Original Article

# *In vitro* efficacy of *Cymbopogon citratus* (lemon grass) essential oil on *S. mansoni* adult worms: A study on motility, tegument ultrastructural changes, and oxidative stress biomarkers

**Nancy M Harba, Engy V Beshay, Mona M Ghanem, Doaa I M Abogalalah**

Medical Parasitology Department, Faculty of Medicine, Menoufia University, Menoufia, Egypt

## ABSTRACT

**Background:** Since Praziquantel (PZQ) is not effective against juveniles, much attention was directed to natural products. Anti-parasitic activities were reported for lemon grass essential oil.
**Objective:** To assess the *in vitro* effect of lemon grass (*Cymbopogon citratus*) essential oil on *S. mansoni* adults.
**Material and Methods:** One hundred and fifty *S. mansoni* adults, of both sexes, were divided into five equal groups, categorized as normal control group (GI), PZQ (1 µg/ml) exposed group (GII), and *C. citratus* oil extract (CCO)-exposed groups (III, IV, and V) at concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml, respectively. All groups were incubated and observed after 24 h and 48 h to evaluate worm motility, tegumental morphological changes, the activity of oxidative stress biomarkers glutathione peroxidase (GPx), superoxide di*Sm*utase (SOD), and malondialdehyde (MDA); as well as gene expression of ubiquitin protein ligase Hul5 (*Sm*Hul5) and deubiquitinating enzyme Ubp6 (*Sm*Ubp6).
**Results:** It was demonstrated that CCO was effective against *S. mansoni* adults *in vitro* in a concentration-dependent manner. It significantly reduced worm motility, and induced obvious changes in tegumental ultrastructure. Additionally, there was a notable decrease in GPx activity, accompanied by an increase in SOD activity at 10, and 50 concentrations, while the higher concentration (100 µg/ml) led to significant SOD inhibition. Activity of MDA showed marked increase. Due to oxidative stress, and damaged proteins, gene expression of *Sm*Hul5, and *Sm*Ubp6 displayed up-regulations.
**Conclusion:** It is concluded that CCO is a promising schistosomicidal agent for future studies.

**Keywords:** *C. citratus; in vitro*; motility; oxidative stress; proteosome gene expression; schistosomiasis; tegument ultrastructure.

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**Corresponding Author:** Doaa IM Abogalalah; **Tel.:** +20 48 2274136; **Email:** doaaibrahem43@yahoo.com

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## INTRODUCTION

Among the neglected tropical diseases, schistosomiasis affects more than 779 million people worldwide[1]. It is considered the main cause of morbidity and mortality after malaria[2]. This disease is caused by flat worms of the genus *Schistosoma*. The most common species causing human schistosomiasis are *S. mansoni*, *S. japonicum* and *S. haematobium*[3]. During their complex life cycle, *Schistosoma* worms encounter the important stressful conditions of reactive oxygen species (ROS) generated by the host as one of the defense mechanisms against infection[4]. This oxidative stress is harmful to worms because it can affect normal functions of important enzymes, proteins and cellular macromolecules inducing cell death[5]. Accordingly, to survive, *Schistosoma* worms rely on effective defense mechanisms, in which antioxidant enzymes play a crucial role[6]. The key enzymes involved in this process include detoxifying enzymes, such as GPx and SOD[7]. Typically, when worms combat stressors like certain medications, ROS rises, and worms would try to maintain survival by their antioxidant enzymes[8]. During oxidative stress, lipid peroxidation results in accumulation of its final product MDA, *i.e.,* an important indicator for oxidative stress[8].

In the same context, during stressful conditions, cytoplasmic proteasome system breaks down the damaged proteins to maintain protein homeostasis. Protein oxidation is a key feature of oxidative stress, and the cytoplasmic proteasome system breaks down the damaged proteins to maintain protein homeostasis. Within *S. mansoni* proteasome system, the ubiquitin protein ligase Hul5 (*Sm*Hul5) and the deubiquitinating enzyme Ubp6 (*Sm*Ubp6) were identified as significant components as proteins are degraded by the 26S proteasome in an ATP-dependent and ubiquitin dependent manner. Moreover, the expression of *Sm*Hul5 and *Sm*Ubp6 genes is distinctly regulated in response to chemical stress, oxidative damage, and heat shock[9].

The primary anti-schistosomal drug is PZQ, which is effective against all species of *Schistosoma*. Despite

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its efficacy, PZQ is not efficient against the juvenile schistosomula, and it does not prevent reinfection[10]. Moreover, depending on only one drug necessitates the development of a new alternative[7]. On this background, considerable efforts have been made to discover new active compounds against schistosomiasis, with a focus on those derived from natural products due to their greater safety and reduced side effects[11]. The bioactive properties of natural plants are attributed to their major active secondary metabolites[12]. These effects arise either from the high concentration of certain molecules or the synergistic interaction among the primary components[13]. Even minor essential oil elements participate in the biological functions of these oils due to synergistic interactions[14].

Lemon grass (*C. citratus*), a member of the Poaceae family, is commonly cultivated in tropical and subtropical regions[15]. Its essential oil includes many phytochemicals among which citral serves as the primary component that plays a key role in the bioactive properties[16]. The components of its essential oil are categorized into tannins, saponins, phenols, flavonoids and alkaloids which include nerol, myrcene, geraniol, citronellol, limonene, α-terpineol, elemicin, catechol, quercetin, and glycosides[17]. Due to its chemical composition, CCO exhibited a wide range of pharmacological properties including antioxidant, anti-inflammatory, antimutagenic, antimicrobial, and antifungal[18]. It also showed giardicidal[19], anti-leishmanial[20], antimalarial[21], anti-trypanosomal activities[22], as well as insecticidal[23], larvicidal[24], and miticidal acaricidal effects[25].

Based on the previous literature, the present study aimed to evaluate the *in vitro* effects of CCO on *S. mansoni* adult motility, tegumental ultrastructure, oxidative stress biomarkers, and proteosome gene expression.

## MATERIAL AND METHODS

This randomized control study was conducted at the Schistosome Biological Supply Program (SBSP) Unit, Theodor Bilharz Research Institute (TBRI) (Giza, Egypt) during the period from May 2023 to July 2025.

**Study design:** Freshly collected *S. mansoni* adults were incubated in RPMI-1640 culture media, and divided into 5 groups (non-exposed, exposed to PZQ, and three different CCO concentrations). Worms were observed after 24 h and 48 h to evaluate motility; ultrastructural tegmental changes; oxidative stress biomarkers of GPx, SOD, and MDA; and proteosome expression of the genes encoding *Sm*Hul5, and *Sm*Ubp6.

**Plant material:** The CCO extract was obtained from Natural Resources Department, Botany Environmental Studies and Research Institute (University of Sadat City, Egypt). Steam distillation technique was used for the extraction of CCO[26].

**Control drug (PZQ):** It was purchased from the Egyptian International Pharmaceutical Industries Company, EIPICO, Egypt. It was dissolved in 0.1% DSMO and supplemented with RPMI medium at a dose of 1 µg/ml[11].

**Analysis of the chemical composition of CCO[27]:** The chemical composition of the extracted oil was analyzed at Main Laboratories of Chemical Warfare (Almaza, Nasr city, Cairo Governorate, Egypt) by gas chromatography and mass spectrometry (QP2010SE, Shimadzu Co., Japan).

**Study groups:** Worms were divided into five equal groups, categorized as normal control group (GI), PZQ (1 µg/ml)-exposed group (GII), and three groups exposed to different concentrations of CCO extract. These groups were GIII (10 µg/ml), GIV (50 µg/ml), and GV (100 µg/ml).

***In vitro* culture:** Worms, kindly obtained from SBSP at TBRI, were first washed in RPMI-1640 media (Sigma-Aldrich, USA) supplemented with penicillin (100 UI/ml), streptomycin (100 µg/ml) (Sigma-Aldrich, USA), and 10% bovine fetal serum (Cultilab, Brazil). Thirty worms (15 male, 15 female) were assigned to each group. Normal control group (GI) was cultured in RPMI-1640 containing 0.1% DSMO (Sigma-Aldrich, USA). The same concentration of DSMO was used as a solvent for PZQ and CCO. Drug control group (GII) was cultured in RPMI media supplemented with PZQ (1 µg/ml). All groups were maintained in supplemented PRMI-1640 media, and incubated in 5% $CO_2$ at 25–27°C.

**Motility assay:** The incubated worms were monitored at 24 h and 48 h using a stereomicroscope (SMZ 1000, Nikon). Assessment of viability was based on the standard procedures for screening schistosomicidal compounds established by the WHO Special Program for Research and Training in Tropical Diseases[28]. Accordingly, motility was evaluate as 3: normal activity, 2: slight loss of movement with active tail, suckers, and gynecophoral canal membrane, 1: movement of tails and suckers alone, and 0: no movement. Worms were considered as dead when no movement was identified after 3 min of stereomicroscopic observation[28].

**Scanning electron microscope (SEM)[29]:** Ten worms from each group were fixed in 5% glutaraldehyde-phosphate buffer (ph 7.4) at 4°C, and then fixed in 1% osmium tetroxide buffer for 1 h. They were dehydrated through graded series of ethanol and dried using liquid $CO_2$ as a transitional medium. After drying, they were mounted on stubs and coated with gold in an ion-sputtering apparatus. They were examined and photographed using an ISI-60 SEM (JEOL, JSM 5400 LV, Japan) operating at 25–36 KV using back scattered

electron imaging at Electron Microscope Unit, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

**Oxidative stress biomarkers assay:** From each group, 10 worms were collected and stored at -20°C for GPx, SOD, MDA activity assays. Techniques were performed at the Central lab, Faculty of Medicine, Menoufia University.

**Preparation of worm homogenates:** For each group, the stored worms were subjected to 4 cycles of sonication. Each cycle was set at 0.75 sec with an amplitude of 40%, in phosphate buffer (pH 7.4) at 4°C for 2 min. The homogenates were centrifuged for two rounds at 5000 g and 15500 g for 15 min at 4°C. Protein concentrations were quantified and the clear supernatants were stored at –70°C[7].

- **For GPx,** the assay kit (Biodiagnostic, Egypt) was used[30]. Briefly, 0.01 ml of each sample of worm homogenate was added to 1 ml phosphate buffer (pH 7), 0.1 ml of NADPH reagent (lyophilized) and 0.1 ml of diluted hydrogen peroxide (1:100) then mixed well. Absorbance was measured using a spectrophotometer at 340 nm after 3 min and the results were expressed as U/mg.
- **For SOD,** the assay kit (Biodiagnostic, Egypt) was used[31]. From each sample of worm homogenate, 0.1 ml was added to 1 ml of working reagent (phosphate buffer pH 8.5, nitroblue tetrazolium, and NADH in ratio of 10:1:1 ml, respectively). Then, 0.1 ml of phenazine methosulphate (PMS) was added to the mixture and mixed well. Absorbance was measured using a spectrophotometer at 560 nm after 5 min at 25°C and the results were expressed as U/mg.
- **For MDA,** the assay kit (Biodiagnostic, Egypt) was used[32]. In brief, 0.2 ml of each sample of worm homogenate was mixed with sodium dodecyl sulfate, acetate buffer (pH 3.5) and added to 1 ml of the chromogen (thiobarbituric acid), then mixed well. The mixture was heated in a water bath at 95°C for 30 min then cooled. Absorbance was measured using a spectrophotometer at 534 nm and the results were expressed as nmol/mg.

**Real-time PCR for evaluation of proteasomal enzymes *Sm*Hul5 and *Sm*Ubp6 gene expression:** From each group, 10 worms were collected and stored at -80°C for molecular study. Total RNA from adult *S. mansoni* was isolated using Directzol TM RNA Miniprep Plus (Zymo Research, USA)[7]. Then, they were reverse transcripted using Quanti-Tect Revers Transcription Kit (Qiagen, Applied Biosystems, USA)[7]. After that, 10 µl of extracted RNA were added to the reverse-transcription master mix to achieve a reverse-transcription reaction total volume of 20 µl. The obtained complementary DNA (cDNA) was stored at -20°C for further amplification. Amplification was performed using Quanti-Tect SYBR Green PCR Kit (Biokit, Egypt). Table (1) shows the forward and reverse primers[9] (Biokit, Egypt).

**Table 1.** Real-time PCR primers.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| ***Sm*Hul5** | CAACTGGCTTAGCTGAAGTTGG | GCAGATGCTTGTGGATTTGG |
| ***Sm*Ubp6** | ACCTGGCCTCGTAAATCTTG | GCGATATTTCGTCGAGCTT |
| **GAPDH** | TCGTTGAGTCTACTGGAGTCTTTACG | AATATGAGCCTGAGCTTTATCAATGG |

The primers of *Sm*Hul5, *Sm*Ubp6, and the reference gene GAPDH were reconstituted before use in Tris-EDTA buffer (Biokit, Egypt). For each reaction, a mixture of 5 µl of cDNA, 1 µl of each primer, 12.5 µl of SYBR Green master mix (Biokit, Egypt), and 5.5 µl of RNase-free water were added in an Eppendorf tube. An initial denaturation step at 95°C for 15 min was followed by 35 PCR cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Reaction was then terminated by a final extension at 72°C for 15 min with cooling of samples down to 4°C. Melting curve analysis of the PCR yield was obtained using software version 2.0.1 incorporated in the cycler (Applied Biosystems, USA).

**Statistical analysis:** Data were collected, tabulated and statistically analyzed by an IBM compatible personal computer with the statistical package for social science software (SPSS) Version 26 (SPSS Inc., Chicago, IL, USA). Qualitative data were represented as No. and %. Quantitative data were represented as mean±SD. To compare quantitative normally distributed variables, ANOVA (*F* test) was used followed by a post hoc test to detect the significance between two individual groups. Kruskal-Wallis (*K* test) was used to compare quantitative non-normally distributed variables followed by a post hoc test to detect the difference among the groups. Statistical significance is considered if $P \leq 0.05$.

**Ethical consideration:** The study was approval by the ethical committee of the Faculty of Medicine, Menoufia University (IRB: 6/2023PARA25).

## RESULTS

**Chemical composition of CCO:** The chemical analysis of CCO revealed that citral is the predominant component, accounting for 32.43% of the oil's composition. Other significant constituents include 3,7-dimethyl, 2,6-Octadienal (25.47%), beta-myrcene (5.71%), and geraniol (3.93%). Lesser components include 2-butenoic acid, butyl ester (3%), levomenthol (2.78%), isoneral (2.56%), isogeranial (2.38%), along with numerous trace elements (Table 2).

**Table 2.** Chemical analysis of CCO by gas chromatography/mass spectrometry.

| No. | Compounds | Retention time (min) | Percentage (%) |
|---|---|---|---|
| **1** | Beta-Myrcene | 17.329 | 5.71 |
| **2** | Cyclohexanone, 5-methyl-2-methylethyl | 23.910 | 2.10 |
| **3** | Isoneral | 24.193 | 2.56 |
| **4** | Levomenthol | 24.601 | 2.78 |
| **5** | Isogeranial | 24.875 | 2.38 |
| **6** | 3,7-dimethyl , 2,6-Octadienal | 27.394 | 25.47 |
| **7** | Geraniol | 27.816 | 3.93 |
| **8** | Citral | 28.572 | 32.43 |
| **9** | Methacrylic acid, tridecyl ester | 32.984 | 1.91 |
| **10** | 2-Butenoic acid, butyl ester | 33.687 | 3.00 |
| **11** | Mono(2-ethylhexyl) phthalate | 59.274 | 1.20 |

**Motility assay:** The normal control worms (GI) maintained their activity during the experiment. While in the group exposed to PZQ (GII), all parasites died within 24 h. The *S. mansoni* adults treated with CCO showed a significant decline ($P$<0.001) in motility which was influenced by both time and concentration. At the highest concentration of 100 µg/ml (GV), there was a significant ($P$<0.001) inhibition of worm motility when compared to the normal group (GI), with a decrease of 44.33% in male and 46.67% in female motility after 24 h. After 48 h, the decrease in motility was 95.67% for males and 93.33% for females. This decrease was statistically significant ($P$<0.001) when compared to the normal control (GI) while, when compared to the PZQ-exposed group (GII) the difference was not significant (Tables 3, 4).

**Table 3.** Comparison between the five studied groups according to male *S. mansoni* motility score at 24 h and 48 h.

| | Male motility score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24 h | | | | 48 h | | | |
| | Mean±SD (Range) | % decrease | Statistical analysis | | Mean±SD (Range) | % decrease | Statistical analysis | |
| | | | *K* test | Post hoc | | | *K* test | Post hoc |
| **GI** | 3±0 (3-3) | --- | 56 <0.001* | *P*1 < 0.001* *P*2 = 1 | 3±0 (3-3) | --- | 53.08 <0.001* | *P*1 < 0.001* *P*2 < 0.001* |
| **GII** | 0±0 (0-0) | 100% | | *P*3 < 0.001* *P*4 < 0.001* | 0±0 (0-0) | 100% | | *P*3 < 0.001* *P*4 < 0.001* |
| **GIII** | 2.80±0.41 (2-3) | 6.67 | | *P*5 < 0.001* *P*6 < 0.001* | 0.87±0.74 (0-2) | 71% | | *P*5 < 0.001* *P*6 = 0.024* |
| **GIV** | 2.20±0.77 (1-3) | 26.67 | | *P*7 < 0.001* *P*8 = 0.031* | 0.60±0.83 (0-2) | 80% | | *P*7 = 1 *P*8 = 1 |
| **GV** | 1.67±0.82 (0-3) | 44.33 | | *P*9 < 0.001* *P*10 = 0.081 | 0.13±0.35 (0-1) | 95.67% | | *P*9 < 0.003* *P*10 = 0.168 |

Data were expressed as mean ± SD; **GI:** Non-exposed; **GII:** Exposed to PZQ; **GIII:** Exposed to 10 µg/ml CCO; **GIV:** Exposed to 50 µg/ml CCO 50 µg/ml; **GV:** Exposed to 100 µg/ml CCO; ***K:*** Kruskal-Wallis test for comparing between all groups; Post hoc test for comparing between individual groups. ***P*1:** Comparison between groups I and II; ***P*2:** Comparison between groups I and III; ***P*3:** Comparison between groups I and IV; ***P*4:** Comparison between groups I and V; ***P*5:** Comparison between groups II and III; ***P*6:** Comparison between groups II and IV; ***P*7:** Comparison between groups II and V; ***P*8:** Comparison between groups III and IV; ***P*9:** Comparison between groups III and V; ***P*10:** Comparison between groups IV and V; ***:** Statistically significant ($P$<0.05).

**Table 4.** Comparison between the five studied groups according to female *S. mansoni* motility score at 24 h and 48 h.

| | Female motility score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24 h | | | | 48 h | | | |
| | Mean±SD (Range) | % decrease | Statistical analysis | | Mean±SD (Range) | % decrease | Statistical analysis | |
| | | | *K* test | Post hoc | | | *K* test | Post hoc |
| **GI** | 3.0±0 (3-3) | --- | 54.86 <0.001* | *P*1<0.001* *P*2:1 | 3.0±0 (3-3) | --- | 52.70 <0.001* | *P*1 < 0.001* *P*2 < 0.001* |
| **GII** | 0±0 (0-0) | 100% | | *P*3:0.362 *P*4<0.001* | 0±0 (0-0) | 100% | | *P*3 < 0.001* *P*4 < 0.001* |
| **GIII** | 2.80±0.35 (2-3) | 6.67% | | *P*5<0.001* *P*6<0.001* | 1.0±0.85 (0-2) | 66.67% | | *P*5 < 0.001* *P*6 < 0.001* |
| **GIV** | 2.53±0.74 (1-3) | 15.67% | | *P*7<0.001* *P8*=1 | 0.80±0.77 (0-2) | 73.33% | | *P*7 = 1 *P*8 = 1 |
| **GV** | 1.60±1.06 (0-3) | 46.67% | | *P*9<0.001* *P*10<0.001* | 0.20±0.41 (0-1) | 93.33% | | *P*9 < 0.001* *P*10 = 0.036* |

Data were expressed as mean ± SD; **GI:** Non-exposed; **GII:** Exposed to PZQ; **GIII:** Exposed to 10 µg/ml CCO; **GIV:** Exposed to 50 µg/ml CCO 50 µg/ml; **GV:** Exposed to 100 µg/ml CCO; ***K:*** Kruskal-Wallis test for comparing between all groups; Post hoc test for comparing between individual groups. ***P*1:** Comparison between groups I and II; ***P*2:** Comparison between groups I and III; ***P*3:** Comparison between groups I and IV; ***P*4:** Comparison between groups I and V; ***P*5:** Comparison between groups II and III; ***P*6:** Comparison between groups II and IV; ***P*7:** Comparison between groups II and V; ***P*8:** Comparison between groups III and IV; ***P*9:** Comparison between groups III and V; ***P*10:** Comparison between groups IV and V; ***:** Statistically significant ($P$<0.05).

**Results of SEM studies:** In GI (NC), *S. mansoni* females showed an elongated cylindrical body with triangular oral sucker and numerous spines on the rim of the oral and ventral suckers. The tegumental surface showed parallel cytoplasmic ridges with hemispherical papillae and lack of spines, while the area around the excretory pore showed numerous anteriorly directed spines with few hemispherical papillae. Males showed the ventral longitudinal cleft (gynecophoral canal) and lack of tubercles in the anterior region of the tegumental surface opposite the oral and ventral suckers. The tegumental surface showed the characteristic cytoplasmic ridges with abundant oval tubercles covered by spines and papillae in between. The posterior region of the tegument showed few numbers of tubercles and spines (Fig. 1).

Scanning electron microscopic study of PZQ-exposed group (GII) showed abnormally retracted oral and ventral suckers, contraction and coiling of the body with areas of tegumental peeling, destruction of tubercles and spines, rupture of papillae and some tubercles, and deformity of gynecophoric canal (GC) (Fig. 2).

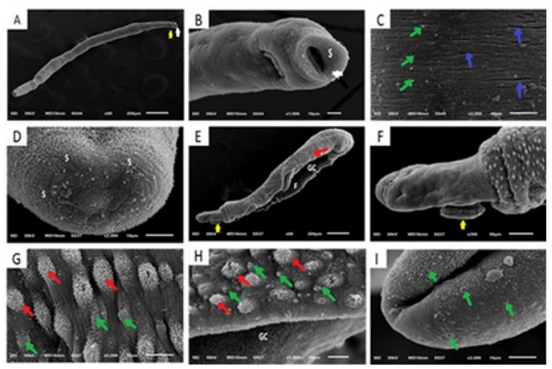

**Fig. 1.** Scanning electron micrographs of *S. mansoni* worms of normal control group (GI). **(A)** Elongated cylindrical female with oral OS (white arrow) and ventral suckers VS (yellow arrow) (x80). **(B)** Triangular OS (white arrow) with spines on the rim (S) (x1000). **(C)** Female tegmental surface with cytoplasmic ridges (blue arrows) and hemispherical papillae (green arrows) (x2000). **(D)** Female area around excretory pore with numerous spines (S) (x2000). **(E)** Adult male showing GC and tegmental surface with oval tubercles covered by raised spines (red arrow) (x80). **(F)** Male anterior region of tegumental surface opposite the oral and ventral (yellow arrow) suckers with lack of tubercles and spines (x350). **(G-H)** Numerous papillae (green arrows) between the tubercles (red arrows) (x2000 and x1500, respectively). **(I)** Male posterior region of the tegument with a few tubercles and spines, numerous macular papillae (green arrows) (x2000).

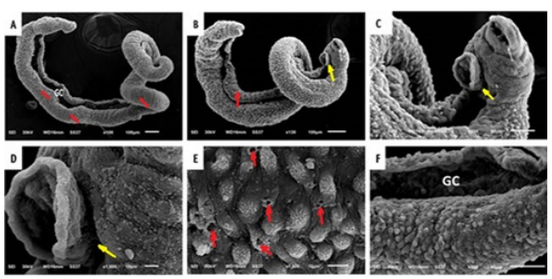

**Fig. 2.** Scanning electron micrographs of adult *S. mansoni* male worms of PZQ-exposed group (GII). **(A)** Contraction and coiling of the body with areas of tegmental peeling (red arrows) and deformity of gynecophoric canal (x100). **(B, C, D)** Contraction and distortion of oral and ventral suckers showing a distinct neck-like area (white and yellow arrows respectively) (x130, x350, x1000, respectively). **(E)** Destruction of tubercles and spines and rupture of papillae (red arrows) (x1500). **(F)** Rough edges of gynecophoral canal with loss of spines on the rim (x500). **GC:** Gynecophoric canal.

Scanning electron microscopic study of *S. mansoni* males treated with CCO (GIII, GIV, GV) showed concentration dependent morphological changes with different degrees of contraction and destruction of both oral and ventral suckers, appearance of edematous wrinkles and pitting of the tegument, flattening of tubercles with loss of the spines, difficultly seen ridges, rupture of papillae, obliteration of gynecophoric canal with disturbed edges and loss of the spines on the edges (Figs. 3, 4, 5).

## Oxidative stress biomarkers assays

Exposure to CCO showed an initial stimulation of GPx activity at the low concentration (10 µg/ml) (GIII). However, it was a non-significant increase when compared to the control group (GI). While higher concentrations (50, and 100 µg/ml) in groups IV and V caused inhibition of this enzyme activity. The results obtained in GV were significant when compared to both control (GI) and PZQ-exposed group (GII) ($P$<0.001) (Table 5, Fig. 6).

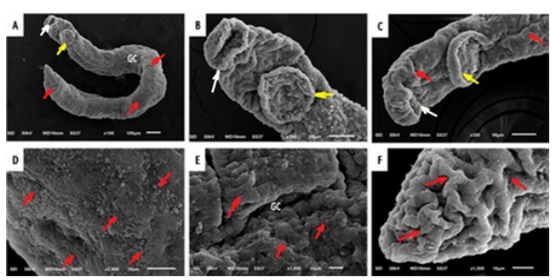

**Fig. 3.** Scanning electron micrographs of male *S. mansoni* worms of CCO (10 µg/ml)-exposed group (GIII). **(A)** Pitting of the tegument, flattening of tubercles with loss of the spines, ill-defined ridges (red arrows), obliterated gynecophoric canal (x100). **(A-C)** Contraction and destruction of both oral and ventral suckers (white and yellow arrows respectively) (x100, x350, x350, respectively). **(D)** Loss of tubercles and spines, pitting of tegumental surface (red arrows) (x2000). **(E)** Obliteration of gynecophoric canal, disturbed edges with loss of spines on the rims. Wrinkling and pitting of the tegumen , flattening of tubercles with loss of spines (red arrows) (x1000). **(F)** Edematous wrinkles of tegument at the posterior end (red arrows) (x1500). **GC:** Gynecophoric canal.

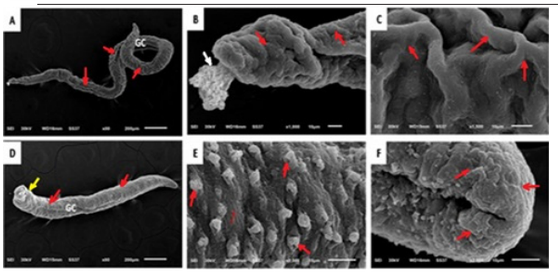


**Fig. 4.** Scanning electron micrographs of female *S. mansoni* worms of CCO (50 µg/ml)-exposed group (GIV). **(A)** Elongated coiled body, with wrinkling of the tegument (red arrows) and obliteration of gynecophoric canal (x80). **(B)** Expulsion of oral sucker (white arrow), edematous tegument with wrinkles and loss of the spines (red arrows) (x1000). **(C)** Edematous wrinkled tegument (red arrows) (x1500). **(D)** Destruction of ventral sucker (yellow arrow), obliterated gynecophoric canal with loss of tubercles and spines (red arrows) (x80). **(E)** Loss of tubercles and spines over them, rupture of some tubercles (red arrows) (x2000). **(F)** Edematous wrinkled posterior end with loss of the spines (red arrows) (x2000). **GC:** Gynecophoric canal; **VS:** Ventral sucker.

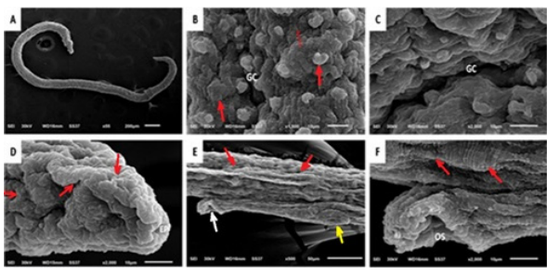


**Fig. 5.** Scanning electron micrographs of adult *S. mansoni* worms of CCO (100 µg/ml)-exposed group (GV). **(A)** Elongated body (x55). **(B)** Obliterated gynecophoric canal with destructed edges, loss of tubercles and spines (red arrows) (x1500). **(C)** Obliterated gynecophoric canal with destructed edges, loss of spines on rims (x2000). **(D)** Posterior end around the excretory pore, wrinkled tegument with loss of the spines (red arrows) (x2000). **(E)** Elongation between oral (white arrow) and ventral suckers (yellow arrow), pitted wrinkled tegument (red arrows) (x500). **(F)** Destroyed oral sucker and loss of spines, loss of normal ridges (red arrows) (x2000). **GC:** Gynecophoric canal; **OS:** Oral sucker.

Groups exposed to CCO exhibited significant increases in SOD activity compared to both the control (GI) and PZQ-exposed (GII) groups. The highest significant value was in CCO-exposed group at a concentration of 50 µg/ml (GIV) (12.7±0.49) (P<0.001). However, a significant decrease in SOD activity was noted at the higher concentration (100 µg/ml) (GV) (6.95±0.51) (*P*<0.001) (Table 5, Fig. 6).

The highest increase of MDA was in PZQ-exposed group (GII) (16.05±0.69) which was statistically significant when compared to all other groups (*P*<0.001). While CCO exposed groups showed significant increases in a concentration-dependent manner when compared to control group (GI) (*P*<0.001). The difference was significant when GV was compared to all other groups (Table 5, Fig. 6) (*P*<0.001).

**Gene expression of *Sm*Hul5 and *Sm*Ubp6:** All studied groups showed up-regulation of *Sm*Hul5 and *Sm*Ubp6 gene expression in comparison with the normal control group (GI). The higher concentration of CCO (100 µg/ml in GV) showed significant decrease in the fold of change of both up-regulated gene expression when compared to the lower concentrations of CCO (10 µg/ml and 50 µg/ml in GIII and GIV respectively) (*P*<0.001). However. this decrease in gene expression was still up-regulated and the differences were significant (*P*<0.001) when compared to the normal control group (GI) (Fig. 7).

**Table 5.** Oxidative stress biomarkers assays among the studied groups.

| Biomarkers | Study groups | | | | | Statistical analysis |
|---|---|---|---|---|---|---|
| | GI | GII | GIII | GIV | GV | |
| **GPx (U/mg)** | 3.28±0.42 | 2.44±0.38 | 3.50±0.32 | 2.70±0.33 | 1.11±0.14 | ***F*** = 39.43, ***P*** < 0.001* |
| **SOD (U/mg)** | 5.02±0.22 | 3.07±0.22 | 8.02±0.38 | 12.7±0.49 | 6.95±0.51 | ***F*** = 435.93, ***P*** < 0.001* |
| **MDA (nMol/mg)** | 4.01±0.49 | 16.05±0.69 | 5.90±0.36 | 7.20±0.65 | 12.40±0.84 | ***F*** = 314.93, ***P*** < 0.001* |

***:** Significant (*P*<0.05).

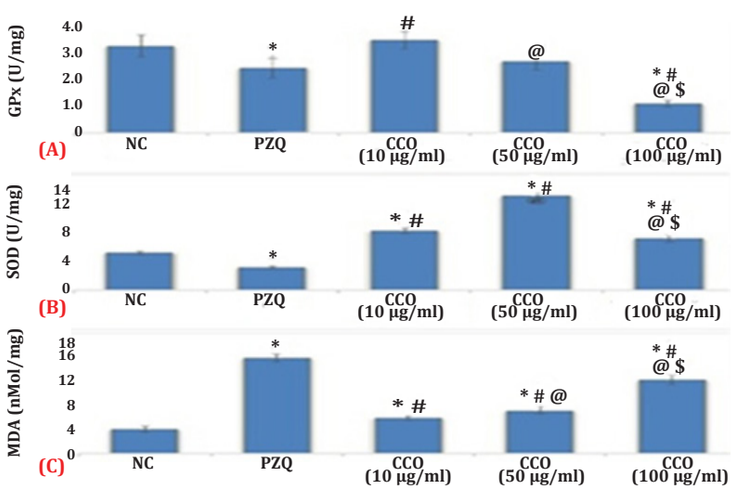


**Fig. 6.** Mean±SD of the oxidative stress biomarkers assays among the study groups. **A)** GPx, **B)** SOD, and **C)** MDA. ***:** Significant when compared to GI; **#:** Significant when compared to GII; **@:** Significant when compared to GIII; **$:** Significant when compared to GIV.

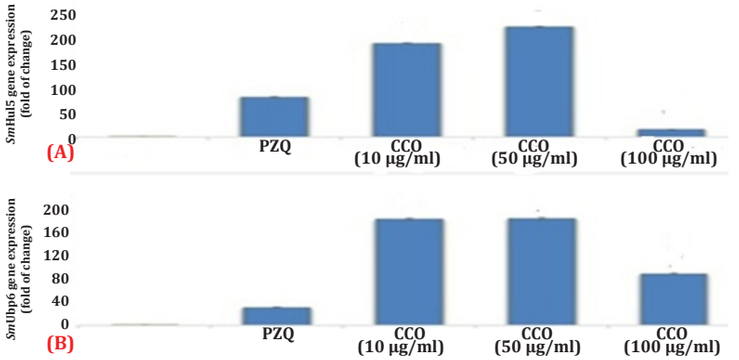


**Fig. 7.** Proteosome gene expression among the studied groups. A) *Sm*Hul5 and B) *Sm*Ubp6. ***:** Significant when compared to GI; **#:** Significant when compared to GII; **@:** Significant when compared to GIII; **$:** Significant when compared to GIV.

## DISCUSSION

Natural plants are regarded as promising sources for the development of new drugs to overcome praziquantel drug resistance and failure of treatment. Therefore, this study aimed to evaluated the *in vitro* anti-schistosomal activity of CCO extract on *S. mansoni* adult worms. In the current study, the chemical analysis of lemon grass oil identified citral as the dominant primary component accounting for 32.43%, along with other trace elements. This result was in agreement with Macedo *et al.*[33] who reported that citral concentration in CCO was 36.75%. In accordance with the present work, other studies reported citral as the main component of CCO but with different percentages. For instance, Macedo *et al.*[34] reported 97.7% of citral in essential oil. Additionally, Mansour *et al.*[35] showed that the Egyptian CCO is composed mainly of geranial (citral A) (40.72%), neral (citral B) (34.98%), geraniol (8.30%) and linalool (5.60%). Soliman *et al.*[36] also reported that citral constitutes 79.69% of the essential oil of the Egyptian CCO, while other significant components included myrcene (8.05%), geraniol (3.22%), and cis-verbanol (1.84%). Similarly, Majewska *et al.*[14] reported that geranial was 48.14% while neral was 38.32% in their analysis. Moreover, Gonzales *et al.*[13] reported that citral A and citral B represent 45.7% and 33.9% of CCO, respectively. Furthermore, Li *et al.*[37] found that CCO is composed of geranial (37.40%), neral (31.97%) and myrcene (15.65%). Aly *et al.*[16] also reported that α-citral (36.08%), β-citral (34.22%), and β-myrcene (13.84%) are the main volatile oils of CCO. But in contrast to the current analysis, Moutassem *et al.*[38] reported a low citral content of 0.99% in lemon grass essential oil cultivated in Nigeria. This variation in the chemical composition of lemon grass could be attributed to the influence of several factors, including genetic variations, geographical location, climate and seasonal changes, plant nutrition, the application of fertilizers, plant maturity, post-harvest drying, storage conditions, and the extraction method[13,14,33,39].

To the best of our knowledge, this is the first study conducted on the efficacy of CCO against *S. mansoni*. However, anti-parasitic effects of CCO were reported in several previous studies on other parasites. For example, Macedo *et al.*[34] found that CCO was able to reduce the parasite load of *Haemonchus contortus* by 38.6% in experimentally infected Mongolian gerbil. Gonzales *et al.*[13] demonstrated that varying concentrations of CCO (100, 200, 300, 400, and 500 mg/ml) achieved 100% efficacy against *Notozothecium janauachensis* (a monogenean parasite of fish) *in vitro* in a concentration dependent manner. Li *et al.*[37] also revealed that CCO at concentrations of 10% and 5% successfully eliminated all *Sarcoptes scabiei* within 10 and 25 min, respectively. Moreover, Méabed *et al.*[39] demonstrated a dose-dependent efficacy of CCO against *Giardia lamblia* and Emiru *et al.*[40] found a similar effect against *T. congolense*.

Interestingly, in a study conducted by Gomes *et al.*[41], citral was found effective for the treatment of *S. mansoni* reducing its motility and increasing its mortality. However, in their study, citral alone showed better results than *Lippia alba* essential oil, suggesting that the interaction between the major and smaller compounds present in this oil can lead to an antagonistic effect reducing its schistosomicidal activity.

In another study, Baccega *et al.*[42] reported that citral and geraniol, the major compounds of CCO, have potential antiparasitic activity against *Trichomonas gallinae in vitro*. They explained the mechanism of action of these compounds by the ability to induce damage to the cell membrane and alteration of its integrity and permeability. Citral acts in a similar way to other aldehydes through the inhibition of activity of the thiol group enzymes in the cytoplasmic membrane[43]. In addition, essential oils are lipophilic and can penetrate into the cell membrane interacting with polysaccharides, fatty acids, and phospholipids, eventually leading to cell death due to loss of ions and cellular content[41]. Furthermore, our findings aligned with previous *in vitro* studies that demonstrated the anti-schistosomal activity of essential oils and molecules from various natural plants against adult worms of *S. mansoni*. These reports mainly described the ability of natural products to decrease worm motility and induce death[44]. For instance, higher concentrations

of licochalcone A at 12.5 µM elicited no motility of 100% of *S. mansoni* adult worms *in vitro* at 24 h[45]. The essential oil of *Dysphania ambrosioides* at 25 and 12.5 µg/ml presented remarkable schistosomicidal action *in vitro* and killed 100% of adult worm pairs within 24 and 72 h, respectively[46].

The tegument of *S. mansoni* plays a vital role in nutrient absorption and the secretion of certain substances. Additionally, it serves to shield the parasite from the host's immune system. Hence, it is an important target for the development of anti-schistosomal drugs[28,44]. Regarding the SEM results of the present study, the normal control group revealed an intact tegument without any morphological alterations. The tegumental morphological damage of PZQ-exposed group were similar to those reported in several studies[11,28,47].

In the CCO exposed groups, all tested concentrations caused significant tegumental damage in a concentration-dependent manner. These findings are in agreement with Matos-Rocha *et al.*[28] who studied the effect of monoterpene rotundifolone (ROT) at different concentrations against *S. mansoni in vitro* and revealed destruction of suckers, loss of tubercles and formation of blebs all over the body. Moreover, the study conducted by Gonzales *et al.*[13], revealed tegumental perforation of monogenean parasites exposed to 500 mg/ml of CCO. Our results also approximate those reported by El-Morsy *et al.*[48], who evaluated the anti-schistosomal effects of *Ficus carica* leaf extract. They observed swelling in the oral and ventral suckers as well as obliteration of the gynaecophoral canal, swelling of the tegumental surface and tubercles, with partial or complete loss of spines in a dose dependent manner.

Furthermore, results of the present study agree with Gomes *et al.*[41] who studied the essential oil of *Lippa alba* against *S. mansoni* adults. The authors reported destruction of the tegument and disorganization of the reproductive system of both male and female worms. They attributed their results to the lipophilic nature of essential oils which allows them to cross cell membranes of parasite, increase lipid peroxidation and induce oxidative stress which ultimately leads to serious damage to the cell membrane that leads to cell death[49]. Interestingly, the changes observed in motility and death of adult *S. mansoni* worms caused by the natural products in several previous studies were mainly associated with morphological alterations in worm tegument[1,44].

The present study revealed that exposure to CCO initially stimulated the activity of GPx, at the lower concentration (GIII) but inhibition occurred at higher concentrations (GIV, GV). Similarly, Aguiar *et al.*[7] observed a reduction in GPx activity in adult *S. mansoni* worms after incubation with curcumin. Concerning the effect of herbal extracts on GPx, Al-Olayan *et al.*[50] reported that the schistosomicidal activity of *Ceratonia siliqua* pod extract was through the induction of an oxidative stress that was evidenced by decreased activity of GPx enzyme. However, the initial increase with lower concentrations in our study, could be explained as an initial protective response of the parasite that could not be sustained at higher concentrations of CCO[51].

In the present study, the groups exposed to CCO (GIII, GIV) exhibited a significant increase in SOD activity compared to GI and GII. However, a decrease in SOD activity was observed at the higher concentration (GV). The initial elevation of SOD activity may represent trials by adult worms to combat the state of oxidative stress. While, the reduced activity of SOD at the higher concentration could be attributed to enzyme exhaustion or an excessive production of ROS that exceeded the worms' capacity to manage[8]. The initial increase of SOD in the current study with lower concentrations of CCO is in accordance with Aguiar *et al.*[7] who reported occurrence of oxidative stress, apoptosis, and increase of SOD activity in *S. mansoni* adults exposed to curcumin. Moreover, Agbafor *et al.*[52] reported an anti-oxidant effect of *C. citratus* ethanolic extract with significant increases in SOD and glutathione perioxidase activities in mice infected with *Plasmodium berghei*. However, the reduced SOD activity in GV is in agreement with Souza *et al.*[45] who reported that licochalcone A increased the superoxide anion level and decreased SOD activity in *S. mansoni* adult worms *in vitro*.

In the present study, CCO exposed groups showed significant increase in MDA activity which was also concentration dependent. This finding is in accordance with Agbafor *et al.*[52] who reported significant reduction of MDA of the *C. citratus*-treated mice when compared to *P. berghei* untreated mice. The results of the current work could be explained by the ability of essential oils to induce oxidative stress, reduce parasite ability to resist ROS, and increase lipid peroxidation[1,49].

Regarding the expression of *Sm*Hul5 and *Sm*Ubp6, our results revealed significant up-regulations of these genes expression in CCO exposed groups (GIII, GIV, GV) when compared to the control group (GI). However, the higher concentration 100 µg/ml (GV) resulted in a lesser fold of increase when compared with other concentrations (GIII and GIV). This result was in accordance with De Paula *et al.*[9] who reported up-regulation of *Sm*Hul5 and *Sm*Ubp6 gene expression with oxidative stress ensuring the importance of the proteasome system in the cellular response during stress.

In conclusion, CCO was found effective against adult *S. mansoni in vitro* by inducing oxidative stress. This oxidative damage and accumulation of metabolites led to tegumental ultrastructure alterations and

reduction of worm motility and viability. However, these promising results require further studies for more evaluation and validation of CCO as a possible candidate for development of a new anti-schistosomal drug.

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**Author contribution:** All authors conceived and designed the research topic. Beshay EV analyzed and interpretated research data. Ghanem MM wrote the draft of the manuscript. Harba NM, Beshay EV, Abogalalah DIM critically revised the article for publication. All authors approved the final revision before publication.

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