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Chemical Profiling, Molluscicidal Impact of Ethanolic Extract of *Saussurea costus* Root against *Biomphlaria alexandrina* Snails, Intermediate Host of *Schistosoma mansoni*

Hebat-Allah A. Dokmak[1], Amina M. Ibrahim[1], Tamer S. Mohamed[2], Asmaa S. Mohamed[2]*

[1]Medical Malacology Department, Theodor Bilharz Research Institute, Kornaish El Nile, Warrak El-Hadar, Imbaba (P.O. 30), Giza 12411, Egypt
[2]Medicinal Chemistry Department, Theodor Bilharz Research Institute, Kornaish El Nile, Warrak El-Hadar, Imbaba (P.O. 30), Giza 12411, Egypt
*Corresponding Author: asmaasalah25@yahoo.com

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ABSTRACT

The trematodes, specifically the genus *Schistosoma*, are responsible for causing the parasitic disease known as schistosomiasis. In this research, the aim was to utilize the extract of *Saussurea costus* root as a molluscicidal substance to target the snails, which act as the intermediary host for the parasite species and disrupt the life cycle of the parasite. During the investigation, *Biomphalaria alexandrina* snails were treated with *S. costus* root extract for twenty four hours, before undergoing a 24-hour recuperation phase. According to the results, the extract was found to have a molluscicidal potential, with a lethal concentration (LC_{90}) of 113.70ppm. Hemocyte abnormalities in *B. alexandrina* snails were brought about by exposure to ethanolic extract's LC_{10} and LC_{25}. Furthermore, histopathological changes were recognized in the hermaphrodite and digestive glands which were confirmed through transmission electron microscope imaging. The genotoxic effects of the extract were also investigated using the RAPD-PCR technique, revealing a decrease in genomic stability (GTS) to 25% at the LC_{25} concentration. Moreover, the root extract of *S. costus* has negative effects on the viability and infection of *Schistosoma mansoni* miracidia and cercariae. The study examined the effects of exposing the infected *B. alexandrina* snails with *S. mansoni* miracidia to the LC_{10} and LC_{25} concentrations of the root extract after 3 and 21 days. The analysis using GC/MS identified seven compounds in the extract. Therefore, the root extract of *S. costus* as a bio control agent could replace the chemical molluscicides in reducing schistosomiasis.

INTRODUCTION

Schistosomiasis is a parasitic infection disease transmitted through freshwater snails in many developing countries **(WHO, 2022)**. The life cycle, development, and transmission of the disease depend on the presence of these snails. Approximately 260 million individuals are estimated to have been exposed to infective Schistosome

cercariae, which emerge from snails, in freshwater habitats **(WHO, 2023)**. One of the key strategies to combat this disease is to end the cycle of human-snail-human transmission, and this involves implementing snail control measures such as the use of molluscicides **(McCullough *et al*., 1980; Lardans & Dissous, 1998; Sokolow *et al*., 2015; Sokolow *et al*., 2016)**. Molluscicides are chemical substances or natural extracts that are utilized to eliminate snails and control their population **(Aber, 2003)**.

Saussurea costus (family Asteraceae) is a perennial aromatic plant **(Waly, 2009)**. Its root is yellowish finger-sized. The common name "Kustha" reflects its Indian origin **(Polunin & Stainton, 1984)**. It is also known as "Al-Kost Al-Hindi" in the Arab countries. *S. costus* is traditionally used for its anti-trypanosomal activity **(Julianti *et al*., 2011)**, and its stimulating, disinfectant, sedative, bronchodilator effects **(Wani *et al*., 2011)**. Moreover, its roots are used in treating diseases characterized by an excessive complement activation, such as respiratory distress, rheumatoid arthritis and systemic lupus erythematosus **(Polunin & Stainton, 1984)**. Additionally, *S. costus* has shown an encouraging anticancer activity on examined cell lines **(Fan *et al*., 2014)**. Furthermore, wide antibacterial action against a variety of human infections has been demonstrated by ethanol extract of *S. costus* **(Hasson *et al*., 2013)**. Further research has documented the bioactive characteristics of *S. costus* roots, encompassing anti-inflammatory, antiviral, anti-immune and anti-ulcer effects **(Zahara *et al*., 2014)**. The content of flavonoid and phenolic of *S. costus* root extract could be responsible for the biological activities that have been identified. Strong antioxidant properties are attributed to flavonoids and phenolic acids, which are found in plant tissues. These anti-oxidant phenolics are essential for healthy development, infection prevention, and defense against both internal and external sources of harm **(Ara & Nur, 2009; Tibiri *et al*., 2010)**. Building on these researches, the primary goal of the present research was to explore the molluscicidal and cercarecidal influences of *S. costus* against *B. alexandrina* and *S. mansoni* cercariae with highlighting its bioactive constituents by using GC-MS analyses.

MATERIALS AND METHODS

1. Organisms

The Center for Schistosome Biological Supplies and the Department of Medical Malacology at Theodor Bilharz Research Institute (TBRI) in Imbaba, Giza, Egypt are the sources of all the organisms employed in this study. *Biomphalaria alexandrina* snails measured 3- 5mm were cultivated using the method described by **Liang *et al* (1987)**. The miracidia were hatched in dechlorinated water under light at 25± 1°C and used for experimental tests. While, the *S. mansoni* cercariae were obtained from the life cycle involving hamster rats or CD mice as terminal hosts and the snail species as transitional hosts.

2. Plant processing

2.1. Extraction of Saussurea costus root

S. costus roots were purchased from a nearby market and authenticated in Al-Azhar University's Department of Taxonomy. The plant's roots were pulverized into a fine powder after being allowed to dry naturally. A 100g sample of dry powder was extracted using 1L of 90% aqueous ethanol four times, followed by a 72-hour maceration period at 25°C. The extract was filtered, and a gummy residue (8.3g) was obtained by concentrating the filtrate using a rotatory evaporator at 50°C and decreased pressure (Buchi Rotavapor RE; Switzerland), with 60– 80C petroleum ether, and the sticky residue was defatted before being examined. Moreover, the extracted defatted material (6.1g) was maintained at 4°C.

2.2. Preliminary phytochemical assessments

To determine what kind of secondary metabolites are in the defatted ethanol extract, two-dimensional paper chromatography and preliminary phytochemical assays were applied **(Brain & Turner, 1975; Harborne, 1992)**.

2.3. Total flavonoid content (TFC) and total phenolic content (TPC) determination

Using the phosphomolybdenum test, the ethanolic extract's total phenolic content was determined **(Mossalem *et al*., 2021)**. The technique outlined by **Ezzat *et al.* (2022)** was also reported to evaluate the total flavonoid content.

3. Bioassay

3.1. Toxicity of ethanolic S. costus root extract

The toxicity of ethanolic *S. costus* root extract was analyzed following the methodology described by **Nolan *et al*. (1953)**. Probit analysis, based on the approach suggested by **Finney (1971)**, was employed to determine the LC_{50}, LC_{90}, the lethal concentration at which 50% LC_{50} and 90% LC_{90} of the test organisms were affected by the extract, and slope function of the extract using the **Litchfield** and **Wilcoxon (1949)** method.

3.2. Impact of ethanolic S. costus root extract on hemocyte morphology of B. alexandrina snails

Hemolymph samples were collected from control and treated *B. alexandrina* snails following the methods outlined in **Abdul-Salam and Michelson (1980)**, **Nduku and Harrison (1980)**, **Ibrahim *et al*. (2018)** and **Ibrahim *et al*. (2023)**.

3.3. Impact of ethanolic extract of S. costus roots on molecular aspects of B. alexandrina snails

A DNA extraction process called CTAB/Chloroform-Isoamyl Alcohol was employed, and the dream taq green master mix kit (2X) was used to study molecular aspects like PCR and sequencing in *B. alexandrina* snails. Genomic DNA from a variety of organisms is frequently isolated using this technique **(Doyle & Dickson, 1987; Cullings, 1992)**.

3.4. Impact of S. costus roots extract on ultrastructure of digestive and hermaphrodite glands (TEM) in B. alexandrina snails

TEM samples were prepared at RCMB, Al-Azhar University using a standard protocol **(Abdel-Aziz *et al*., 2017; Yosri *et al*., 2022)** and imaging with a JEOL JEM 1010 transmission electron microscope.

3.5. Miracicidal and cercaricidal activity of S. costus root extract on S. mansoni miracidia and miracidia production

Miracicidal and cercarial bioassay tests were conducted following the methods described by **Obare (2016)**. In order to promote miracidial hatching, *S. mansoni* eggs were placed in light coming from a 60-watt bulb. After 30min, 5ml of H_2O containing 30 newly hatched miracidia were combined with 5ml of water that had twice the concentration of the selected concentrations (LC_{25} ppm and LC_{50} ppm) in a divided Petri dish. A triplicate setup was prepared, and each setup was observed under a microscope at 10-minute intervals for a period of one and a half hour (10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes). Miracidia that were motionless or dead were counted and noted. A control group with 5ml of dechlorinated tap water containing 30 newly hatched miracidia was also prepared. For the cercarial bioassay, the same technique was applied, but 5ml of water containing 30 cercariae of *S. mansoni* was used instead of miracidia.

3- 5mm snails were subjected to a number of miracidia (5- 8 *miracidia*/ snail/ 2ml dechlorinated water) and maintained at a controlled temperature (24± 1°C). Sublethal concentrations of the extract were administered to the snails at seven and twenty one days post-exposure. Control groups of untreated snails were included for comparison. Every week, cercariae were counted, and the prepatent, patent, and lifetime of the infected snails were noted. It was also determined how many cercariae each snail produced on average and the infection rate **(Pflüger *et al*., 1984; Dokmak *et al*., 2024)**.

4. Antioxidant activity

4.1. ABTS radical scavenging assay

The extract was evaluated for its capacity to scavenge ABTS radicals through an approach outlined by **Re *et al*. (1999)**. This evaluation employed ascorbic acid as a standard. Furthermore, the inhibitory concentration (IC_{50}) of the extracts was found to be the amount required to scavenging 50% of ABTS radicals.

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4.2.Scavenging free radicals using DPPH assay

A technique reported by **Manzocco *et al*. (1998)** was employed to assess the extract's potential to scavenge DPPH. The formula {(A0–A1)/A0} ×100 was used to determine the percentage inhibitory activity, where A0 denotes the control's absorbance and A1 the extract's or standard's absorbance. Regression analysis was utilized to ascertain the IC_{50} value **(Mohamed *et al*., 2022)**.

5. GC-MS conditions

Using a gas chromatograph (Shimadzu 2010) with electron impact ionization (70 eV), the chemical constituents of the ethanolic extract were identified. The apparatus utilized was a VF-5 fused silica capillary column (30m × 0.25mm, 0.25µm film thickness). With helium as the carrier gas, and the rate of flow was 1ml/ minute. For one minute, the temperature was set to 50°C. It was then progressively raised to 120°C (5°C/minute), 120– 190°C (2°C/ minute), kept for one minute at 190°C, 190– 250°C (10°C/ minute), and held at 280°C for three minutes. The ions' mass ranged from 35 to 500m/ z, and the entire run took 60 minutes. By using an autosampler, 1µl of the tested samples and a standard combination (1mg/ 1ml n-hexane) were injected. By comparing the volatile constituents' retention times (t R), retention indices to (C8–C20) n-alkane standards, and mass spectra to corresponding data from the Wiley and NIST electronic libraries, it was possible to characterize the volatile constituents.

6. Examining data with statistics

The statistical package for the social sciences (SPSS) application and the windows version of "GraphPad Prism software" were used to statistically analyze the data. An analysis of variance (ANOVA) was used to examine the significance of differences between the treatment and control groups. The results were expressed as mean ± standard deviation (SD).

RESULTS

1. Molluscicidal activity of *S. costus* roots upon *B. alexandrina* snails

The findings in Table (1) demonstrated that the extract's LC_{90} value, obtained after a 24-hour exposure period, was determined to be 113.70ppm. The steep slope values of the extract indicate that the probability lines for lethal concentrations (LC) were steep as well, revealing that *B. alexandrina* snails' sensitivity to the *S. costus* root extract is dependent on concentration.

2. Impact of *S. costus* roots on hemocytes morphology in *B. alexandrina*

The study found that the control group had granulocytes (G), small hemocytes (S), and hyalinocytes (H) (Fig. 1A). Treatment with LC_{10} of *S. costus* root extract increased hyalinocytes and showed abnormalities in nucleus numbers, including cells with two nuclei (Fig. 1B). Granulocytes had abundant granules and some formed pseudopodia (PS) (Fig. 1C). Snails treated with LC_{25} exhibited hemocytes with numerous granules in granulocytes and abnormal cell membranes. Some hyalinocytes showed eccentric shrinkage or division into two nuclei (Fig. 1D).

3. Impact of ethanolic extract of *S. costus* roots on molecular aspects in *B. alexandrina* snails

Genotoxicity was assessed in snails exposed to *S. costus* root extract. Five primers were used, resulting in clear and reproducible DNA bands (Table 2). Genomic template stability (GTS) decreased, indicating genotoxic effects. DNA band patterns were analyzed in *B. alexandrina* snails treated with LC_{25}, sub lethal concentrations of the extract, revealing DNA damage (Fig. 2). Primer P2 generated the most PCR fragments (nine bands), while P6 and P8 produced fewer (two bands). DNA fragments ranged from 100 to 3000 base pairs, with the appearance of new bands and normal ones vanish, indicating polymorphism. A study used RAPD-PCR to analyze the effects of a root extract on genomic stability. Five primers produced 21 amplified bands, with only six being polymorphic (20.4% of total). Table (3) exhibits disappearance of bands and emergence of new ones. The extract caused new band formation, indicating DNA damage. Similarity coefficient between treated and control groups was 48%. GTS, a measure of genomic stability, decreased to 25% at LC_{25} concentration, confirming extract's damaging effect on DNA.

4. Impact of *S.costus* roots on digestive and hermaphrodite glands in *B. alexandrina*

In the electron microscopy study, the digestive gland of untreated *B. alexandrina* snails displayed normal *microvilli* at the apex of the digestive cells, with nuclei located adjacent to the basement membrane (Fig. 3A, B). There were normal tight connections between neighboring digestive cells in the upper lateral side, secretory cells, and large

vacuoles. Many mitochondria and golgi apparatus were dispersed close the nucleus of the digestive cell. However, *B. alexandrina* snails exposed to LC_{25} from the ethanol extract of *S. costus* roots exhibited distortion of the tip of *microvilli* in the digestive cells and destruction of digestive gland (Fig. 3C). Regarding to the hermaphrodite gland, the normal gland of non-treated *B. alexandrina* snails are composed of many vesicles called acini that are isolated from one another by a thin layer of vascular connective tissue. Male and female reproductive gametes are produced in each acinus, with mature ova situated at its periphery and bundles of male sperm distributed in its middle. Sections of this gland from snails treated with LC_{25} of *S. costus* roots ethanol extract showed deteriorations in the form of connective tissue, atrophy and rupture, as well as ova and sperm in the gland acini. Furthermore, it was evident that certain vacuolated ova and degeneration sperms had sustained the most significant damages (Fig. 4A - C).

5. Impact of *S. costus* roots on *B. alexandrina* with *S. mansoni* and cercarial production

The treatment was administered seven days and twenty one days after infection with *S. mansoni miracidia*. The results showed that the treated snails experienced negative effects on various biological parameters at first cercarial shedding phase in comparison with the untreated infected snails (control group) (Fig. 5A- D). Survival rate of snails treated with LC_{10} at 7 and 21 days of exposure was significantly decreased to 20.2 and 22.57%, respectively, compared to 90% in the control group. Snails treated with LC_{25} could not tolerate the stresses and died before cercarial shedding stage (survival rate = 0%). The infection rates of snails treated with LC_{10} after seven and twenty one days of exposure to miracidia were also lower compared to the control group, recording 28.5.2 and 31.1%, respectively, compared to 61.6% in the control group. However, the prepatent period and duration of cercarial shedding were not notably different between the groups receiving treatment and those not. On the other hand, the life span and cercarial production per infected snail were significantly lower in the snails subjected to LC_{10} after seven and twenty one days of miracidial course compared to the nontreated snails ($P< 0.05$).

6. Miracicidal and cercaricidal potential of *S. costus* roots against *S. mansoni* miracidia and cercariae

The study found that the rate of mortality of miracidia elevated gradually with time and with higher concentrations of the ethanolic extract, for example, when miracidia were subjected to 58.77mg/ L (LC_{25} for *B. alexandrina* snail), 53% of the miracidia died after 40 minutes, and 81% died after one hour and 20 minutes (Table 4 & Fig. 6A). Increasing the concentration to 77.71mg/ L (LC_{50} for *B. alexandrina*) further increased the mortality rates. After 40 minutes, the mortality rate increased from 53 to 67%, and it reached 100% after one hour (Table 5 & Fig. 6B). The same concentration-dependent and

time-dependent effect was observed with the cercariae of *S. mansoni*; a positive relationship was found between the concentration and time of exposure to the extract, leading to an increased mortality. In the group exposed to 77.71mg/ L, all cercariae were observed dead within 60 minutes of exposure (Table 6 & Fig. 7A, B). Interestingly mentioned to the variability, movement of cercariae of *S. mansoni* exposed to LC_{25} of root extract showed decrease in variability and decrease in its movement comparing with control. Lethal times (LT_{50} and LT_{90}) indicate how long it takes to kill 50 and 90% of cercariae and miracidia, respectively, were recorded for 58.77 and 77.71mg/ L. For miracidia, LT_{50} and LT_{90} were 44.10 minutes and 88.94 minutes in the 58.77mg/ L group, and 25.43 minutes and 56:02 minutes in the 77.71mg/ L group (Table 5). For cercariae, the corresponding values were 44:9 and 87:45 minutes in the 58.77mg/ L group, and 27:0 and 53:08 minutes in the 77.71mg/ L group (Table 7).

7. Phytochemical screening

The phytochemical screening of *S. costus* roots indicated the presence of a diverse range of constituents, including alkaloids, tannins, flavonoids, coumarins, phenols, steroids, and terpenoids, while saponins and lipids were absent in the extract. Our findings agree with previous studies of **Pandey *et al*. (2007)** and **Chaudhary (2015)**. The results of this screening are summarized in Table (8), which provides a comprehensive list of these identified constituents.

8. Assessing the antioxidant potential, total flavonoid content (TFC), and total phenolic content (TPC) quantitatively in a *S. costus* root extract

Plants produce flavonoids and phenolics which have strong biological activities as anti-oxidant activity. In the present study the value for TPC was recorded for ethanolic root extract (357.88± 0.28mg GAE/ g ext.) of *S. costus*, while TFC (312.47± 1.17mg RE/ g ext) was found in the ethanolic extract of roots (Table 9). Moreover, the antioxidant potential of *S. costus* root extract is presented in Table (9); the DPPH free radical scavenging activity was exhibited by the ethanol extract of *S. costus* root with IC_{50} value of 12.71µg/ mL, while in the ABTS radical scavenging assay, the IC_{50} value was 8.27µg/ mL.

9. Gas chromatography –mass spectrometry (GC-MS)

As shown in Table (10), the active compounds are listed together with their peak area (%), retention duration, molecular weight, and molecular formula. We noted that the predominant components in the ethanolic extract of *S. costus* roots (83.35%) were: furan-2(3H) -one, 3a, 4, 6a, 7, 8, 9, 9a, 9b -octahydro-6-methyl-3,9bis (methylene)-,[3aS(3a.alpha. ,6a. alpha.,9a.alpha.,9b.beta.)]; cis-Thujopsen (5.25%); Tricyclo [8.6.0.0 (2,9)]Hexadeca-3,15-diene, trans-2,9-anti-9,10-cis-1,10 (4.85%); Dihydro dehydro lactone [dihydro (3S,3aS,6aR,9aR,9bS)-3-Methyl-6,9-dimethylenedecahydroazuleno[4,5-

b]furan-2(9bH)-one] (4.31%); 13-Docosenamide, (Z) (3.96%);alpha.-Guaiene(3.64%); 1,4-Methanocycloocta[d]pyridazine,1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-, (1.alpha. ,4.alpha.,4a.alpha.,10a.alpha.) (-5.36%).

Table 1. Molluscicidal activity of *S. Costus* roots extract against *B. alexandrina* snails after 24h exposure

***S.costus* roots extract**	**LC_{10} (ppm)**	**LC_{25} (ppm)**	**LC_{50} (ppm)**	**LC_{90} (ppm)**	**slope**
24h	41.72	58.77	(46.38-105.11)	113.70	1.21

Table 2. RAPD primers used to evaluate genotoxicant-induced alterations, total bands and polymorphic bands in control and exposed *B. alexandrina* snails to extract of *S. costus* roots

Primer code	Primer name	Primer sequence (5′→ 3′)	Length (bp)	Total bands	Polymorphic bands	% of polymorphism	Similarity coefficient
P2	SCOT-14	ACGACATGGCGACCACGC	18	9	2	22.2	0.44
P5	SCOT-31	CCATGGCTACCACCGCCT	18	5	3	60	0
P6	SCOT-33	CCATGGCTACCACCGCAG	18	2	0	0	1
P7	SCOT-34	ACCATGGCTACCACCGCA	18	3	1	0	0
P8	SCOT-52	ACAATGGCTACCACTGCA	18	2	0	20	1
Total				21	6	20.4	% 48

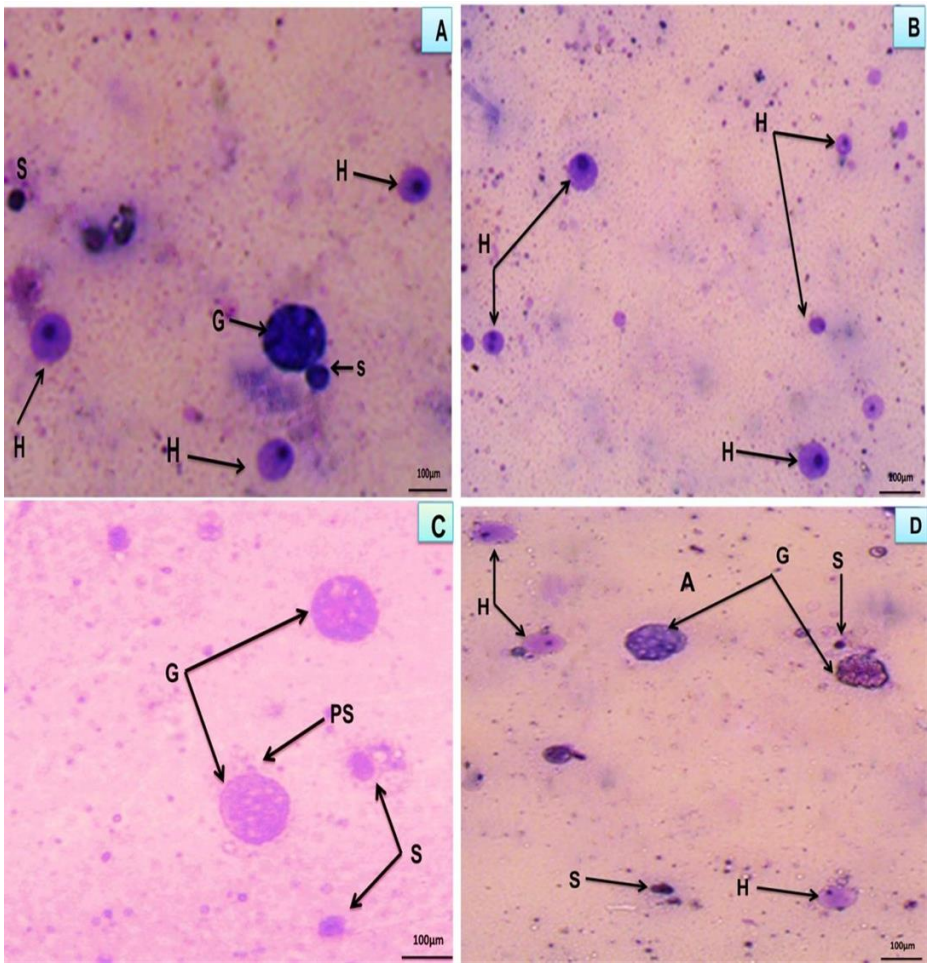

Fig. 1. Photomicrographs (x40) revealing the effects of ethanol extract of *S. costus* root on various types of *B. alexandrina* snails' hemocytes. **A)** Control snails' hemocytes; **B** and **C)** Snails treated with LC_{10} of *S. costus* root extract; **D)** Snails treated with LC_{25}

Table 3. RAPD profile changes and % GTS in freshwater snail *B. alexandrina* under sublethal concentration (LC_{25}) ppm of ethanol extract of *S.costus* roots

Primer number	Primer	Control (1)	Exposed (2)	
				d
1	P2	5		1
2	P5	5		2
3	P6	3		0
4	P7	5		2
5	P8	2		0
	Total bands	20	0	5
	a		15	
	a\n		0.75	
	1-(a/n)		0.25	
	% GTS		25	

a is polymorphic profile (a = d+p)
p is appearance band in treated-sample
d is disappearance band in treated-sample
(*n*) the number of total bands in the non-treated cells
GTS% = (1 − a/n) × 100

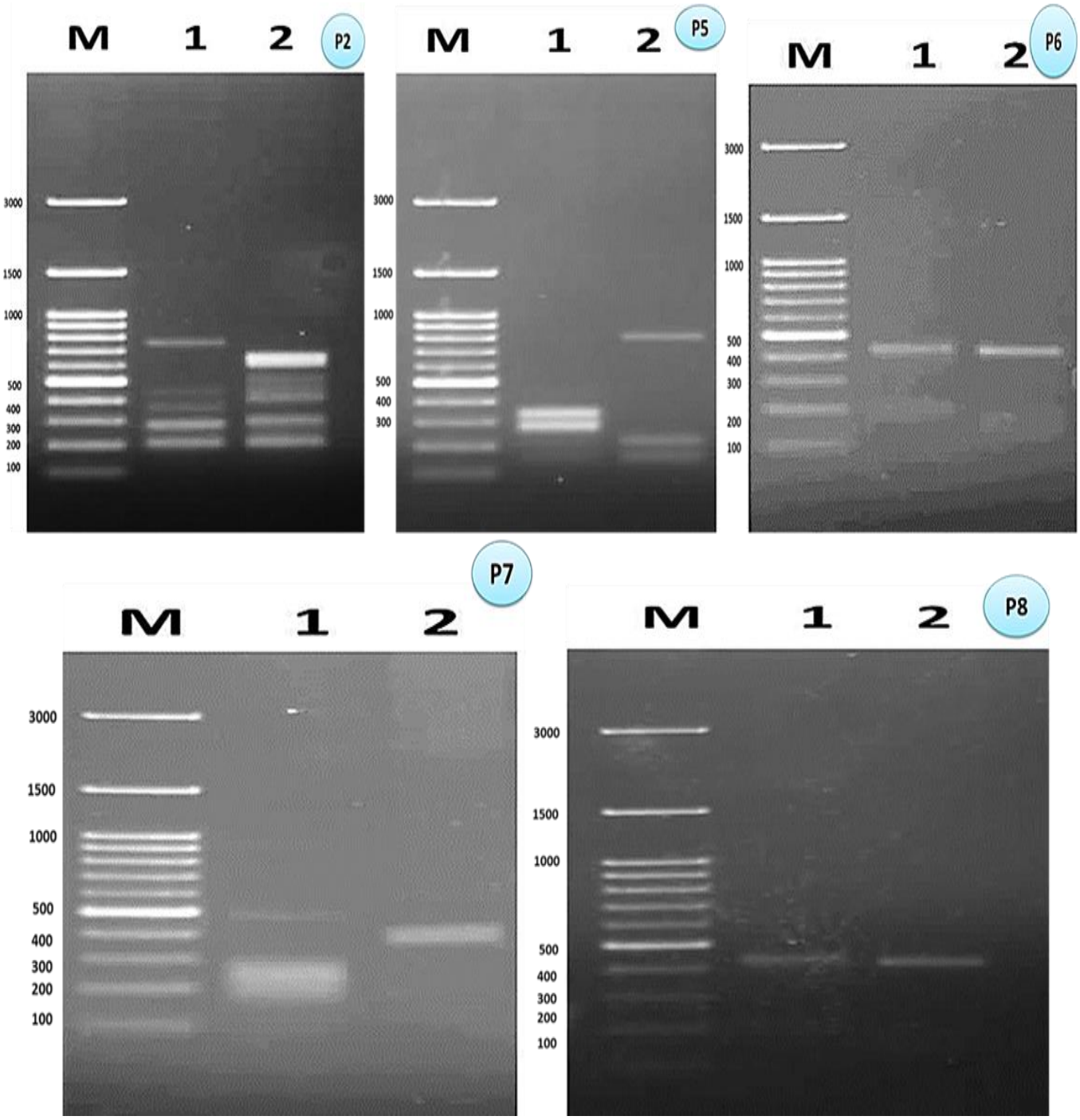

Fig. 2. RAPD fingerprints of the freshwater snail *B. alexandrina* Lanes; M: 100bp plus DNA ladder; 1: Control group, 2: LC_{25} ppm of ethanol extract of *S. costus* roots

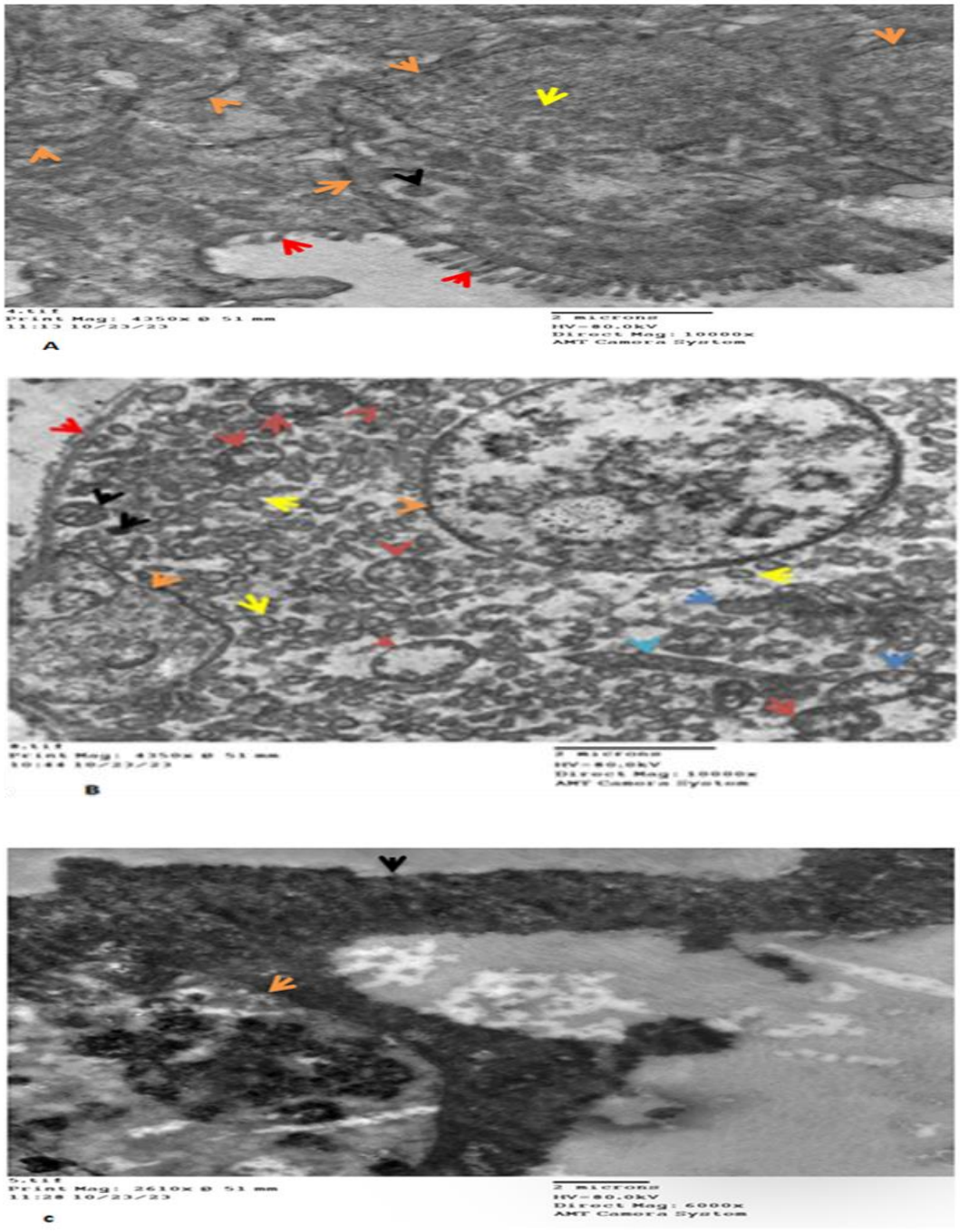

Fig. 3. Transmission electron microscope (TEM) photograph showing the ultrastructure of normal digestive glands of *B. alexanderina* snails: **(A, B)** Normal *microvilli* (red arrow), nucles (black arrow), secretory cell (yellow arrow), adjacent digestive glands (orange arrow), golgi apparatus (blue arrow), mitochondria (dark red arrow), large vacuoles (orange headarrow), and **(C)** Treated digestive gland with LC_{25} shows deformation *microvilli* (dark black arrow), deformation and destruction digestive gland (orange arrow)

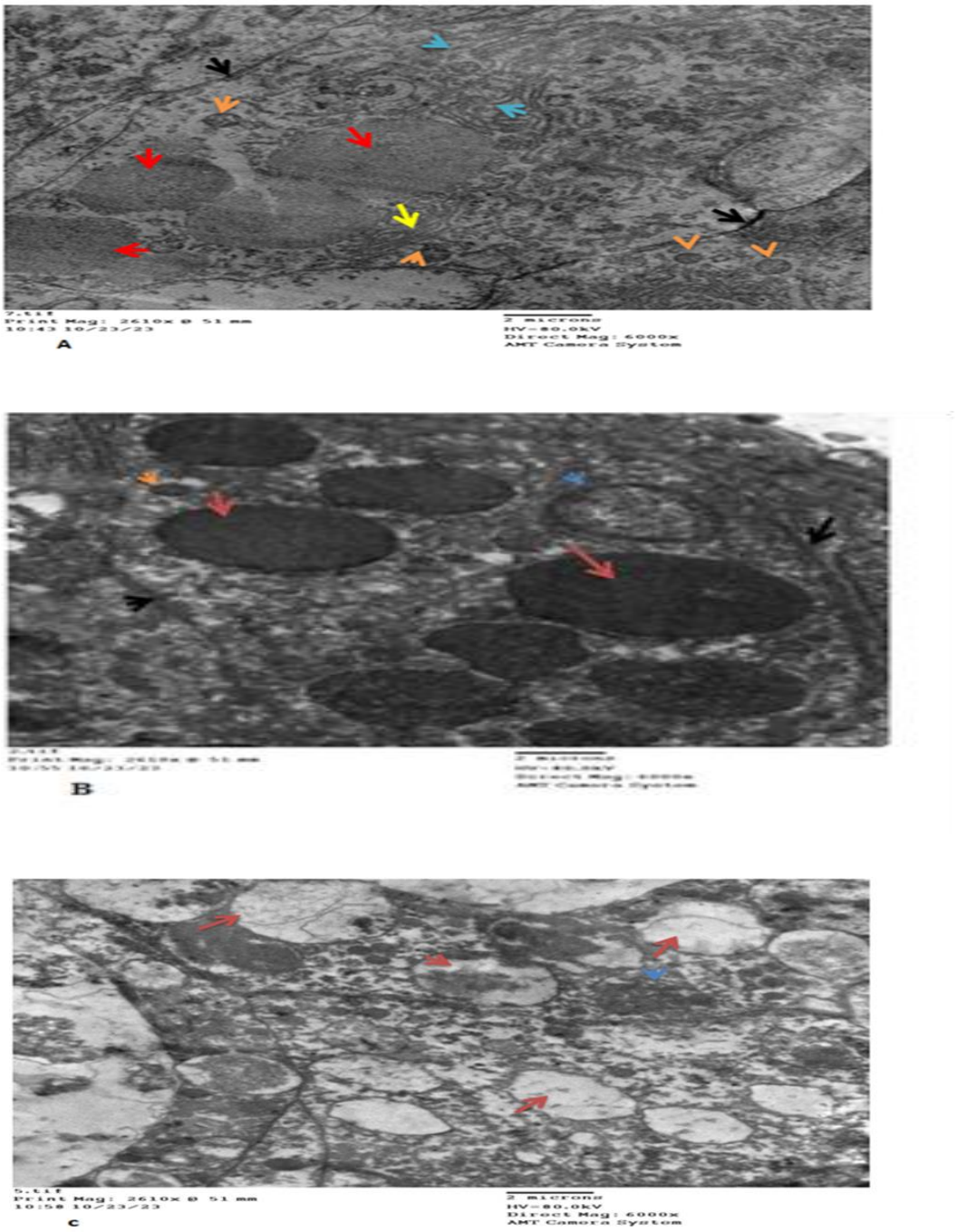

Fig. 4. Transmission electron microscope (TEM) showing the ultrastructure of normal hermaphrodite glands of *B. alexanderina* snails **(A & B)** Normal mature ova (red arrow), sperm (blue arrow), nucles (orange arrow), endoplasmic reticulum (yellow arrow), mitochondria (blue headarrow), compact acini (red arrow), cell membrane (black arrow), and **(c)** Treated hermaphrodite gland with LC_{25} shows deformation mature ova prominent damages were clear for several vacuolated ova (red arrow), degeneration sperm (blue headarrow)

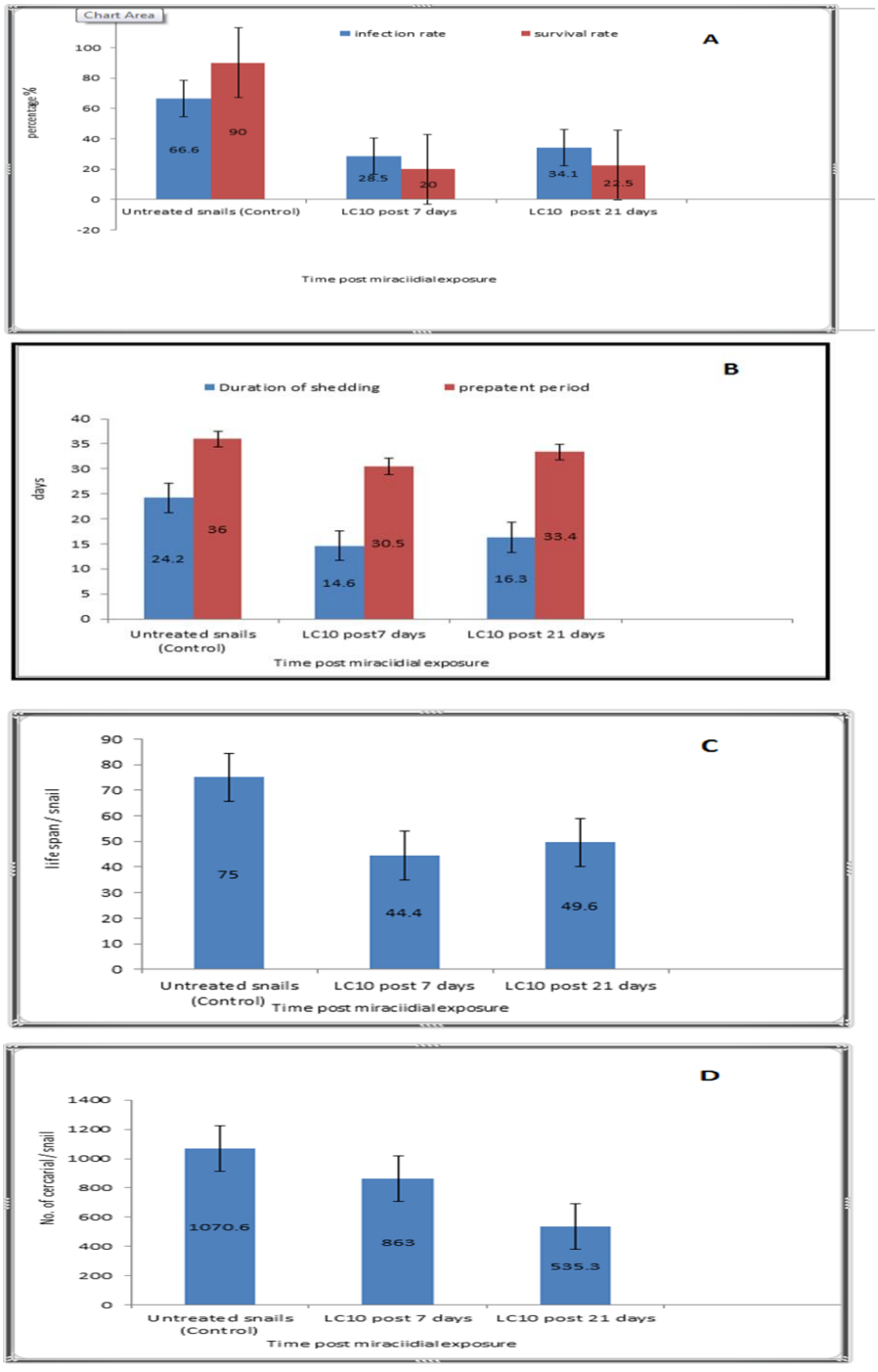

Fig. 5. Impact of ethanol extract of of *S. costus* root on survival rate, **(A)** Infection rate, perpatent period; **(B)** Duration of shedding; **(C)** Life span, and **(D)** Cercarial production of *B. alexandrina* exposed to *Schistosoma mansoni* miracidia at 1st cercarial shedding stage

Table 4. Miracicidal activity of *S. Costus* roots extract against miracidea of *S. mansoni*

Exposure time (min)	**Mortality %**		
	Control	**LC_{25} (ppm)**	**LC_{50} (ppm)**
10	0	16.00 ±0.92	33.00 ±2.44
20	0	22.66 ±1.41	42.33 ±1.92
30	0	42.00 ±1.84	54.66 ±0.53
40	0	53.00 ±2.44	67.00 ±0.92
50	0	55.00 ±0.92	84.00 ±0.92
60	20±00	62.30 ± 2.32	100.00±00
70	20±00	70.30 ± 1.41	
80	20±00	81.60 ± 1.41	
90	20±00	100.0 ±00.	

Table 5. Lethal time (LT_{50}& LT_{90}) of *S. mansoni* miracidia post exposure to *S. costus* roots extract

Conc. (ppm)	**LT_{50} (min)**	**LT_{90} (min)**
LC_{25}	44:.10	88:49
LC_{50}	25:43	56:02

Table 6. Cercaricidal activity of *S.Costus* roots extract against cercarea of *S. mansoni*

Exposure time (min)	**Mortality %**		
	control	**LC_{25} (ppm)**	**LC_{50} (ppm)**
10	0	15.5 ±1.09	30.3±1.0
20	0	21.60 ±0.53	40.6±2.6
30	0	40.60 ±1.06	53.3±2.8
40	0	49.00 ±0.92	66.0±0.92
50	0	52.00 ±0.92	83.0±2.2
60	25±00	60.30 ± 0.53	100±00
70	25±00	68.30 ± 0.53	
80	25±00	82.60 ± 2.44	
90	25±00	100.0 ±00	

Table 7. Lethal time (LT_{50} & LT_{90}) of *S. mansoni* cercariae post exposure to *S. costus* roots extract

Con.(ppm)	LT_{50} (min)	LT_{90} (min)
LC_{25}	44:.9	87:45
LC_{50}	27:00	53:.08

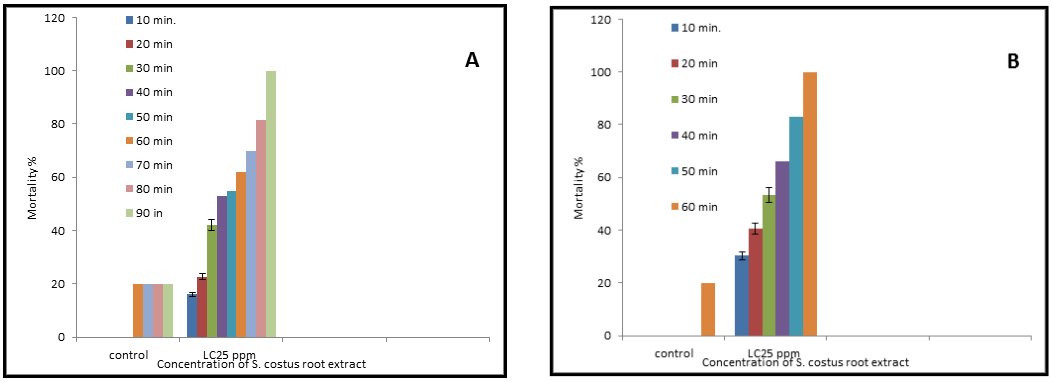

Fig. 6. Inhibitory activities of *S. Costus* roots extract against miracidia of **(A)** *S. mansoni B. alexandrina* treated with LC_{25} , and **(B)** *B. alexandrina* treated with LC_{50} of extract at different intervals of time

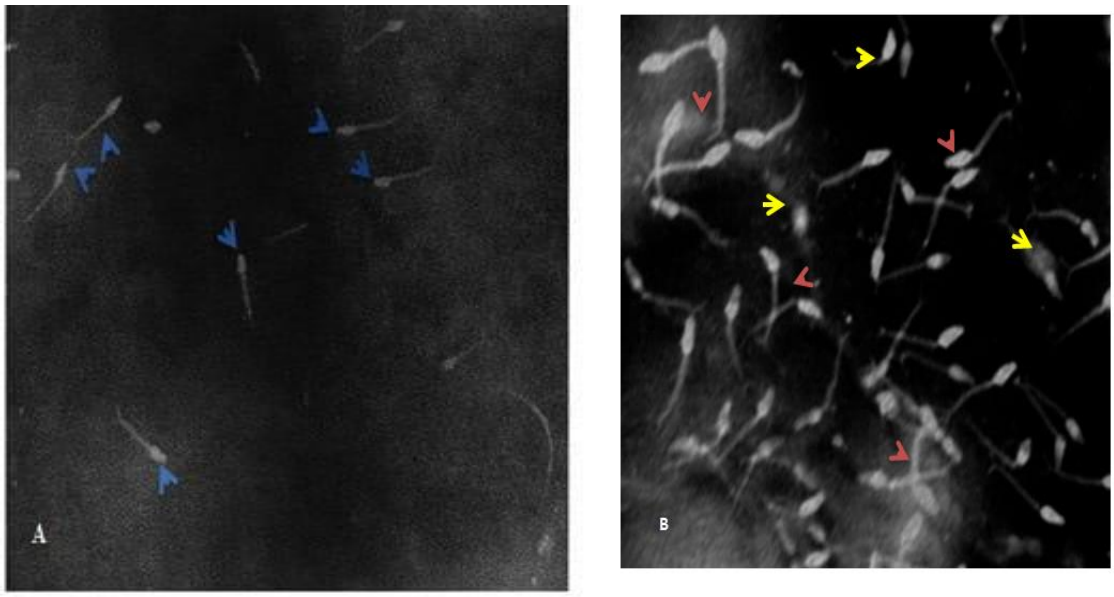

Fig. 7. Light photomicroscope (x40) showing cercarial movement of **(A)** control cercariae of *S. mansoni* exhibited active movement (blue arrow). **(B)** Treated cercariae of *S. mansoni* with LC_{25}, exhibited reduced viability after 40 minutes of exposure to to ethanolic extract of *S. costus* roots (yellow and red arrow)

Table 8. Phytochemical screening of *S. costus* roots

Phytoconstituent	**Result**
Flavanoids	+
Phenolics	+
Tanins	+
Alkaloids	+
Terpenes	+
Saponin	-
Lipids	-
Steroids	+
Glycosides	+
Coumarins	+
+ = test positive, - = test negative	

Table 9. Total phenolics content (TPC), total flavonoids content (TFC) and the antioxidant activity of *S. costus* roots

TFC (mg RE / g ext.)	**TPC (mg GAE / g ext.)**	**ABTS (IC_{50} µg/ml)**	**DPPH (IC_{50} µg/ml)**
312.47 ± 1.17	357.88 ± 0.28	8.27±1.11	12.71±0.93
Ascorbic acid	-	1.99±1.07	3.21±0.55

Table 10. Chemical compounds identified in *S. costus* ethanol extract by GC-MS analysis

Peak No.	Name of component	Base m/z	Mol. Formula	Retention time Sec	Area percent %
1	Alpha.-Guaiene	105.15	$C_{15}H_{24}$	27.620	3.64
2	Tricyclo[8.6.0.0(2,9)]hexadeca-3,15-diene, trans-2,9-anti-9,10-cis-1,10	108.15	$C_{16}H_{24}$	28.091	4.85
3	Cis-Thujopsene	105.15	$C_{15}H_{24}$	29.947	5.25
4	Dihydrodehydrocostus lactone ; (3S,3aS,6aR,9aR,9bS)-3-Methyl-6,9-dimethylenedecahydroazuleno[4,5-b]furan-2(9bH)-one	91.10	$C_{15}H_{20}O_2$	33.134	4.31
5	Azuleno[4,5-b]furan-2(3H)-one, 3a,4,6a,7,8,9,9a,9b-octahydro-6-methyl-3,9-bis(methylene)-, [3aS-(3a.alpha.,6a.alpha.,9a.alpha.,9b.beta.)]	91.10	$C_{15}H_{18}O_2$	34.226	83.35
6	1,4-Methanocycloocta[d]pyridazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-, (1.alpha.,4.alpha.,4a.alpha.,10a.alpha.)	107.15	$C_{13}H_{20}N_2$	37.467	-5.36
7	13-Docosenamide, (Z)-	59.10	$C_{23}H_{42}NO$	41.237	3.96

DISCUSSION

The present study demonstrates that the root extract of *S. costus* exhibits molluscicidal activity against *B. alexandrina* snails in laboratory conditions. This work is the first documented use of *S. costus* root extract against *Schistosoma mansoni*, the parasite that causes schistosomiasis. However, there have been numerous studies on the molluscicidal, miracicidal, and cercaricidal effects of medicinal plants. Some of these plants are inexpensive, safe, and suitable for local snail control, as reported by **Adewumi *et al*. (2013)** and **Dai *et al*. (2014)**. Therefore, there is a growing interest in identifying safe molluscicides of plant origin, with particular emphasis on their miracidicidal and cercaricidal activities, as highlighted by **Santos *et al*. (2014)** and **Duval *et al*. (2015)**. The observed molluscicidal activity of the extract (at a concentration of 113.70ppm) in this study suggests that its effectiveness may be dependent on the concentration used. These findings are consistent with previous studies conducted by **Dokmak *et al*. (2021)** and **Ibrahim *et al*. (2023)**, which reported a concentration-dependent toxic molluscicidal activity of saponin and chlorophyllin against the intermediate host of *Schistosoma haematobium*. It is possible that the active compounds present in the *S. costus* extract exerted an impact on molluscan tissues during the snails' exposure, or they might have been transformed into more toxic metabolites within the snails' tissues. Overall, this study contributes to the growing body of research on the potential of medicinal plants as molluscicides, particularly against the intermediate hosts of schistosomiasis. Further investigations are warranted to explore the mechanism of action of *S. costus* extract and its potential application in controlling snail populations and interrupting the transmission of schistosomiasis. Many studies (**Mohaptra & Nayak 1998; Singh *et al*., 2004; Singh *et al*., 2005; Singh *et al*., 2010)** have examined the insecticidal, piscicidal, and molluscicidal properties of compounds from different classes. According to the study's findings, snails might be killed by concentrations of *S. costus* root extract, as well as by *S. mansoni* miracidia and cercariae. Previous researches **(Le Clec'h *et al*., 2016; Baroudi *et al*., 2020)** have demonstrated that snails have a dual immune system consisting of cellular and humoral components. In snails, hemocytes, which control immunological responses, can take on three different morphological forms. As shown with *B. alexandrina* snails, immunological responses are among the biomarkers of exposure to environmental stresses in snails **(Ibrahim & Sayed, 2020)**. The treatment involving the use of an LC_{25} concentration of *S. costus* root extract resulted in an increase in hyalinocytes and the emergence of abnormalities in nucleus numbers, such as cells with two nuclei. Additionally, granulocytes exhibited numerous granules, abnormal cell membranes, and some even formed pseudopodia. Pseudopodia formation is an immunological action employed by these cells to eliminate foreign materials **(Guria, 2018; Ibrahim *et al*., 2023)**. The observed abnormalities in the morphological characteristics of these cells could be attributed to the snails' deteriorating health and changes in physiological

parameters due to continuous exposure to the phytochemical compounds present in the plant extract.

RAPD PCR is a sensitive and reliable approach used to detect DNA damage in *B. alexandrina* snails exposed to a sublethal concentration (LC_{25} ppm) of *S. costus* root extract. This method helps identify genotoxin-induced DNA damage in snails by observing changes in RAPD profiles, such as new bands emerging or vanishing in comparison to control samples **(Padmesh *et al*., 1999)**. Bands form on the DNA when a primer is unable to bind to a particular location that the genotoxic material has changed may disappear. Conversely, new bands may appear when changes in the DNA structure (such as insertions, deletions, or breaks) make certain sites accessible to the primer **(Pietrasanata *et al*., 2000; Enan, 2006)**. Since DNA lesions can cause structural alterations, decrease DNA polymerization, or inhibit Taq DNA polymerase activity, they can negatively impact RAPD profiles **(Nelson *et al*., 1996; Atienzar, 2000)**. One example of a DNA lesion is bulky complexes. Extensive DNA damage can lead to a decrease in band intensity or the complete absence of amplified products. In this study, the sublethal concentration required for DNA changes to occur was determined as 24 hours of exposure followed by an additional 24 hours of recovery **(Ibrahim *et al*., 2022)**. The genotoxic activity of the root extract may be attributed to its toxic effect on DNA or the generation of reactive oxygen species, which can impair the antioxidant defense system and impact the lifespan of organisms exposed to *S. costus* root extract **(Lai & Singh, 2004; Lee *et al*., 2004; Guier *et al*., 2006)**. The current study of investigation by TEM indicated severe damages of the *S. costus* extract to the cell constituents of treated *B. alexandrina* snails. Visualization of such alterations entailed sections from the digestive and hermaphrodite glands of treated and untreated snails were used in histological investigations. When compared to the control group, which represented normal structure, the tissues of the digestive gland suffered from severe damage to the secretory and digesting cells as well as damage to the *microvilli*. This could be explained by the possibility that the plant extract from the treated snails may have a more sensitive gland, which could have a toxic impact and increase the mortality of the snails. Similarly, the studied molluscicidal agents induced histo- pathological changes to the hermaphrodite gland cells of treated *B. alexandrina* snails. The most prominent damage in the hermaphrodite gland cells was deformation ova and shrink of connective tissues and disintegration in the hermaphrodite gland compared to the control group that showed normal structure.

Numerous therapeutic plants generate a variety of groups of secondary metabolites, these compounds can interfere with metabolic processes within cells, disrupt cellular membranes, or alter gene expression or signal transduction pathways **(Omojate *et al*., 2014; Mohamed *et al*., 2017)**. According to studies, extracts from the roots of *S. costus* contain larvicidal properties **(Lui 2012; Obare *et al*., 2016)**. The penetrating extract may disrupt the fine tegument of miracidia, and the authors speculated that this interference

may also interfere with the expression of proteins essential for cellular processes, which could account for the death of miracidia. Another theory is that the parasite **(Zelck & Von Janowsky, 2004)** down-regulated antioxidant enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione-S-transferase (GST), which are involved in defense against oxidative damage. Similar results were observed in a trial in which the water surface was treated with red cedar wood oil for a short period of time. This caused cercariae to alter their behavior, swimming rapidly to the bottom before becoming immobile or twitching their tails occasionally **(Naples *et al.*, 2005)**. The mobility and penetration of *S. mansoni* cercariae were shown to be significantly reduced by **Abd El Ghaffar *et al*. (2018)** when the infected mice's skin was exposed to methanol extracts of *S. nigrum* and *C. citrinus*. Similar findings were made by **Ahmed and Rifaat (2005)**, who found that *S. mansoni* cercariae were greatly attenuated and less able to pierce the skin of mice when *S. nigrum* leaves were boiled in water.

S. costus is a plant that is high in antioxidants. Its main constituents are sesquiterpene lactones, such as dehydrocostus lactone and costunolide, and it has a variety of biological activities, such as immune-modulatory, hypo-glycemic, anti-hepatotoxic, hypolipidemic, anti-inflammatory, antiviral, and anticancer properties **(Zahara *et al.*, 2014; Amara *et al.*, 2017)**. According to the study's data, the infection of *B. alexandrina* snails with *S. mansoni* miracidia and the subsequent cercariae generation were influenced by sublethal quantities of the organic solvent extract of *S. costus* root. After being exposed to miracidials for seven and twenty-one days, snails treated with the extract at LC_{25} concentration perished before releasing cercariae. This implies that the snails died before they could reach the cercarial shedding stage because the stress of both the infection and the *S. costus* extract was too much for them to handle. The compound in the extract may have a lethal effect on the infected snails, which could reduce their immune response. The gastropod immune system protects the snail intermediate host against schistosome infection, a process that is aided by humoral and hemoptysis **(Pila *et al.*, 2017)**. Alternatively, the extract may accumulate in the head-foot region of the infected treated snails. According to **Malek (1980)**, the mother sporocysts of the parasite are usually located in this area during the early prepatent period, which occurs seven days following miracidial exposure. However, in contrast to the control group, snails treated with the extract at an LC_{10} concentration showed lower survival rates during the initial shedding, a shorter cercarial shedding time, and a diminution in the overall quantity of cercariae produced per infected snail. The extract may have inhibited the snails' capacity to manufacture and release cercariae even at sublethal quantities, according to this evidence. The results of this investigation show that *B. alexandrina* snails subjected to the sublethal dose of *S. costus* extract had hemocytes with aberrant morphological forms.

Our findings of phytochemical screening match those recorded in previous studies **(Pandey *et al.*, 2007; Chaudhary, 2015)**. The rich profile of phytochemicals in ethanol

extract of *S. costus* roots suggests their potential for various biological activities and therapeutic applications. However, *S. costus* was reported to possess significant anti-oxidant activity (high phenolic and flavonoid content) and is known to be used for medicinal purpose **(Qiu *et al*., 2010)**. Alternatively, oxidative stress and free radicals are mostly associated. Free radicals are created, and when these radicals come into contact with crucial biological components including proteins, DNA, and the cell membrane, they may cause harm **(Archana Gupta *et al*., 2020)**. According to **Moon and Shibamoto (2009)** and **Shantabi *et al*. (2014)**, antioxidants react with free radicals and neutralize them, potentially preventing harm before it occurs. A broad variety of secondary metabolites produced by plants can be used as natural antioxidants. Therefore, two i*n-vitro* assays, the DPPH free radical scavenging assay and the ABTS radical scavenging assay, were used in our investigation to measure antioxidant activity. Via comparing antioxidants to an ascorbic acid standard, the ABTS technique assesses how well they can scavenge the ABTS radical that is produced in the aqueous phase. Strong oxidizing agents, such as $KMnO_4$ or $K_2S_2O_8$, react with the ABTS salt to produce ABTS **(Ilyasov *et al*., 2020)**. In addition, the DPPH approach is used to estimate the antioxidant activity based on the technique through which antioxidants inhibit lipid oxidation, the DPPH free radical is extremely stable, reacts with hydrogen-containing molecules, which decolorizes the solution of DPPH after a reduction process. The antioxidant potential to reduce the DPPH radical is measured in this assay resulting in DPPH free-radical capture and then estimate free-radical scavenging activity **(Valko *et al*., 2007)**.

Plant extracts containing bioactive chemicals can be identified with GC-MS, an efficient method. According to the area % beneath each individual peak and their retention time (Rt), which are obtained from the GC-MS chromatogram of the ethanolic roots extract of *S. costus*, there were seven components present in the chromatogram. These components' mass spectra matched the peaks of recognized compounds listed in NIST17 Libraries of spectral databases LIB. According to the GC-MS analysis, the major constituent present in *S. costus* extract is Azuleno[4 ,5-b]furan -2(3H)-one, 3a, 4,6a, 7,8,9 ,9a,9b-octahydro-6 -methyl-3,9bis(methylene),[3aS(3a.alpha.,6a.alpha.,9a.alpha,9b. beta.)] at 83% level. Azuleno functions as an anti-allergic, ACE-inhibitor, and alpha-reductase inhibitor, Carboxylase – Inhibitor, Acetyl – choline - antagonist, Anesthetic Adrenocorticotrophic, , Anaphylactic **(Arunmathi & Malarvili, 2017)**.

In addition, Cis-Thujopsene that was detected in ethanol extract of *S. costus* roots (5.25%) is a non-oxygenated tri-cyclic sesquiterpene that demonstrated strong antibacterial action against Phytophthora ramorum. It has also been noted to lower heart rate and blood pressure and to have sedative effects. According to **Hu *et al.* (2006)** and **Zheng and colleagues (2012)**, alpha-guaiene compounds (3.64%) exhibit anti-inflammatory properties. Furthermore, a naturally occurring sesquiterpene lactone with numerous biological activities, including antifungal, anti-immune, anti-cancer, anti-

inflammatory, and spasmolytic properties, is called dihydro dehydro costus lactone **(Wedge *et al.*, 2000; Hsu *et al.*, 2009; Liu *et al.*, 2021)**. Additionally, a negative concentration means that the calibration curve does not intersect the Y-axis at zero or is not extrapolated to zero. In the case of 1,4-Methanocycloocta[d]pyridazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl (1α.,4α.,4aα.,10aα.) (-5.36%), analyte concentrations below your lowest calibration point may result in negative values if the curve is extrapolated and intersects the Y-axis above zero.

CONCLUSION

The root extract of *S. costus* was found to be both genotoxic and molluscicidal against *B. alexandrina* snails and caused alterations on hemocytes and histopathology. Additionally, it has larvicidal activity on the free larval stages of *S. mansoni*. Therefore, *S. costus* ethanolic extract could be used as a safe, plant-based molluscicide to stop the transmission of schistosomiasis through controlling the intermediate host.

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Arabic Summary

هبه دقماق ، امينة ابراهيم ، تامر سامي ، اسماء صلاح
معهد تيودور بلهارس للابحاث، مصر

الديدان المسطحة (وتعرف أيضاً بالمثقوبات)، وتحديدًا جنس البلهارسيا، هي المسؤولة عن التسبب في المرض الطفيلي المعروف باسم داء البلهارسيات. كان الهدف في هذا البحث هو الاستفادة من المستخلص الايثانولي لجذور نبات القسط الهندي و تجربته كمبيد للرخويات و ذلك عن طريق استهداف القواقع التي تعمل كعائل وسيط لأنواع الطفيليات وتعطيل دورة حياة الطفيليات

أثناء البحث و التجارب، تمت معالجة حلزونات بيومفلاريا الكسندرينا بالمستخلص الايثانولي لجذور نبات القسط الهندي لمدة أربع وعشرين ساعة، قبل الخضوع لمرحلة تعافي لمدة 24 ساعة و قد أظهرت النتائج أن المستخلص له قدرة قاتلة للرخويات، حيث بلغ التركيز المميت LC_{90}= 113.70 جزء في المليون.

و من المثير للاهتمام حدوث تشوهات في الخلايا الدموية في حلزونات بيومفلاريا الكسندرينا بعد التعرض للمستخلص الإيثانولي للنبات بتركيزات LC_{10} وLC_{25} علاوة على ذلك، تم التعرف على التغيرات النسيجية المرضية في الغدد الخنثى والغدد الهضمية والتي تم تأكيدها من خلال التصوير بالمجهر الإلكتروني النافذ.

تم أيضًا دراسة التأثيرات السمية الجينية للمستخلص باستخدام تقنيةRAPD-PCR ، مما كشف عن انخفاض في الاستقرار الجيني (GTS) إلى 25٪ عند تركيز LC_{25}. كما أن المستخلص الايثانولي لجذور نبات القسط الهندي له آثار سلبية على حيوية وعدوى البلهارسيا المنسونية في الاطوار ميراسيديا والسيركاريا.

تناولت الدراسة تأثير تعريض حلزونات بيومفلاريا الكسندرينا المصابة بالبلهارسيا إلى تركيزات LC_{10} و LC_{25}من المستخلص الايثانولي لجذور نبات القسط الهندي بعد 3 و21 يومًا. و كذلك تم تحديد كفاءة المستخلص كمضاد للاكسدة و ذلك لاحتواءه علي نسة كبيرة من الفينولات و الفلافينوديدات. تم تحليل المستخلص الايثانولي لجذور نبات القسط الهندي بالطرق الكروماتوجرافية و الكيميائية حيث اظهرت النتائج احتوائه علي كثير من الايضيات الثانوية المتنوعة مثل القلويدات والفلافونويدات و التنينات والكومارين، والفينول، و الاستيرويدات، والتيربينويدات. و كذلك حدد التحليل باستخدام كروماتوجرافية الغاز المتصل بمطياف الكتلة احتواء المستخلص علي سبعة مركبات بتركيزات مختلفة مثل ازولينوفيوران(83.35%) و الفا جوانين (3.64%) و سيز سوجوبسن (5.25%) .

ولذلك، فإن المستخلص الايثانولي لجذور نبات القسط الهندي يعد كعامل مكافحة حيوي يمكن أن يحل محل مبيدات الرخويات الكيميائية في تقليل داء البلهارسيات.