effect began to appear in the lowest concentration after 30 min and the death rate increased to reached 100% at a concentration of 50 mg/ml after 120 min of exposure to the extract. It had the same effect on miracidia in two species of schistosomes. While, *Salvia fruticosa* showed a lower effect against miracidia for the two species of schistosomes, than the two other plants at (LC_{50}= 49 mg/ml and LC_{90}= 177 mg/ml on *S. mansoni* miracidia, at LC_{50}= 34 mg/ml and LC_{90}= 164 mg/ml on *S. haematobium* miracidia), the effect began to appear in the lowest concentration after 30 min and gradually increased until reached to100% at a concentration of 100 mg/ml at 120 min in *Schistosoma mansoni* and it reached 100% at a concentration of 100 at 90 min in *Schistosoma haematobium* (Table 5). Whereas, the positive group showed complete death of miracidia (100%) after 30 min of incubation. On the contrary, the negative group was still living until the experiment was ended.

Table (5): Miracicidal activity of *Origanum majorana, Ziziphus spina-christi* and *Salvia fruticosa* aqueous extracts against miracidia after 180 min of exposure under laboratory conditions

Miracidia	Plant extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
Miracidia of *S. mansoni*	*Origanum majorana*	7	24	39	74
	Ziziphus spina-christi	11	31	49	96
	Salvia fruticosa	15	49	91	177
Miracidia of *S. haematobium*	*Origanum majorana*	5	23	35	65
	Ziziphus spina-christi	8	29	45	84
	Salvia fruticosa	10	34	73	164

Cercaricidal activity

When cercariae was exposed to different concentrations of plant extracts, the effect began to appear after 15 min of exposure to the lowest concentration of the two schistosomes species, and the mortality rate increased gradually with the increase in the period of exposure. *Origanum majorana* was the most active in 100% mortality of cercariae after 90 min at (LC_{50}= 17 mg/ml and LC_{90}= 66 mg/ml on *S. mansoni* cercariae, and at LC_{50}= 16 mg/ml and LC_{90}= 54 mg/ml on *S. haematobium* cercariae). *Origanum majorana* showed the same effect on cercariae in two species of schistosomes, it started the effect at the lowest concentration after 15 min, and the mortality

rate began to gradually rise until reached 100% at a concentration of 50 mg/ml at 90 min. As for *Ziziphus spina-christi*, it also had a mortality effect on cercariae at (LC_{50}= 17 mg/ml and LC_{90}= 66 mg/ml on *S. mansoni* cercariae, and at LC_{50}= 23 mg/ml and LC_{90}= 67 mg/ml on *S. haematobium* cercariae), the mortality began to appear when exposed to a low concentration of 6.25 mg/ml at 15 min and the death rate increased to reach 100% at a concentration 50 mg/ml after 120 min of exposure to the extract. It had the same effect on cercariae in two species of schistosomes. Whereas, *Salvia fruticosa* showed an effect against cercariae for the two Egyptian species of *Schistosoma*, but it was less than previous at (LC_{50}= 31 mg/ml and LC_{90}= 193 mg/ml on *S. mansoni* cercariae, LC_{50}= 14 mg/ml and LC_{90}= 140 mg/ml on *S. haematobium* miracidia), The effect began to appear in the lowest concentration after 60 min and gradually increased until it reached to100% at a concentration of 100 mg/ml at 90 min in *Schistosoma mansoni* but the effect started to appear at low concentration of 6.25 mg/ml at 15 min and reached to 100% at a concentration of 50 at 120 min in *Schistosoma haematobium* (Table 6). While the treated group showed complete death of cercariae (100%) after 30 min of incubation. On the contrary, the untreated group was still living until the experiment was ended.

Table (6): Cercaricidal activity of *Origanum majorana, Ziziphus spina-christi* and *Salvia fruticosa* aqueous extracts against cercariae after 180 min of exposure under laboratory conditions

Cercariae	Plant extract	LC_{25}	LC_{50}	LC_{75}	LC_{90}
Cercariae of *S. mansoni*	*Origanum majorana*	9	17	30	66
	Ziziphus spina-christi	9	17	30	66
	Salvia fruticosa	8	31	73	193
Cercariae of *S. haematobium*	*Origanum majorana*	1	16	25	54
	Ziziphus spina-christi	9	23	33	67
	Salvia fruticosa	6	14	48	140

DISCUSSION

Schistosomiasis is widespread in sub-Saharan Africa, particularly in poor provincial society, imposing a great social and economic load. Due to the presence of a single drug approved by the World Health Organization, effective strategies for controlling this disease were essential, it is an important approach to intensify control measures to eliminate the aqueous stages, which are snails and miracidia, as well as cercariae in the life cycle of schistosomiasis or at least reduce it. Niclosamide, is the synthetic material used by the World Health Organization to control host aquatic snails, miracidia and cercariae, but it is very expensive, also toxic to non-target organisms such as fish and the snail resistance is very possible. It is important to research the biological agents that can reduce the shedding of cercariae and kill both miracidia and cercariae. Several researchers have tested some biological control agents as well as biopesticides

including snail predators, plant extracts, fungi, and bacteria (Younes *et al*., 2017; Abd El-Ghany *et al*., 2018).

Due to the diversity in biological activities and molecules that natural products provide, they are a good alternative to conventional or chemical therapeutic compounds. This biological diversity is the result of a large number of as yet unknown active compounds. Despite advances in medicinal chemistry, biotechnology, and genomics, the discovery of new drugs for schistosomiasis treatment remains challenging (Silva *et al*., 2017; Lago *et al*., 2018). The aqueous extract of *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* was chosen because water is a safe and non-toxic, widespread solvent avoiding high toxicity of the organic solvent (such as methanol, acetone, chloroform and dichloromethane) to live organisms (Kinuthia *et al*. 2015).

It was observed in this study the continuous movement of the snails and also the aggregate on the water-air interface. Brackenbury (1999) and Adetunji and Salawu (2010) agreed with these results and noticed that when placing bulinid snails with lethal doses of the extract, the snails remain in a constant state of movement to try to get out of the extract, which causes them to have an irritating behavior and also floats on the surface of the water that does not make their body in close and continuous contact with the extract, they try to avoid the extract. In this study the lethal concentrations LC_{50} of aqueous extracts of *Origanum majorana* was 42 mg/ml on *B. alexandrina* and 35 mg/ml on *B. truncates, for Ziziphus spina-christi* it was 43 mg/ml on *B. alexandrina* and 35 mg/ml on *B. truncates*, while *for Salvia fruticosa* it was 69 mg/ml on *B. alexandrina* and 56 mg/ml on *B. truncates* after 24 hours of exposure. Several researchers have examined the effect of medicinal plants as molluscicidal, Kiros *et al*. (2014) who confirmed that the aqueous extract of *Glinus lotoides* fruits had an anti-snail activity on *Biomphalaria pfeifferi*. Whereas, Al-Snafi (2015) confirmed that the *Anagallis aryensis* extract has strong efficacy as a molluscicide. This activity is due to the presence of two compounds called anagakkoside B and desgluco-anagaloside B, which were found to have similar activity to that of the Niclosamide (synthetic molluscicide). Ibrahim and Ghoname (2018) confirmed that the *Anagallis arvensis* aqueous extract had a molluscicidal effect against *Biomphalaria alexandrina* and state that hormonal activity, reproductive rates and reduction in survival were reduce after treatment. Jia *et al*. (2019) documented that the pentacyclic triterpenoid saponin derived from *Camellia oleifera* has molluscicidal activity against *Oncomelania hupensis*, *Biomphalaria alexandrina* and *Bulinus truncatus*. Matos *et al*. (2020) proved that the curcumin extract has molluscicidal activity against *Biomphalaria glabrata* and stated that the embryo development, egg hatching and fecundity rate of adult snails were decreased.

According to the World Health Organization, the lethal concentration (LC_{50}) of any molluscicide should not exceed 100 ppm (WHO, 2019). In the present study, the active extracts caused a clear swelling of a cephalopodal mass, and an emergence of hemorrhagic "blisters" on the ventral surface of the footsole and its failure to respond to the mechanical stimulation with a sharp needle. Mucous secretion was also observed in the foot. Snails exhibited many behavioral responses after exposure to the active plant extracts examined in this study, including the "distress syndrome" which refers to intoxication and which has been described in other species such as the planorbid type by Sullivan and Cheng (1975), Van Aardt and Coertze (1981) and Brackenbury and Appleton (1999). The swelling of the tissues was not limited to the entire cephalopodal mass, but also included the tentacles. According to Brackenbury and Appleton (1999) when the snail is exposed to the lethal concentration of plant extracts, it causes haemorrhae in the cephalopodal mass due to the accumulation of water in the soft body of the

snail. However, according to that study, an imbalance in the permeability of the foot sole epithelium was seen by preventing its normal osmo-regulatory function and attributing this to the toxic principles of the active plant extracts Brackenbury and Appleton (1999). However, upon starting the recovery period by transferring the snail into water-free of plant extracts, the toxic effect of sublethal doses of plant extracts was reversible. This observation was recorded in several results, including Harry and Aldrich (1963), and Van Aardt and Coertze (1981) said after exposure to copper sulphate the snails (*Bulinus tropica*) can move again in sublethal dose.

Phytochemical analysis showed the presence of many active substances such as Cedren, Phenol, Butanone, Eudesmol, Propanol, Capsaicin, Guanosine, Glycine Thienopyridine, Spathulenol, Purinol, Spirosten. According to Mandefro's result (2017) the chemical analysis of the plants he used contained many active substances such as saponins and carbohydrates, but he confirmed that the main snails killing compounds in the aqueous extract of this plant is saponins. Several other studies have also documented that saponins are a highly effective molluscicide. Mandefro explained that the saponin causes damage to the animal cell membrane by forming a complex compound with the plasma and the membrane.

The infectivity of both species of schistosomes (*Schistosoma mansoni* and *Schistosoma haematobium*) miracidia were greatly reduced by *Origanum majorana, Ziziphus spina-christi* and *Salvia fruticosa*. This may be explained as the LC_{50} of plant extracts of the three tested plants had weakened the ability of the miracidia to penetrate the soft tissues of the snails. Several studies have investigated the effect of plant extracts on miracidia, Bakry *et al*. (2002) they observed that when *Schistosoma mansoni* miracidia were exposed to LC_{25} extract of methanol from *Euphorbia lacteal*, it reduced the ability of miracidia to infect the intermediate host *Biomphalaria alexandrina* snails. El-Emam *et al*. (1986) reported that the infection rate was reduced when treated with 50 ppm from *Calendula micrantha* extract. There are also many studies with the same result, including Tantawi *et al*. (2000) who studied the extract from *Solanium dudium*, Sharaf El-Din *et al*. (2001) studied the extract from *Zygophyllum simplex*, Bakry *et al*. (2004) obtained the extracts from *Agave franzosinii* and methanol extracts from *Furcraea selloea* and *Ophioglossum reticulatum*. Also, these results were consistent with several investigations using a chemical compound or plant extract as a molluscicides such as Rawi *et al*. (1995) investigated the extracts from *Ammi majus* and *Calendula micrantha officinalis* against *Bulinus truncatus* and *Biomphalaria alexandrina*, Mohamed *et al*. (2000) studied the effect of Abamectin on *Biomphalaria alexandrina*, Abdel Aziz *et al*. (2011) observations of the effect of methanolic extract of *Plectranthus tenuiflorus* revealed similar conclusions. Thus, Ansari *et al*. (2000) observed that the effect of *Artemisia maritima* caused a significant decrease in cercarial shedding and cercarial production in *Biomphalaria alexandrina* the intermediate host of *S. mansoni*. Sharaf El-Din *et al*. (2001) treated *Biomphalaria alexandrina* with sublethal concentrations of aqueous extraction of *Zygophyllum simplex*, he obtained similar reduction in cercarial shedding and cercarial production. Shortening in cercarial shedding period may be due to the effect of the extract on the soft tissues of the snails, as well as the penetration of the miracidia into the tissues of the snails. This leads to a disturbance in the physiological functions of the treated snail body, which leads to a shorter period of shedding as well as the life span (Bakry *et al*., 2017).

When the miracidia attacks the snail, and stabilizes in the soft tissues of it, it undergoes asexual reproduction to produce cercariae, the infectious stage of schistosomiasis. According to previous investigations, a single miracidia may be divided to give more than 20000 cercariae (Grimes *et al*., 2015). The present study showed that the aqueous extract of the tested plants had a

cercariacidal activity against *S. mansoni* and *S. haematobium* cercariae, where the activity was dependent on both time and dose. This observation is similar to that of Rug and Ruppel (2000) who examined the toxic activity of aqueous and methanol extract of *Jatropha curcas* and found that the aqueous extract had cercaridcidal activity at 100 ppm killed 100% of the cercariae after 2 h and was more efficient than the methanol extract, whereas Mohamed *et al*. (2005) tested the *Nigella sativa* seeds on three stages of *S. mansoni*, the result indicates its strong biocidal effects against miracidia, cercariae, and adult worms, Medina *et al*. (2009) who demonstrated the kaurenoic acid derived from *Croton floribundus* has cercaricidal activity on cercariae of *S. mansoni* when exposed to 100 $\mu g/mL^{-1}$, after one hour the mortality rate was 100%, also Chen *et al*. (2012) who reported the curcumin had high activity against *S. japonicum* cercariae, at a concentration of 8 $\mu g/mL^{-1}$, after 30 minutes the mortality rate was 100%. Kiros *et al*. (2014) observed the aqueous extract of *Glinus lotoides* has cercariacidal activity after 2 h of exposure at LC_{50} and LC_{90} values were 18.7 and 41.7 mg/l, respectively.

Conclusion

By studying the effect of *Origanum majorana*, *Ziziphus spina-christi* and *Salvia fruticosa* on the intermediate host of *Schistosoma mansoni* and *Schistosoma haematobium*, as well as on different life stages. *Origanum majorana*, *Ziziphus spina-christi* had strong molluscicidal, miracicidal and cercaricidal activity for both Egyptian species schistosomes *in vitro*, while *Salvia fruticosa* was less effective than the previous ones. This requires more studies to know the active substances that had this fatal effect on snails, miracidia, and cercariae.

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