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Original Article

The in Vitro Antischistosomal Activity and Genotoxicity of the Active Ingredients of *Allium sativum* (allicin) and *Curcuma longa* (curcumin)

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Abstract

Background: In this study, we assessed the in vitro antischistosomal activity of the active ingredients of *Allium sativum* (allicin) and *Curcuma longa* (curcumin) on *Schistosoma mansoni*.

Methods: This study was conducted in Faculty of Science, Port said University, Egypt (2018). Adult worms were exposed to a range of concentrations of AL or CU, and worm survival was assessed 24 h post-exposure to calculate the lethal concentration of the compounds. Scanning electron microscopy was used to assess ultrastructural changes in the surface of AL- or CU- treated worms. The genotoxicities of AL and CU on *S. mansoni* were determined by DNA fragmentation analysis.

Results: We determined the concentrations of AL and CU required to kill 50% of *S. mansoni* (LC_{50}). The LC_{50} of AL was 8.66 μL/mL, whereas 100% mortality of *S. mansoni* was achieved by AL at concentrations of 50 μL/mL. The LC_{50} of CU was 87.25 μL/mL, with the highest mortality of 91.3% seen after 24 h exposure to 100 μg/mL CU. Ultrastructural studies revealed that exposure to either AL or CU led to mild or severe surface damage to *S. mansion*, respectively. The degree of damage in the worms was sex-dependent. Interestingly, while CU exposure resulted in DNA fragmentation in *S. mansoni* worms, we observed no genotoxic effects of AL.

Conclusion: Both AL and CU exhibit antischistosomal activity; the study provided evidence suggesting that these compounds act through distinct mechanisms. These promising results encourage further investigation into these compounds as potential antischistosomal agents, either alone or as complementary treatments to praziquantel.

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Introduction

Schistosomiasis is a disease caused by parasitic worms and is considered a high-priority, neglected tropical disease by WHO (1). It can result in acute and long-term pathological disorders (2,3). There are limited drugs available for the treatment of *Schistosoma* spp. in humans, and while the current drug of choice is praziquantel (PZQ) (4), PZQ efficacy has been reduced (5-7). Such drugs may be found in natural resources, for example in medicinal plants recommended for the treatment of schistosomiasis (8-11).

Allicin (AL) is the main active ingredient of *Allium sativum* (commonly known as garlic) and is considered responsible for many of the beneficial effects associated with this plant (12-16). While many in vitro studies have examined the efficacy of garlic extract as an antischistosomiasis drug (17,18), few have studied the effects of AL in isolation (19). Curcumin (CU), the active ingredient of *Curcuma longa* (commonly known as turmeric) is a phenolic compound and a yellow pigment often used as a spice and food colorant (20). Curcumin has multiple therapeutic properties (21-24). Most of the studies dealt with turmeric crude as antischistosomal agent (25-27). In vitro studies on the antischistosomal activity of curcumin have focused on its genotoxic effects on the adult stages (28,29).

It is important to assess the effects of drugs against *S. mansoni* in a variety of ways, rather than focusing solely on mortality. We herein examined ultrastructural changes in the worm tegument by scanning electron microscopy; these analyses enable us to better understand the mechanism of action of each drug (30,31). The molecular applications were more recently used in the evaluation of the therapies (29, 32). Furthermore, PZQ is less effective at treating immature developmental stages of *S. mansoni* than it is at treating adult worms (33). Thus, it is important to find new drugs that are also effective against earlier stages of *S. mansoni* (34-36).

Little is known of the antischistosomal activity of allicin or curcumin. In this study, we evaluate the in vitro antischistosomal activity of these compounds on different developmental stages of *S. mansoni*. In response to allicin and curcumin, we assayed worm mortality, ultrastructural surface changes, and genotoxicity in adult worms.

Methods

This study was conducted in the Faculty of Science, Port said University, Egypt in 2018.

Active ingredients

Allicin (AL; $C_6H_{10}OS_2$) was obtained in liquid form from Sciencemed (Egypt). Curcumin (CU; $C_{21}H_{20}O_6$) was obtained as a powder from Sigma Aldrich (St. Louis, MO, USA) and dissolved in phosphate buffer saline (PBS).

Miracidiacidal and cercaricidal activity of AL and CU

Biomphalaria alexandrina snails were maintained at a temperature of 25 ± 2 °C (37). Infected snails were kept in glass jars (up to 10 animals per jar) containing: sand, 100 mL deionized and dechlorinated water, and snail food. Snails were incubated with a range of concentrations of AL or CU to determine the LC_{50} of these compounds. The calculated LC_{50} values of these compounds were then used as a stock concentration for ensuing miracidiacidal and cercaricidial assays.

For miracidiacidal assays, 20 freshly hatched miracidia were maintained in each well of a tissue culture plate with 1 mL dechlorinated water. A series of dilutions of the calculated LC_{50} values of AL and CU were prepared, and a different concentration was added to each well. Each drug concentration was tested in triplicate wells. Untreated control miracidia

were incubated in 1 mL dechlorinated water alone. Viability of miracidia was assessed visually by dissecting microscope, and the time of miracidia death was recorded (38).

For cercaricidal assays, 1 mL water containing immediately emitted cercariae were placed in each well of a tissue culture plate. A series of dilutions of the calculated LC_{50} values of AL and CU were prepared, and a different concentration was added to each well. Each drug concentration was tested in triplicate wells. Untreated control freshly shed cercariae were incubated in 1 mL dechlorinated water alone (39). Viability of cercariae was assessed visually by stereomicroscope. The viability of cercariae was determined by exposing the worms to AL or CU for 5 min and replacing it with fresh water and monitoring them within 60 minutes.

In vitro preparation of worms

Ten male hamsters (*Mesocricetus auratus*) of similar age and mass (100-140 g) were obtained from the Schistosome Biological Supply Center (Theodor Bilharz Research Institute, Giza, Egypt). Hamsters were incubated with 80-100 *S. mansoni* cercariae for 1 h using a partial immersion technique, as previously described (40). At 8 wk post-infection, adult worms were recovered from the animals by hepatic portal vein perfusion (41). Adult *S. mansoni* worms were then incubated at 37 °C in 24-well plates, with 24-30 worms per well. Worms were maintained in 1 mL RPMI-1640 media supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), 10 % fetal calf serum (Gibco), 2 g/L glucose, 0.39 g/L glutamate, and 20 g/L NaHCo₃ (39).

Assessing mortality of adult S. mansoni worms for three successive days

A range of concentrations of either AL or CU was added to each well of a tissue culture plate containing adult *S. mansoni* worms, maintained as described above. Worm mortality was assessed visually by stereomicroscope for up to 3 days (42,43).

Determination of the LC50 values of AL and CU against adult S. mansoni

Adult worms were incubated in wells of a tissue culture plate with a range of concentrations of either AL or CU. Worms were monitored for 24 h after exposure to a given compound, and mortality assessed visually by stereomicroscope. The LC50 values of AL and CU were determined using SPSS statistical program (ver.20, Chicago, IL, USA).

Scanning electron microscopy of adult S. mansoni

S. mansoni worms were exposed to the determined LC_{50} of AL or CU for 24 h. Worms were fixed in equal volumes of 4% glutaraldehyde and cacodylate 0.2 M for 2 hours. Worms were then washed in equal volumes of sucrose 0.4M and cacodylate 0.2 M for 2 h and postfixed in osmium 2% and cacodylate 0.3M for 1 h. Fixing was carried out at 4 °C. Samples were washed with distilled water and gradually dehydrated in increasing concentrations of ethyl alcohol for 5 min each (30%, 50%, 70%, and 90%) and finally, in absolute alcohol for 10 minutes. Samples were then air-dried and mounted on copper stubs using double-sided adhesive tape, coated with gold using an S150A sputter coater (Edwards, UK). Images were captured and were analyzed using a Philips XL30 scanning electron microscope (Philips, Eindhoven, Netherlands) operated at 10-30 kV, at the Electron Microscopy Unit of the Theodor Bilharz Research Institute.

Assessing DNA fragmentation by agarose gel analysis

DNA was extracted from either untreated control *S. mansoni* adult worms or worms exposed to the determined adult schistosomal LC_{50} of AL or CU for 24 hours (44). DNA was separated by gel electrophoresis on a 2% agarose gel containing 1% GelRed (1:500). DNA fragmentation was visualized using a BIO-RAD Gel DOC ™ XR+.

Results

The molluscicidal LC_{50} values for AL and CU here were 315 µL/L and 5690 µg/L, respectively. We then assayed the miracidiacidal and cercaricidial activity of AL and CU using a series dilution of the molluscicidal LC_{50} (Table 1).

Table 1: Miracidiacidal and cercaricidal activity of AL and CU

Drug	Dose	Miracidia (N=20)		Cercariae (N=20)	
		Mortality rate (%)	Death time (min)	Mortality rate (%)	Death time (min)
AL	LC_{50}	100	8	100	5
	$1/2^{th}$ LC_{50}	100	20	100	9
	$1/4^{th}$ LC_{50}	No effect after 60 min		100	60
	$1/8^{th}$ LC_{50}	No effect after 60 min		No effect after 60 min	
CU	LC_{50}	100	20	100	50
	$1/2^{th}$ LC_{50}	100	40	100	60
	$1/4^{th}$ LC_{50}	No effect after 60 min		No effect after 60 min	

N: number of miracidia or cercariae. **LC_{50}:** lethal concentration 50

At the determined LC_{50} of AL, we observed 100% killing of both miracidia and cercariae within 8 and 5 min, respectively. No effects on miracidia viability were observed after 1 h incubation with $1/4^{th}$ LC_{50}. No antischistosomal activity of AL was seen against miracidia or cercariae when applied at a concentration of $1/8^{th}$ LC_{50}. At the determined LC_{50} of CU, we observed 100% killing of both miracidia and cercariae within 20 and 50 min, respectively. No effects of CU on either miracidia or cercariae viability were observed after 1 h incubation with $1/4^{th}$ LC_{50}.

Next assessed the mortality of *S. mansoni* adults after exposure to a range of concentrations of AL or CU for three successive days (Table 2). Exposure of adult worms to concentrations of 30 µL/mL AL resulted in 100 % mortality after 24 hours. Exposure to concentrations of 100 µg/mL CU resulted in 100 % mortality of *S. mansoni* adults by day two. Viability assays were used to determine the LC_{50} of AL and CU following 24 h exposure of adult *S. mansoni* worms. In this case, the LC_{50} of AL was found to be 8.66 µL/mL for AL and 87.25 µg/mL for CU (Table 2).

Table 2: Mortality rate (%) of *S. mansoni* worms exposed to different concentrations of AL and CU for three consecutive days in vitro

Group AL (µL/mL)	First day	Second day	Third day	Group CU (µg/mL)	First day	Second day	Third day
5	4.33	73.33	86.67	5	NU	NU	NU
10	54.55	81.82	100	10	0	33.3	66.67
20	71.43	100	100	20	0	37.5	75
30	100	100	100	30	NU	NU	NU
40	100	100	100	40	NU	NU	NU
50	100	100	100	50	4	40	80
60	NU	NU	NU	60	4.17	45.83	95.83
80	NU	NU	NU	80	12	64	100
100	NU	NU	NU	100	91.3	100	100
LC_{50}	8.66			LC_{50}	87.25		

**NU:** not used. **LC_{50}:** lethal concentration 50

We examined the surface ultrastructure of drug-treated worms using scanning electron microscopy. Here, we observed mild to severe changes in the surface ultrastructure of adult *S. mansoni* worms exposed to an LC$_{50}$ of AL or CU in comparison with control worms (Fig. 1A, 1B, 1G, and 1J). The damage was specifically seen in the tegument of *S. mansoni* worms exposed to AL or CU. Male worms exposed to AL displayed significant atrophy of the tubercles, with loss of spines and intertubular ridges, and swelling between tubercles (Fig. 1C and 1D). Exposure of male worms to CU was associated with severe erosion of the tubercles (Fig. 1E) and the peeling of the tegument (Fig. 1F). While few protuberances were seen in the gynecophoral canal of male worms exposed to AL (Fig. 1H), damage to the gynecophoral canal edge (Fig. 1I) was apparent in males exposed to CU. Furthermore, though female worms exposed to AL showed wrinkling of

the tegument and small tegument dimples (Fig. 1K), wrinkling was more pronounced following exposure to CU (Fig. 1L).

To assess the genotoxic effects of AL and CU on *S. mansoni*, we used DNA fragmentation analysis on separated genomic DNA (Fig. 2). Lane profile analysis of DNA from either untreated control worms or worms exposed to an LC$_{50}$ of AL or CU is shown in Fig. 3. DNA fragmented bands were not detected in control worms and those exposed to AL (Fig. 3B and 3C). Here, we observed DNA fragmentation of *S. mansoni* upon exposure to CU, where separation yielded 4 distinct fragments (Fig. 3D). The analysis of these bands is illustrated in Table 3 in comparison with the reference ladder. The sizes of these bands were 800 bp, 625.8 bp, 454.4 bp, and 284 bp with a relative front of 0.64, 0.68, 0.73, and 0.80, respectively.
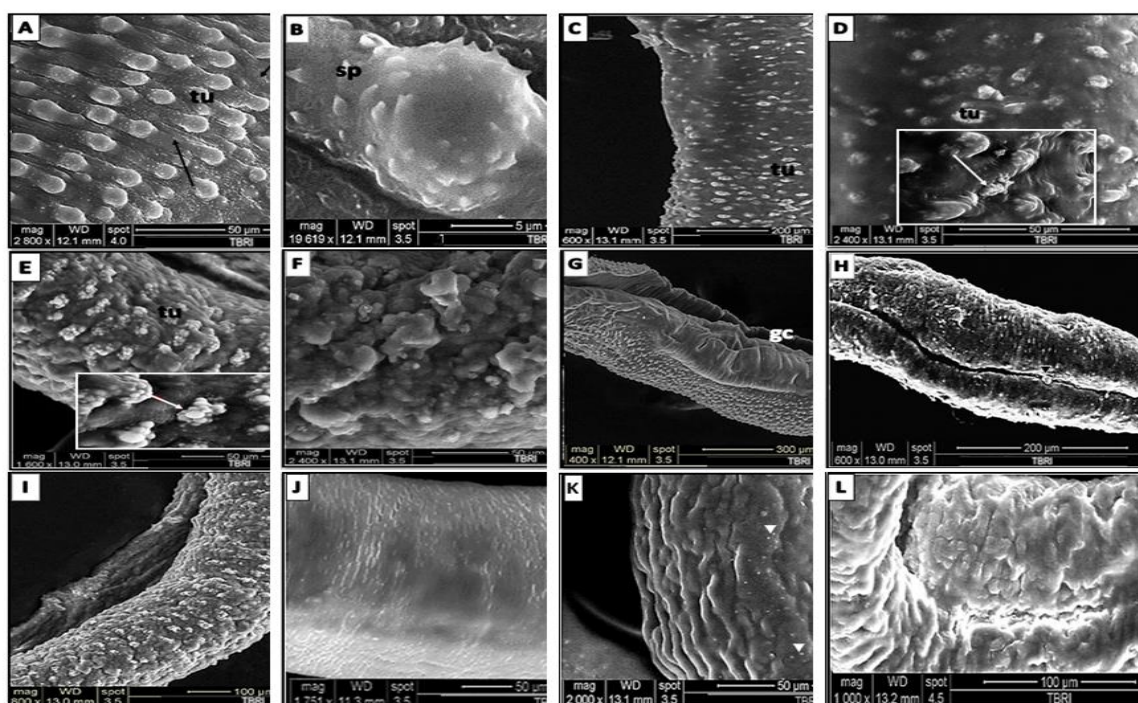
Fig. 1: Scanning electron micrographs of untreated control S. mansoni and S. mansoni exposed to an LC50 of AL or CU. Images are shown of the male tegument (A-F) and gynecophoral canal (G-I), and the female tegument (J-L). tu - tubercles, black arrows - intertubular ridges, sp - spines, white arrow - swelling, gc - gynecophoral canal, black arrow head - protuberances, white arrow head - dimples. Higher magnifications are shown as inset images (7988X, scale bar, 10μm) and (6000X, scale bar 20 μm).
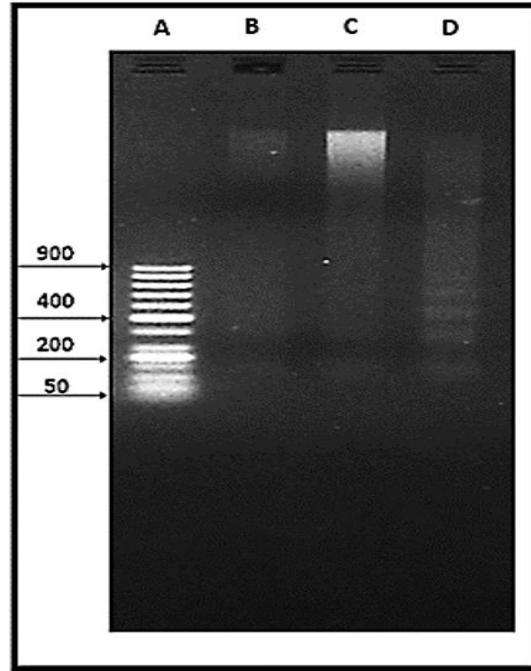
Fig. 2: Electrophoretic separated genomic DNA from S. mansoni. 50 bp DNA ladder (A). Untreated control S. mansoni (B). S. mansoni worms exposed to an LC50 of AL (C). S. mansoni worms exposed to an LC50 of CU (D).
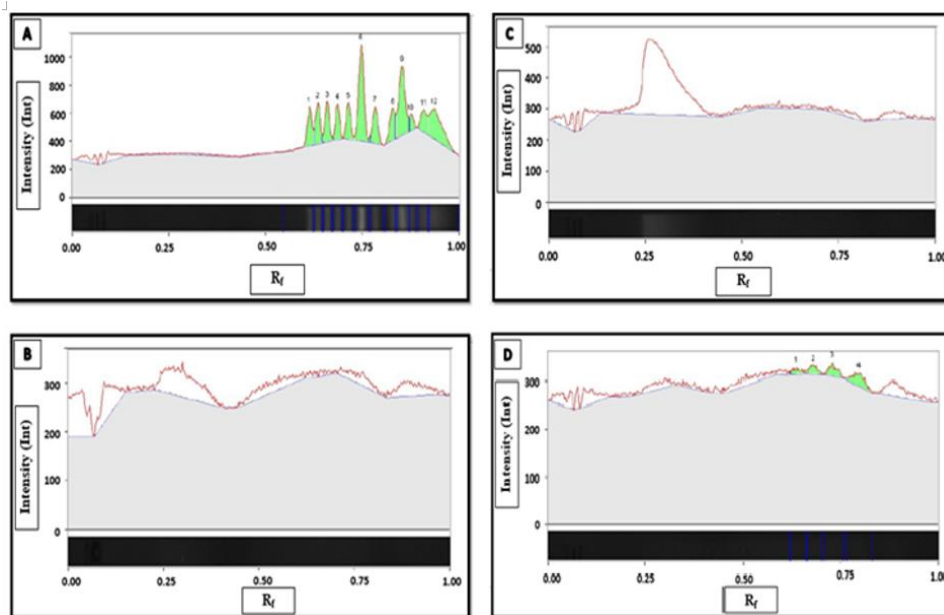
Fig. 3: Lane profile analysis of S. mansoni DNA fragments. Samples were run alongside a 50 bp DNA ladder (A). Untreated control S. mansoni (B). S. mansoni worms exposed to an LC50 of AL (C). S. mansoni worms exposed to an LC50 of CU (D).

Table 3: Band profile analysis of DNA fragments

Lane	Band number	Base pair (bp)	Relative front (R_f)	Volume (Int)	Band (%)	Lane (%)
A	1	900	0.61	55.14	6.6	5.9
	2	800	0.64	55.68	6.7	6
	3	700	0.66	53.92	6.5	5.8
	4	600	0.69	46.62	5.6	5
	5	500	0.72	49.63	6	5.3
	6	400	0.75	153.12	18.5	16.5
	7	300	0.78	57.09	6.9	6.1
	8	250	0.83	48.93	5.9	5.3
	9	200	0.85	134.62	16.2	14.5
	10	150	0.88	19.30	2.3	2.1
	11	100	0.91	43.04	5.2	4.6
	12	50	0.94	112.80	13.6	12.2
D	1	800	0.64	5.31	15.5	3.5
	2	625.8	0.68	6.59	19.2	4.3
	3	454.4	0.73	9.25	27	6
	4	284	0.80	13.12	38.3	8.5

A: ladder. **D:** CU

Discussion

This is one of the first studies to examine the antischistosomal activity of AL and CU on the different developmental stages of *S. mansoni* (miracidia, cercariae, and adult). In this study, we also assay the antischistosomal effect of AL and CU on adult worms in a variety of ways, by assessing: animal mortality, ultrastructural morphology, and genotoxicity upon exposure.

We determined the molluscicidal LC_{50} values of AL and CU to be 315 μL/L and 5690 μg/L, respectively. Both the molluscicidal LC_{50} and 1/2 LC_{50} of AL and CU were efficient in the elimination of 100% of miracidia and cercariae, while 1/4 LC_{50} of AL was only effective against cercariae. Together these results indicate that AL exerts a higher biocidal effect against miracidia, cercariae, and adult worms than CU (45). CU showed a marked effect against cercariae in vitro and resulted in ultrastructural damage, which resulted in deficiencies of cercariae ability to infect mice (46).

Continuous exposure of adult *S. mansoni* worms to either AL or CU for three days revealed higher mortality rates in response to AL than CU. Here, exposure to 30 μL/mL of AL resulted in 100% mortality from the first day, while the highest recorded mortality rate for CU exposure was 91.3% at the higher concentration of 100 μg/mL. The impressive antischistosomal activity of AL is in agreement with previous results obtained using *A. sativum* extract. *A. sativum* displayed the highest antischistosomal activity when compared with four other plant extracts (*Dryopteris filixmas, Tanacetum vulgare, Juglans nigra, Syzygium aromaticum*) (17). In contrast with the present study, exposure of *S. mansoni* to turmeric extract at a concentration of 50 μM resulted in 100% mortality after 24 h (47). The mortality rates affected consequently on LC_{50} of both tested drugs, as it was 8.66 μL/mL for AL and 87.25 μg/mL for CU. Of note, the LC_{50} values of both AL and CU were higher than those of mefloquine and praziquantel, which were 3.96 and 6.67 μg/mL, respectively (48).

The tegument is the primary interface between the parasite and the host environment,

thus analyzing this structure upon exposure to a drug allows us to better understand the therapeutic mechanism (49,50). In contrast to observations from our mortality assays, ultrastructural changes in the worms were more apparent upon exposure to an LC_{50} of CU than to an LC_{50} of AL. Here, while *S. mansoni* exposed to AL exhibited moderate surface damage, CU induced severe tegumental deformations in the worms. AL exposure resulted in moderate perturbations to the suckers, though these changes were more pronounced in CU-exposed worms. In agreement with a previous study, we found that male worms exposed to AL displayed atrophied tubercles and loss of spines (19). We also observed erosion and peeling of the tubercles in CU-exposed males. The peeling of the tegument in *S. mansoni* worms was subjected to polyvalent vaccine (30). Female worms exhibited either slight or moderate wrinkling of the tegument in response to AL or CU, respectively. Interestingly, both compounds showed a less pronounced effect on the tegument of females than males. Male *S. mansoni* worms are more susceptible to the venom of *Cerastes cerastes* snake than female worms (31). This may serve to explain why the tegument of male worms showed more damage than those of female worms.

The effects of an LC_{50} of CU were also observable at the molecular level. Lane profile and band analysis revealed that the genotoxicity of CU resulted in DNA fragmentation of *S. mansoni*. These findings are similar to previous observations in which DNA fragmentation of *S. mansoni* was detected in response to in vitro exposure to CU (29). In contrast, *S. mansoni* showed no evidence of DNA fragmentation upon exposure to an LC_{50} of AL.

Conclusion

Both AL and CU exhibit mild antischistosomal activity. Our findings encourage further investigation into the use of these compounds as antischistosomal agents, either alone or as complementary treatments to PZQ. In future studies, we hope to elucidate further the links between AL- and CU-induced ultrastructural changes, genotoxicity, and worm mortality in *S. mansoni*. We also wish to investigate the effects of these compounds on RNA, and better understand their efficacy in vivo.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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