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BIOACTIVITY EVALUATION OF *SARCOPHYTON* CRUDE EXTRACT AS A LARVICIDAL AGENT AGAINST *CULEX PIPIENS* (DIPTERA; CULICIDAE) LARVAE

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Culex pipiens (Cx. pipiens L.) mosquitoes play a crucial role as vectors for many diseases. The persistent application of synthetic insecticides for controlling mosquitoes culminates in the development of insect resistance. The marine ecosystem has garnered considerable interest in exploring new affordable insecticide alternatives with low resistance and high efficiency. Hence, this work was conducted to evaluate the potential larvicidal effect of Sarcophyton soft coral crude extract against third-instar larvae of Cx. pipiens as a safe insecticidal agent. To conduct this objective, the determination of the Sarcophyton extract's phytochemicals and its gas chromatography-mass spectrometric (GC-MS) fatty acid profile occurred. The mortality rate induced by Sarcophyton extract against larvae was assessed in a dose-dependent manner at 24, 48, and 72-hrs post-exposure. Also, the larvae were observed for any behavioral and morphological alterations, along with an estimation of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activities in larvae. The chemical characterization of Sarcophyton extract revealed different bioactive phytochemicals and three primary fatty acids: linoleic acid, oleic acid, and palmitic acid. The median lethal concentrations (LC_{50}) of Sarcophyton extract were recorded at 745.418, 370.064, and 232.017 µg/ml after 24, 48, and 72 hrs of exposure time, respectively. Treatment with 24 hrs-LC_{50} of Sarcophyton extract revealed notable neurobehavioral toxicity and morphological aberrations against Cx. pipiens larvae. The application of 24 hrs-LC_{50} of Sarcophyton extract displayed a neurotoxic effect evidenced by the significant inhibition of AChE activity and suppression of GST detoxification activity. The larvicidal action mechanism of Sarcophyton could be attributed to its bioactive components. Hence, this study concluded that Sarcophyton extract could be a potential natural candidate for controlling Cx. pipiens larvae.

Keywords: *Sarcophyton; Culex pipiens larvae; phytochemicals; fatty acids; acetylcholinesterase; glutathione-S-transferase*

INTRODUCTION

Mosquitoes (Diptera, Culicidae) are widely recognized as hazardous insects. They substantially affect healthcare systems and the economy, leading to significant losses in commercial and labor productivity, especially in tropical and subtropical regions[1]. Mosquitoes are considered the primary biological vectors of several human pathogens and parasites through biting. Additionally, they have the ability to spread several viral infections among animals[2-4].

Culex pipiens (*Cx. pipiens* L.)[5] is a frequently encountered mosquito species in Egypt. It is the vector responsible for transmitting filariasis, West Nile virus, and Rift Valley fever virus[6], causing illness, death, and economic burden. Larval control is an effective tool for mosquito management due to its vulnerability and low mobility, resulting in less dangerous environmental outcomes[7, 8]. Mosquito control largely relies on commercial chemical insecticides, such as carbamates, organophosphates, organochlorines, and Dichloro Diphenyl Trichloroethane (DDT)[2,9,10]. These artificial pesticides have been preferred

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thus far due to their rapid efficacy and convenient application[7]. However, the misuse of synthetic insecticides has led to environmental and human health problems[11, 12], in addition to the emergence of resistant variants[13-15]. Therefore, there is a necessity for alternative and environmentally safe substances that exhibit potential mosquito larvicidal properties. This can be accomplished by isolating natural products from marine organisms due to their biodegradability and minimal toxic effects on non-target species and the environment[16].

Previous studies indicated that secondary metabolites derived from marine invertebrates demonstrate numerous pharmacological properties and can be considered a potential source for drug development[17-19]. Among marine invertebrates, *Sarcophyton* soft coral (order Alcyonacea, family Alcyoniidae) is known to be a prolific producer of marine secondary metabolites, especially terpenoids and steroids[20-23]. These metabolites serve as the organisms' chemical defense mechanisms against any attack and maintain competitive relationships within the benthic habitat[24-27]. The biologically active substances from this soft coral showed pharmacological activities, such as antioxidant[28], antimicrobial[29, 30], and anti-inflammatory activities[31]. Although *Sarcophyton* soft coral is abundant on the Egyptian Red Sea reefs[32], limited studies were carried out on the larvicidal effect of its extract. Thus, this study was suggested to assess the larvicidal activity of *Sarcophyton* extract against third-instar larvae of *Cx. pipiens* to develop an alternative eco-friendly larvicidal agent.

MATERIALS AND METHODS

Sarcophyton collection and identification

The marine soft coral colony of *Sarcophyton* sp. was obtained from the Red Sea, Hurghada, Egypt beach at a depth of 3–6 m using the self-contained underwater breathing apparatus (SCUBA) diving technique **(Fig. 1)**. Samples were identified based on the morphology of colonies and interior sclerites following Janes and Leewis[33], Ezz-AlArab[34], and Cesnales[35]. The fresh specimens of *Sarcophyton* were then washed with distilled water and immediately kept frozen until the extraction process.

Preparation of the *Sarcophyton* crude extract

The *Sarcophyton* soft corals were fragmented and desiccated at ambient temperature. According to Zubair et al.[36], samples were extracted exhaustively by maceration in absolute methanol (1:2, *w/v*) for 3–5 days at room temperature with occasional stirring to facilitate the extraction of the active ingredients. Extract filtration was performed through Whatman filter paper (No. 1). Following that, the obtained filtrate was concentrated at 30–40 °C utilizing a rotary vacuum evaporator to eliminate the solvent from the extract and lyophilized to reach a viscous extract. The crude extract was preserved at a temperature of –80 °C for further analysis.

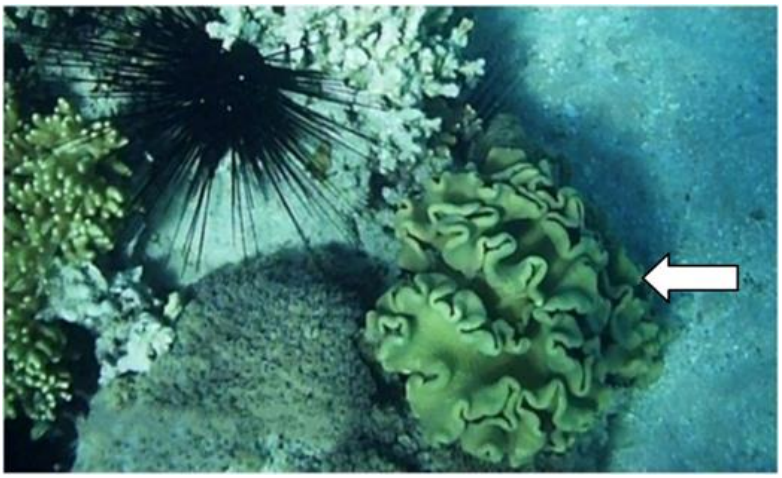


Fig. 1: *Sarcophyton* soft coral colony (white arrow) in its Red Sea habitat

Qualitative tests for phytochemical screening

Phytochemical analyses were conducted qualitatively on the crude methanolic extract of *Sarcophyton* using standard conventional methods described by Harborne[37], Kumar et al.[38], and Morsy[39] to identify its phytochemical constituents, including proteins, lipids, steroids, carbohydrates, alkaloids, phenolics, tannins, flavonoids, terpenoids, diterpenes, saponins, anthraquinones, and coumarins. The crude extract was diluted with distilled water to obtain 1 mg/ml concentration and then used for the qualitative phytochemical tests. The results were investigated by precipitation or color change.

Detection of proteins (Biuret test)

Two milliliters of 20% KOH solution were added and mixed thoroughly with 2 ml of the extract. Then, one ml of 0.5% $CuSO_4$ solution was slowly added to this mixture.

Detection of lipids (Emulsion test)

The extract was first dissolved in ethanol, mixed with water, and shaken vigorously.

Detection of steroids (Sulfuric acid test)

Five drops of concentrated H_2SO_4 were added to one milliliter of the extract.

Detection of carbohydrates (Molisch's test)

About two drops of an alcoholic solution of α-naphthol were added to one ml of the extract in a test tube. Around two milliliters of concentrated H_2SO_4 were added carefully on the side of this test tube.

Detection of alkaloids (Dragendroff's test)

Five milliliters of extract were first acidified with 1 M HCl. This acidic medium was heated, filtered, and then treated with Dragendroff's reagent (solution of potassium bismuth iodide).

Detection of phenolics (Ferric chloride test)

One milliliter of $FeCl_3$ solution was added to 2 ml of extract.

Detection of tannins (Braymer's test)

A few drops of 5% $FeCl_3$ were added to about 0.5 ml of extract and 0.5 ml of the respective solvent.

Detection of flavonoids (Aluminium solution test)

About 0.5 ml of extract was mixed with 0.5 ml of the respective solvent, and then a few drops of 1% $AlCl_3$ were added.

Detection of terpenoids (Salkowski test)

About 0.5 ml of extract was mixed with 0.5 ml of the respective solvent. Then, 1 ml of chloroform and 1 ml of concentrated H_2SO_4 were carefully added.

Detection of diterpenes (Copper acetate test)

A few drops of copper acetate solution were added to the extract.

Detection of saponins (Froth test)

About 0.5 g of extract was added to 3 ml of $NaHCO_3$ solution and shaken vigorously, and then the mixture was allowed to stand for about 20 min.

Detection of anthraquinones (Borntrager's test)

In a test tube, about 12 milliliters of benzene were added to the extract, shaken well, and filtered. About 6 milliliters of 10% NH_4OH solution were added to the filtrated solution, shaken again, and the test tube was left aside for a while. After the two layers were separated, the color of the ammonia phase was observed.

Detection of coumarins (Alkaline hydrolysis test)

Two drops of 1% NaOH solution were added to 1 ml of extract and heated in a water bath for 3-4 min until the solution became clear. After that, four drops of 2% HCl were added to the clear solution.

Determination of fatty acids in *Sarcophyton* crude extract

The fatty acid methyl ester was prepared following the methodology described by Azir et al.[40]. A total of 50 mg of lipid was dissolved in a mixture of 0.8 ml hexane and 0.2 ml sodium hydroxide. Then, the solution was stirred vigorously using a vortex stirrer for one minute. The solution was left on a stand for a duration of 10 min to facilitate the separation of the clear fatty acid methyl ester solution from the cloudy aqueous layer. The upper layer

was carefully gathered. The composition of the fatty acid was injected into gas chromatography-mass spectrometry (GC-MS) using a TG-5MS Zebron capillary column (length 30 m × 0.25 mm ID, 0.25 µm film thickness; Thermo) for analysis. Helium was utilized as a carrier gas with a flow rate of 0.7 ml/min. The oven temperature was raised from 140 °C to 240 °C for 2 min (3 °C/min), and mass spectra were then acquired in EI-scan mode at an energy of 70 eV. The scanning range was from 50 to 600 m/z. The identification of peaks relied on comparing their retention times to those in the National Institute of Standards and Technology's (NIST) mass spectral collection. The proportion of each fatty acid was determined by calculating the ratio of the peak area to the overall chromatographic area.

Cx. pipiens mosquito

Cx. pipiens mosquitoes were acquired from the colony reared at the Zoology Department, Faculty of Science, Damanhour University, Egypt. Mosquitoes were maintained under controlled conditions, specifically at a 14:10 hrs (light-dark) period, a temperature of 27 ± 2 °C, and relative humidity ranging from 75 to 85%.

Evaluation of the larvicidal activity of _Sarcophyton_ crude extract

The larvicidal bioassay was accomplished in accordance with the standard protocols established by the World Health Organization[41], with some modifications. In brief, twenty-five 3rd instar larvae were transferred into disposable plastic cups with 250 ml of dechlorinated water mixed with *Sarcophyton* extract at final concentrations of 375, 600, 750, 900, 1200, 1500, and 1800 µg/ml. Each concentration was conducted in three replicates (n = 3). Mortality rates were documented at the three time points following exposure: 24, 48, and 72-hrs post-exposure. Larvae with no signs of movement upon gentle probing with a glass rod in the siphon or cervical region were considered dead.

Behavioral study

After 24 hrs of exposure, the third-instar larvae of *Cx. pipiens* treated with median lethal concentration (LC_{50}) were examined for behavioral alterations like wriggling speed, vertical and horizontal motions, and self-biting behavior. Behavioral symptoms exhibited by the larvae were documented and photographed. These behavior observations were evaluated in comparison against control larvae[42].

Morphological study

Morphological observations of the control and 24 hrs-LC_{50}-treated 3rd instar *Cx. pipiens* larvae were examined using a stereomicroscope (Olympus SZX7; 1.6 X magnification) equipped with a camera to identify morphological changes.

Preparation of whole-body homogenate for biochemical analysis

A total of 15 whole-body *Cx. pipiens* 3rd instar larvae from the control group and treated groups with 24 hrs-LC_{40} (sub-dose of 24 hrs-LC_{50}) and 24 hrs-LC_{50} of *Sarcophyton* crude extract were washed with sterilized distilled water and then dried over absorbent paper. Using a Teflon hand homogenizer, Eppendorf tubes were homogenized with one ml of icy-cold sodium phosphate buffer (20 mM, pH 7.0). Centrifugation of homogenates occurred at 8000 rpm and 4 °C for a period of 15 min. The deposits were removed, and clear supernatants were utilized for the enzymatic analysis[43]. According to Bradford[44], the protein content in samples was evaluated using a reference standard of bovine serum albumin (BSA, Sigma).

Estimation of acetylcholinesterase activity in the third-instar larvae of _Cx. pipiens_

Acetylcholinesterase (AChE, EC 3.1.1.7), the cholinergic marker, was determined throughout the whole-body homogenate using the Ellman et al.[45] method. AChE reacted with acetylcholine thioiodide (ACTI) as the initial substrate and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) as a second substrate, giving a yellow-colored compound. In the enzyme-linked immunosorbent assay (ELISA) plate (Bio Tec. USA), 150 µl of phosphate buffer (0.1 M, pH 8) was introduced to the ELISA blank well, and 130 µl of phosphate buffer was applied to the ELISA activity wells. Five microliters of substrate ACTI (75 mM in distilled water) were added to both blank and activity ELISA wells. Then, 20 µl of

homogenate supernatant was added to only activity ELISA wells. Prior to adding the second substrate (60 µl of DTNB, 0.32 mM in 10 ml phosphate buffer 0.1 M, pH 8) to the blank and activity ELISA wells, the plate was preincubated for 15 min at 37 °C. The spectrophotometer measured the absorbance at a wavelength of 405 nm every two minutes. The values obtained were analyzed, and blank readings were subtracted from sample readings. The AChE activity in the sample was expressed as µM of ACTI hydrolyzed/min/mg protein.

Determination of glutathione-S-transferase activity in the third-instar larvae of *Cx. pipiens*

The glutathione-S-transferase (GST, EC 2.5.1.18) colorimetric assay procedure is based on the formation of glutathione dinitrobenzene complex and hydrochloric acid. Ten microliters of 1-chloro-2,4-dinitrobenzene (1 mM of CDNB in ethanol) were added as a substrate to 1.37 ml of phosphate buffer (0.1 M, pH 6.5), and the mixture was thoroughly combined using a vortex. About 25 µl of the sample homogenate supernatant or phosphate buffer as a blank (pH 7.4) was added and allowed to undergo incubation at a temperature of 37 °C for 5 min. Subsequently, a volume of 100 µl of reduced glutathione (GSH) at a concentration of 5 mM was introduced to initiate the reaction, which was then allowed to incubate for a duration of 20 min at an ambient temperature. The variation in sample absorbance was recorded at 340 nm against the blank[46]. The GST activity in the sample was expressed as µM of CDNB conjugated/min/mg protein.

Statistical analysis of data

The results were reported as the average values of triplicate samples obtained from independent trials ± standard deviations (SD). The statistical analyses were performed using IBM-SPSS Version 20.0, which is specifically designed for social sciences research. The larval mortality data underwent a log concentration-probit mortality regression analysis[47] to calculate the lethal concentrations of *Sarcophyton* extract with their 95% upper and lower confidence limits along with the Chi-square estimates for the mortality data. Differences in the percentages of larval mortality and enzyme activities between the control and treated groups were analyzed using one-way analysis of variance (ANOVA). Additionally, a post-hoc multiple-comparison test known as the Tukey test was conducted to further examine the differences[48]. The acquired results were considered statistically significant with a p-value of ≤ 0.05.

RESULTS AND DISCUSSION

Results

Qualitative phytochemical analysis for *Sarcophyton* crude extract

The results of the phytochemical screening of the *Sarcophyton* extract revealed positive responses for proteins, lipids, steroids, carbohydrates, alkaloids, phenolics, tannins, flavonoids, terpenoids, diterpenes, and saponins. At the same time, tests for anthraquinones and coumarins showed negative responses **(Table 1)**.

Table 1: Preliminary phytochemical screening results of the *Sarcophyton* crude extract.

Phytochemical constituents	Test name	Observation	Result
Proteins	Biuret test	Pale purple color	+
Lipids	Emulsion test	Cloudy or milky emulsion	+
Steroids	Sulfuric acid test	Red color	+
Carbohydrates	Molisch's test	Violet ring at the junction	+
Alkaloids	Dragendroff's test	Orange or reddish-brown precipitate	+
Phenolics	Ferric chloride test	Blue to the green color	+
Tannins	Braymer's test	Brownish green/blackish color	+
Flavonoids	Aluminium solution test	Yellow color	+
Terpenoids	Salkowski test	Reddish-brown color	+
Diterpenes	Copper acetate test	Emerald-green color	+
Saponins	Froth test	Froth	+
Anthraquinones	Borntrager's test	Pink to violet color	–
Coumarins	Alkaline hydrolysis test	Cloudy solution	–

+ = Present, – = Absent.

Fatty acid composition of the *Sarcophyton* crude extract

The GC-MS fatty acid profile of the *Sarcophyton* crude extract is shown in **Table 2** and **Fig. 2**. There were five different fatty acids in *Sarcophyton* extract, including three saturated fatty acids (SFAs), one monounsaturated fatty acid (MUFA), and one polyunsaturated fatty acid (PUFA). The overall content of UFAs (61.83%) was higher than that of SFAs (32.81%) in *Sarcophyton* extract. Among all fatty acids detected in *Sarcophyton* extract, linoleic acid (C18:2ω6) was the most abundant (31.15%), followed by oleic acid (C18:1ω9) (30.68%) and palmitic acid (C16:0) (27.24%).

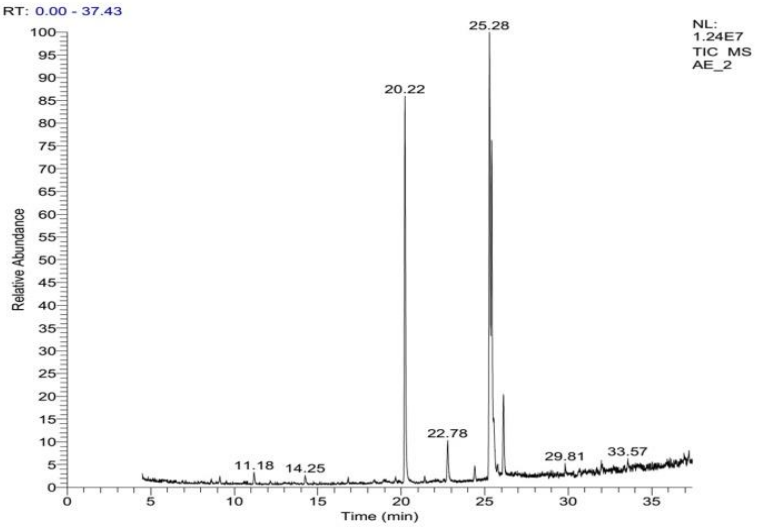
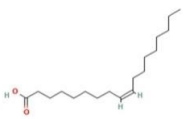
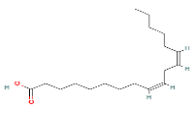


Fig. 2: GC-MS chromatogram of *Sarcophyton* crude extract showing the retention times of the identified fatty acids.

Table 2: Fatty acids detected in *Sarcophyton* crude extract using gas chromatography-mass spectrometry (GC-MS).

Fatty acid name	Chemical structure	Carbon atom (n)	Molecular formula	MW (g/mol)	Type	RT (min)	Area %
Tetradecanoic acid (Myristic acid)		C14:0	$C_{14}H_{28}O_2$	228.37	SFA	14.25	0.17
Hexadecanoic acid (Palmitic acid)		C16:0	$C_{16}H_{32}O_2$	256.42		20.22	27.24
Octadecanoic acid (Stearic acid)		C18:0	$C_{18}H_{36}O_2$	284.5		26.12	5.40
ΣSFA							32.81
(9Z)-Octadecenoic acid (Oleic acid)		C18:1ω9	$C_{18}H_{34}O_2$	282.5	MUFAOmega-9	25.41	30.68
9,12-octadecadienoic acid (Linoleic acid)		C18:2ω6	$C_{18}H_{32}O_2$	280.4	PUFA Omega-6	25.28	31.15
ΣUFA							61.83

MW: molecular weight, RT: retention time, SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, and PUFA: polyunsaturated fatty acid.

Larvicidal activity of *Sarcophyton* crude extract against the third-instar larvae of *Cx. pipiens*

The data in **Fig. 3** reveal that the mortality rates of the third-instar larvae that of *Cx. pipiens* were dependent on the utilized concentration and the exposure time. The mortality percentages in all concentrations and exposure times exhibited significant differences ($p< 0.001$ and $p< 0.05$, respectively). There was a complete absence of larval mortality in the control group. At the same time, the *Sarcophyton* extract at all concentrations surpassed 50% mortality after 48 and 72 hrs of exposure time, with 100% larval mortality at a concentration of 1800 µg/ml after 72 hrs

On 24, 48, and 72 hrs post-treatment, the detected larval death fluctuated between the minimum values (26.666% ± 2.309, 50.666% ± 6.110, and 72% ± 4, respectively) at the lowest concentration (375 µg/ml) and the maximum values (94.666% ± 2.309, 98.666% ± 2.309, and 100% ± 0, respectively) at the highest applied concentration (1800 µg/ml).

The values of lethal concentrations of the *Sarcophyton* extract on the third-instar larvae of *Cx. pipiens*, estimated by the probit regression equation as well as the chi-square test, are presented in **Table 3**. All values of lethal concentrations of *Sarcophyton* extract decreased dramatically with the increasing exposure time. The values of LC_{50} (745.418, 370.064, and 232.017 µg/ml), LC_{90} (2147.337, 1127.771, and 805.707 µg/ml), and LC_{95} (2898.426, 1546.721, and 1146.690 µg/ml) of *Sarcophyton* extract against the *Cx. pipiens* third-instar larvae were recorded after 24, 48, and 72 hrs of exposure time, respectively.

Behavioral abnormalities of the third-instar larvae of *Cx. pipiens* under 24 hrs-LC_{50} treatment with *Sarcophyton* crude extract

Applying *Sarcophyton* crude extract resulted in notable neurobehavioral toxicity effects on 3rd instar *Cx. pipiens* mosquito larvae. Upon exposure, the larvae exhibited heightened levels of energy and restlessness, characterized by vigorous and repetitive wriggling movements. These observed orientation symptoms prompted an alteration of a forceful self-biting anal papillae behavior, developing a circular-shaped positioning structure, as depicted in **Fig. 4**. Nevertheless, before attaining a state of motionlessness, the larvae became sluggish and could not ascend to the water surface.

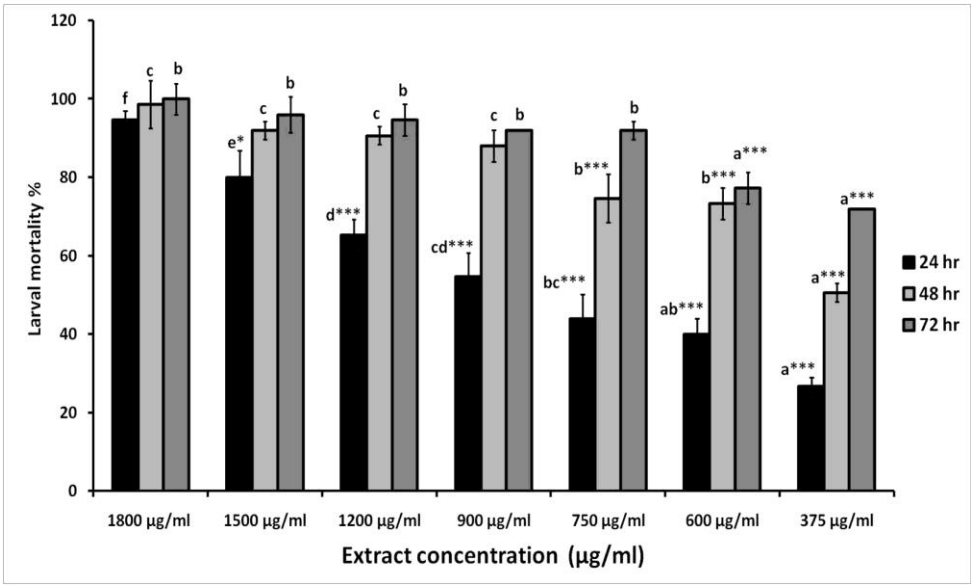


Fig. 3: Mean mortality percentages of the third-instar larvae of *Culex pipiens* under exposure to different concentrations of *Sarcophyton* crude extract at different periods (24, 48, & 72 hr). The value of each bar is presented as a mean of 3 replicates ± SD. Different letters[a, b, c, d, e, and f] are significantly different. *Significant at p-value ≤ 0.05, ** Significant at p-value ≤ 0.01, and *** Significant at p-value ≤ 0.001 (one-way ANOVA).

Table 3: Lethal concentrations of *Sarcophyton* crude extract against the third-instar larvae of *Culex pipiens* during different exposure periods.

Exposure periods (hrs)	Lethal concentration (µg/ml) (95% CL)					Regression equation	Intercept ± SE	χ^2	df	*p*-value
	LC_{25}	LC_{40}	LC_{50}	LC_{90}	LC_{95}					
24 hrs	427.132	604.731	745.418	2147.337	2898.426	y = 3,75 x + −10,375 R^2 Linear = 0.888	8.011 ± 1.455	3.368	5	0.643
LCL – UCL (µg/ml)	278.589 - 539.339	459.027 - 721.745	606.318 - 879.220	1631.687 - 3623.476	2057.938 - 5682.807					
48 hrs	205.861	296.898	370.064	1127.771	1546.721	y = 3,125 x + −7,8125 R^2 Linear = 0.924	6.801 ± 1.629	1.262	5	0.939
LCL – UCL (µg/ml)	78.630 - 311.990	145.705 - 409.292	209.995 - 484.590	906.894 - 1674.821	1172.010 - 2788.624					
72 hrs	120.495	181.401	232.017	805.707	1146.690	y = 2 x + −4,6 R^2 Linear = 0.902	5.607 ± 1.865	1.743	5	0.883
LCL – UCL (µg/ml)	14.676 - 231.164	35.422 - 304.525	59.980 - 360.654	616.878 - 1184.586	864.050 - 2293.952					

LC_{25}, LC_{40}, LC_{50}, LC_{90}, and LC_{95}: lethal concentrations for 25, 40, 50, 90, and 95% mortality of the treated larvae, respectively, 95% CL: 95% confidence limit, LCL: lower confidence limit, UCL: upper confidence limit, y: the probit value of mortality, x: the log concentration of the *Sarcophyton* crude extract, R^2: correlation coefficient, SE: standard error, χ^2: Pearson chi-square, and df: degree of freedom. Note: values of chi-square are not significant (p> 0.05).

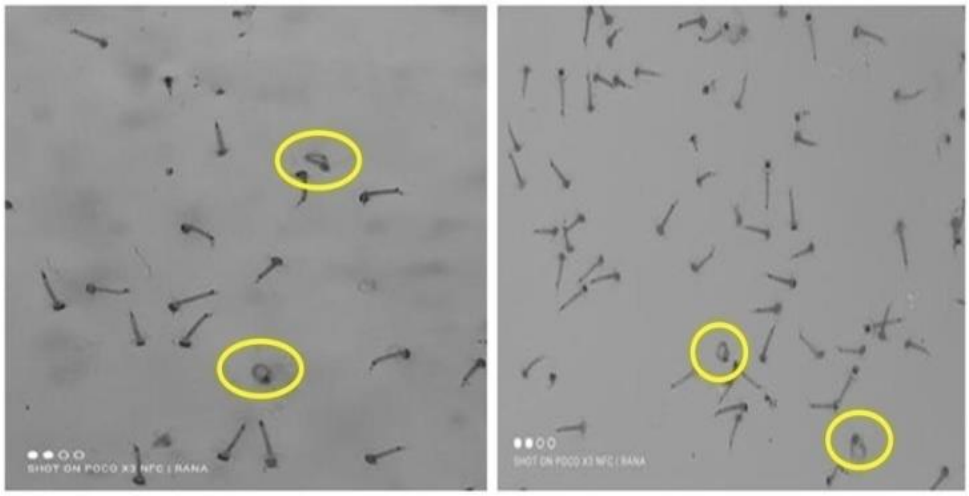


Fig. 4: Digital photograph of third-instar larvae of *Culex pipiens* under 24 hrs-LC_{50} treatment with *Sarcophyton* crude extract showing self-biting anal papillae behavior (yellow circles).

Morphological abnormalities of the third-instar larvae of *Cx. pipiens* under 24 hrs-LC_{50} treatment with *Sarcophyton* crude extract

Compared to control larvae, the treated larvae with LC_{50} of *Sarcophyton* crude extract revealed several morphological aberrations, as illustrated in **Fig. 5**. Control 3rd instar *Cx. pipiens* mosquito larva showed a typical appearance, characterized by distinct and fully developed head, thorax, and abdominal segments throughout the entire body **(Fig. 5a)**. The whole bodies of 3rd instar *Cx. pipiens* mosquito larvae treated with *Sarcophyton* crude extract were severely damaged. The treated larvae showed some aberrations in external features, such as loss of abdominal setae and abnormalities in the head region like darkening pigmentation, abnormal shrinkage, and loss of upper and lower head setae and mouth brushes, as shown in **Fig. (5b-d)**. Larva, in **Fig. 5b**, revealed an indistinct appearance of the thorax and abdominal regions, where the first abdominal segments were shortened and collapsed and seemed to be fused with thoracic segments. A blackened and swollen thorax was noticed also in **Fig. 5c**. Furthermore, in comparison to the typical larvae, a subset of larvae subjected to *Sarcophyton* extract exhibited heightened pigmentation in their anal papillae **(Fig. 5d).**

Fig. 5: Stereomicrographs showing the morphology of the third-instar larvae of *Culex pipiens* under 24 hrs-LC_{50} treatment with *Sarcophyton* crude extract in comparing to the control larvae. **(a)** control larvae and **(b–d)** treated larvae.

Enzyme activities in the third-instar larvae of *Cx. pipiens* under treatment with 24 hrs-LC_{40} and 24 hrs-LC_{50} of *Sarcophyton* crude extract

The results illustrated in **Table 4** and **Fig. 6** show a significant increase ($p< 0.01$) in AChE activity with a change percentage of 27.731% and a highly significant decrease ($p< 0.001$) in GST activity with a change percentage of 50.316% in the third-instar larvae of *Cx. pipiens* treated with 24 hrs-LC_{40} of *Sarcophyton* crude extract compared to the control larvae. While at 24 hrs-LC_{50}, *Sarcophyton* crude extract exhibited a significant decrease in both activities of AChE and GST with reduction percentages of 29.201 ($p< 0.01$) and 64.166% ($p< 0.001$), respectively, compared to the control larvae.

Table 4: Enzyme activities in the third-instar larvae of *Culex pipiens* after 24 hrs-treatment with LC_{40} and LC_{50} values of *Sarcophyton* crude extract in comparing to the control group.

Enzymes	Extract concentration (µg/ml)			F-ratio	*p*-value
	Control	LC_{40}	LC_{50}		
Acetylcholinesterase (AChE) (µM/min/mg protein)	2.178 ± 0.167^{b}	$2.782 \pm 0.100^{c**}$	$1.542 \pm 0.201^{a**}$	44.014	0.000
Change %	-	27.731	–29.201		
Glutathione-S-transferase (GST) (µM/min/mg protein)	15.653 ± 0.074^{c}	$7.777 \pm 0.074^{b***}$	$5.609 \pm 0.061^{a***}$	16899.507	0.000
Change %	-	–50.316	–64.166		

Each value is the mean of 3 replicates ± SD. LC_{40} and LC_{50}: lethal concentrations for 40 and 50% mortality of the treated larvae, respectively. The means in the same row with different superscript letters [a, b, and c] are significantly different.* Significant at p-value ≤ 0.05, ** Significant at p-value ≤ 0.01, and *** Significant at p-value ≤ 0.001 (one-way ANOVA).

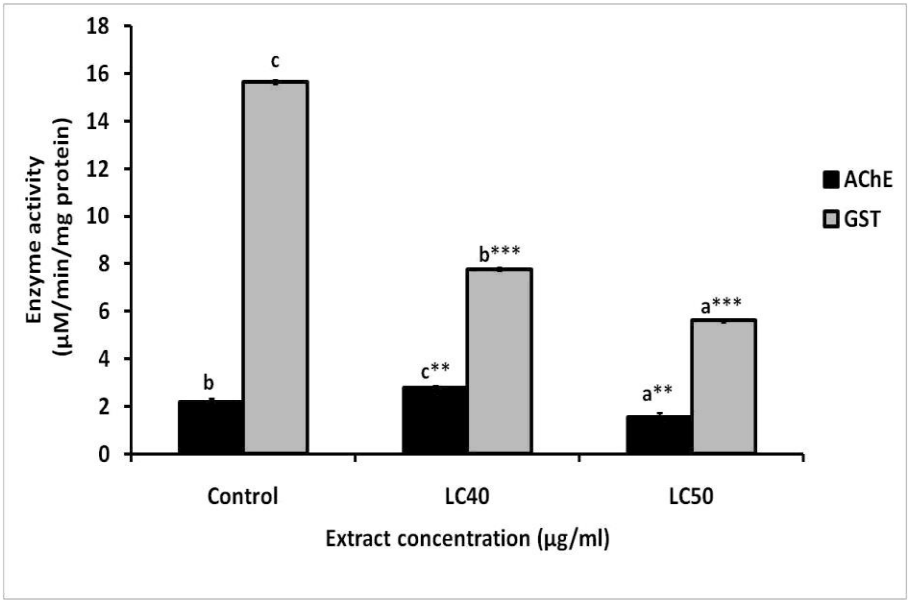


Fig. 6: Enzymatic activities of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) in the third-instar larvae of *Culex pipiens* after 24 hrs-treatment with LC_{40} and LC_{50} values of *Sarcophyton* crude extract in comparing to the control group. The value of each bar is presented as a mean of 3 replicates ± SD. Different letters $^{(a, b, \text{and } c)}$ are significantly different. * Significant at p-value ≤ 0.05, ** Significant at p-value ≤ 0.01, and *** Significant at p-value ≤ 0.001 (one-way ANOVA).

Discussion

Cx. pipiens is the worldwide dispersed mosquito species that causes serious global public health problems[49]. The growing interest in naturally marine-derived insecticides is related to the urgent necessity for identifying new larvicidal alternatives with low resistance and minimal impact on human health and the environment[50]. The wide spreading of *Sarcophyton* soft corals throughout the Red Sea[32] suggests that these organisms potentially contain many bioactive natural products with promising potential properties as insecticidal agents[51-53].

In the present study, phytochemical screening indicated that *Sarcophyton* crude extract contains several bioactive compounds, such as proteins, lipids, steroids, carbohydrates, alkaloids, phenolics, tannins, flavonoids, terpenoids, diterpenes, and saponins. Many previous studies indicated that these compounds, both alone or in combination, have larvicidal efficacy by various action mechanisms and exhibit safety for humans and the environment[2, 54-57].

The larvicidal activity is influenced mainly by the extract's lipid nature[58, 59]; therefore, lipid characterization of the *Sarcophyton* extract was necessary to be determined. The GC-MS analysis of *Sarcophyton* extract revealed the detection of five distinct fatty acids, including three SFAs (palmitic acid, myristic acid, and stearic acid), one MUFA (oleic acid), and one PUFA (linoleic acid). These results were confirmed by the findings recorded by Imbs et al.[60], who documented the same fatty acid types in *Sarcophyton* soft corals. The overall content of UFAs in *Sarcophyton* extract was higher than that of SFAs, where linoleic and oleic acids were the most prevalent fatty acids.

The mortality rates of *Cx. pipiens* third-instar larvae, in the present study, were dependent on the *Sarcophyton* extract concentration and the duration of exposure. As a greater concentration of active compounds in extract corresponds to a greater capacity for larvicidal activity[61]. This result is in the same context as the previous studies investigated by El-Naggar and Hasaballah[18], Singab et al.[62], and Eltak et al.[63] on different extracts applied against the *Cx. pipiens* third-instar larvae. According to Morohashi et al.[64] and Bury et al.[65], the effectiveness against mosquitoes is mainly attributed to the existence of long-chain UFAs rather than SFAs. Similarly, Harada et

al.[66] and Perumalsamy et al.[67] stated that UFAs like linoleic acid and oleic acid were more pronounced acid in toxicity against *Aedes* (*Ae. albopictus* and *Ae. aegypti*) and *Cx. pipiens* larvae than SFAs (myristic acid, palmitic acid, and stearic acid), indicating that UFAs are environmentally safe and effective larvicides.

The current study revealed that the *Sarcophyton* extract surpassed 50% mortality of the 3rd instar larvae of *Cx. pipiens* after 48 and 72 hrs of exposure at all tested concentrations, with 100% larval mortality at 1800 µg/ml after 72 hrs Lethal concentration values of *Sarcophyton* extract against *Cx. pipiens* larvae decreased dramatically by prolonging the exposure time, recording values of LC_{50} (745.418, 370.064, and 232.017 µg/ml), LC_{90} (2147.337, 1127.771, and 805.707 µg/ml), and LC_{95} (2898.426, 1546.721, and 1146.690 µg/ml) after 24, 48, and 72 hr, respectively. A similar potentiality range of *Sarcophyton* extract was investigated by Kamel[68], who found that soft coral *Sarcophyton glaucum* ethanol extract exhibited activity against *Sitophilus oryzae* adults (Coleoptera: Curculionidae) with LC_{50} values of 2273, 1502, and 423.5 µg/ml and LC_{95} values of 6200, 5611, and 2193 µg/ml after exposure times 24, 48, and 72 hr, respectively. Furthermore, Hasaballah and El-Naggar[69] indicated that the methanolic extract of the marine sponge *Callyspongia siphonella* induced larvicidal activity against *Cx. pipiens* with a 24 hrs-LC_{50} value of 610.3 µg/ml.

The neurobehavioral toxicity of *Sarcophyton* extract in the 3rd larval instar of treated *Cx. pipiens* mosquitoes demonstrated heightened activity levels, manifested through increased restlessness as well as larval aggressive movements. The development of neurobehavioral symptoms, such as excitement, paralysis, and death, in *Cx. pipiens* larvae resembles those induced by synthetic nerve poisons, indicating that the *Sarcophyton* extract may function as cytolysins, impacting the neuromuscular coordination inside chemical synapses. Previous studies described the identification of cytolytic toxins from the soft coral *Sarcophyton trocheliophorum*[70]. Another remarkable observation was the aggressive anal and mouth parts biting in treated larvae, forming a circular-shaped structure. This behavior reveals that the cytotoxic effects of *Sarcophyton* extract initiated fatal electrolyte discharges from the anal region, which is consistent with the studies of Ragavendran et al.[43] and Bibi et al.[71]. Pereira et al.[72] recently reported that alkaloids disrupt larvae's nervous system by affecting neurotransmitter receptors, triggering uncontrollable muscle activity, paralysis, and death. According to Rattan[73] and Ahdiyah and Purwani[74], alkaloids have the ability to impact the sodium channels, impeding nerve impulse transmission. This suggests that the neurobehavioral toxicity of *Sarcophyton* could be related to alkaloid-derived interruption of the osmotic and ionic regulation. However, further research is needed to detect the specific mechanism by which *Sarcophyton* extract's bioactive compounds can affect neurotransmission in *Cx. pipiens* larvae.

The morphological observations in the current study revealed that treatment with *Sarcophyton* crude extract caused several aberrations in the head, thorax, and abdominal regions, summarized in abnormal shrinkage, indistinct tagma appearance, and darkening pigmentation. Likewise, *Culex* mosquito larvae that were subjected to *Magnolia denudata* seed extract displayed the same abnormalities documented by Wang et al.[55]. According to Wahab et al.[75], dark pigmentation could be attributed to anomalies in the process of cuticular melanization, which could be induced *via* the suppression of chitin formation. Chapagain and Wiesman[76] recorded that the interaction of the saponins with the larval cuticle causes damage to the cuticle. Therefore, the morphological defects herein may presumably result from hormone disruption or interference with chitin synthesis induced by the bioactive components present in *Sarcophyton* extract. Though this explanation requires more research.

Exploring the manner in which naturally occurring substances perform could provide valuable insights into developing environmentally friendly insecticides with unique targets[67]. The larvicidal strategies primarily depend on AChE and GST as biomarker enzymes to assess neurotoxicity and detoxification processes, respectively[77, 78]. AChE is highly prevalent in the neural tissue of insects, particularly at nerve synapses, playing a crucial role in transmitting nerve impulses across synaptic gaps and the functioning of the

neuromuscular system *via* hydrolyzing neurotransmitter (acetylcholine) into choline and acetate[79-81].

In the current study, the activity of AChE exhibited distinct patterns. More precisely, AChE activity was considerably elevated following exposure to a 24 hrs-LC_{40} concentration of *Sarcophyton* extract compared to control larvae. This induction could be related to the larval metabolic resistance to a sublethal dosage of *Sarcophyton* extract, resulting in an increase in larval metabolism and releasing acetylcholine within the synaptic cleft in an attempt to perform the detox process[82, 83]. Meanwhile, significant AChE inhibition activity was noted in larvae after being exposed to 24 hrs-LC_{50} of the extract compared to control larvae. This could be due to bioactive components in *Sarcophyton* extract, including terpenoids, alkaloids, linoleic acid, oleic acid, and palmitic acid, acting through multiple mechanisms. Lee and Ahn[84] indicated that terpenoids can inhibit AChE in *Ae. aegypti* larvae. Additionally, alkaloids can interfere with the insect's nervous system by acting on neurotransmitter receptors[57, 85-87]. Further, linoleic acid, oleic acid, and palmitic acid primarily target AChE and octopaminergic receptors to exert their larvicidal effects on mosquito larvae[67, 88, 89]. Hence, inhibition of AChE activity could be a consequence result of larval neurotoxicity.

GST is a multi-functional enzyme that performs an essential role in various cellular regulatory processes, including the biosynthesis and intracellular transport of hormones[90-92]. It is involved in phase II of detoxification of various endogenous or exogenous xenobiotics, including insecticides, through catalyzing the binding of the sulfhydryl group of GSH with a broad range of electrophilic toxic xenobiotics, forming a more soluble compound of S-substituted glutathione that can be excreted[93-97]. Insecticide detoxification involves a dealkylation process, where the alkyl portion of the pesticide is combined with GSH by conjugation[98] or *via* a dearylation reaction where GSH interacts with the departing group[99]. Furthermore, GST plays a critical role in the antioxidant defense system by neutralizing lipid peroxides and hazardous oxygen free radical species produced by insecticides, thus protecting against oxidative damage[93, 94, 100, 101].

The GST activities in *Cx. pipiens* larvae treated with 24 hrs-LC_{40} and 24 hrs-LC_{50} of *Sarcophyton* extract were quantified, revealing a significant inhibition in GST activity compared with the control larvae. Similarly, the inhibitory effect in GST activity was recorded in the larvae of *Cx. pipiens* after exposing to different extracts, such as LC_{50} of *Ocimum basilicum*[102], α-cyper, chlorpyrifos, and methomyl[103], and *Lantana camara* extract at the concentration of 1000 µg/ml[104]. The reduction in GST activity could be the consequence of some bioactive substances causing high levels of oxidative stress damage to larval tissues, reducing the larvae's ability to produce antioxidants that mitigate the toxicity[100, 104-107]. Thus, GST inhibition may be related to the presence of phenolic and flavonoid compounds in *Sarcophyton* extract, as declared by Yu and Abo-Elghar[108], Zhao et al.[109], and Wang et al.[110]. Hence, the corresponding reduction in AChE and GST activities suggests that the primary mode of action of *Sarcophyton* extract's bioactive components may be through the suppression of the enzymatic system in mosquito larvae.

Conclusion

This study concluded that *Sarcophyton* extract can induce larval toxicity *via* inhibition of the activities of AChE and GST enzymes, indicating its strong larvicidal effect against *Cx. pipiens* third-instar larvae, which is related to the bioactive compounds and fatty acid composition of this extract. Thus, *Sarcophyton* extract could be a promising alternative tool to control *Cx. pipiens* larvae. However, further research is needed to elucidate the neurotransmission obstruction mechanism of *Sarcophyton* extract in *Cx. pipiens* larvae and to optimize the methods for purifying *Sarcophyton*'s bioactive compounds.

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تقييم النشاط البيولوجي للمستخلص الخام ساركوفيتون كعامل مضاد ليرقات كيولكس بيبينس (ثنائية الأجنحة، كيوليسيدا)

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تلعب بعوضة كيولكس بيبينس دورًا مهمًا كناقل للعديد من الأمراض. إن الاستخدام المستمر للمبيدات الحشرية الاصطناعية لمكافحة البعوض يؤدي إلى تطور مقاومة البعوض لها. ومن ثم، يجري استكشاف النظام البيئي البحري بحثًا عن بدائل جديدة للمبيدات الحشرية تكون ميسورة التكلفة وذات مقاومة منخفضة وكفاءة عالية. وبالتالي، أجري هذا العمل لتقييم التأثير المحتمل القاتل لليرقات للمستخلص الخام من المرجان اللين ساركوفيتون ضد يرقات العمر الثالث لبعوضة كيولكس بيبينس كعامل مبيد حشري آمن. ولتحقيق هذا الهدف، تم تحديد المواد الكيميائية النباتية لمستخلص ساركوفيتون وملف الأحماض الدهنية بالكروماتوغرافيا الغازية–مطياف الكتلة (GC-MS). وتم تقييم معدل الوفيات الناجم عن مستخلص ساركوفايتون ضد اليرقات بطريقة تعتمد على الجرعة بعد ٢٤ و٤٨ و٧٢ ساعة من التعرض. أيضا تمت ملاحظة أي تغيرات سلوكية وشكلية في اليرقات جنبًا إلى جنب مع تقدير أنشطة الأسيتيل كولين استريز والجلوتاثيون–S–ترانسفيراز في اليرقات. وقد كشف التوصيف الكيميائي لمستخلص ساركوفيتون عن وجود مواد كيميائية نباتية نشطة بيولوجيًا وثلاثة أحماض دهنية أولية: حمض اللينوليك وحمض الأوليك وحمض البالمتيك. تم تسجيل تركيزات مستخلص ساركوفايتون القاتلة ل ٥٠% من اليرقات (LC_{50}) عند ٧٤٥,٤١٨ و ٣٧٠,٠٦٤ و ٢٣٢,٠١٧ ميكروجرام/مل بعد ٢٤ و ٤٨ و٧٢ ساعة من وقت التعرض، على التوالي. كشف العلاج باستخدام LC_{50} من مستخلص ساركوفايتون لمدة ٢٤ ساعة عن سمية السلوك العصبي والانحرافات المورفولوجية ضد يرقات كيولكس بيبينس. كما أظهر استخدام تركيز LC50 من المستخلص لمدة ٢٤ ساعة تأثيرًا سامًا للأعصاب، كما يتضح من التثبيط الكبير لنشاط إنزيم الأسيتيل كولين استريز وقمع نشاط إزالة السموم من خلال تثبيط إنزيم الجلوتاثيون–S–ترانسفيراز. يمكن أن تُعزى آلية العمل لمستخلص الساركوفيتون في القضاء على اليرقات إلى مكوناته النشطة بيولوجيًا. وبالتالي، خلصت هذه الدراسة إلى أن مستخلص الساركوفيتون يمكن أن يكون مرشحًا طبيعيًا محتملاً للتحكم فى يرقات بعوضة كيولكس بيبينس.