



***In vitro* Antischistosomal Activity of *Clerodendrum umbellatum* Poir (Labiatae) Leaves Aqueous Extract and Derived Fractions against *Schistosoma mansoni* Adult Worms**

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Authors' contributions

This work was carried out in collaboration among all authors. Authors HBJ, PK and LATT designed the study and wrote the protocol. Authors MCK, HBJ, NGF, ETN and UMF performed *in vitro* experiments. Author ED designed and wrote the phytochemical protocol and author CDT performed it. Author PDDD managed the literature searches. Authors MCK and HBJ performed the statistical analysis and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Treatment against schistosomiasis relies on praziquantel. Its treatment failure and the possible development of resistant schistosomes strains have been reported in the literature. *Clerodendrum umbellatum* leaves are used in Africa for the treatment of intestinal helminthiasis. The aim of this study was to evaluate the *in vitro* activity of *C. umbellatum* leaves aqueous extract and derived fractions on *Schistosoma mansoni* adult worms.

Methodology: Five male and five female *Schistosoma mansoni* adult worms were incubated in each well for 48 h in a GMEM culture medium with *C. umbellatum* aqueous extract (125 to 4000 µg/mL) or its *n*-hexane, ethyl acetate and methanol fractions or the aqueous residue (62.5 to 2000 µg/mL). The main parameters assessed were the worm's mortality and the reduction of motor activity. Phytochemical screening of all our tested substances was also performed. The cytotoxicity assay using mouse melanoma liver cells line was performed on the aqueous extract and on the most active fraction.

Results: Our study shown that *C. umbellatum* leaves aqueous extract and its derived fractions promoted worm mortality. The aqueous extract disclosed a LC₅₀ of 805.21 µg/mL while the LC₅₀ of the methanol fraction was 343.10 µg/mL. With this lowest LC₅₀, the methanol fraction from *C. umbellatum* aqueous extract was therefore the most active. Moreover, it showed low level of toxicity on hepatocytes. Incubation of worms with *C. umbellatum* aqueous extract and fractions also resulted in a significant reduction of the motor activity of survival worms with a 39.54 to 100% reduction after 48h. The phytochemical screening of *C. umbellatum* aqueous extract and fractions revealed the presence of alkaloids, phenols, flavonoids, tannins, saponins and terpenoids.

Conclusion: The present study demonstrated the *in vitro* activity of *C. umbellatum* aqueous extract and derived fractions on *S. mansoni* adult worms and could then justify its empirical use to combat schistosomiasis.

Keywords: *Clerodendrum umbellatum*; schistosomicidal activity; cytotoxicity; *Schistosoma mansoni*.

1. INTRODUCTION

Schistosomiasis, also known as bilharzia, is an infectious disease caused by trematode parasites of the genus *Schistosoma*. People are at risk of schistosomiasis infection mostly in developing countries due to domestic, recreational and agricultural activities which expose them to infested water. The adverse economic and health consequences of this disease are considerable. In children it can cause anaemia, stunting and learning disability, although they can be reversed by prompt treatment. Chronic schistosomiasis in adults can affect their ability to work and lead to infertility which is irreversible. This disease covers 78 endemic countries where more than 229 million people are infected. In tropical countries, it is the second most socioeconomically devastating parasitic disease after malaria. More than 200,000 deaths per year in sub-Saharan Africa are attributable to chronic schistosomiasis [1]. Presently, there is no available vaccine against schistosomiasis and the current treatment relies on praziquantel (IUPAC name: (11*b*S)-2-(cyclohexanecarbonyl)-3,6,7,11*b*-tetrahydro-1*H*-pyrazino[2,1-*a*]isoquinolin-4-one), a low cost anthelmintic which is still the most

effective treatment. Despite its benefits, the intensive use of praziquantel has resulted in reduced cure rates and treatment failure. There are some laboratory reports on the emergence of some resistant strains of *Schistosoma* to praziquantel. Eight isolates of *Schistosoma mansoni* resistant to praziquantel chemotherapy have been in fact obtained from human hosts in Egypt [2 – 5]. It then become urgent to discover new effective schistosomicidal drugs. As a rich source of bioactive molecules, plants have provided a number of useful clinical agents. Recently, several *in vitro* studies have been performed to search for new active compounds from medicinal plants against *S. mansoni* and promising results have been reported [6 – 9]. In Cameroon, the leaves of *Clerodendrum umbellatum* Poir (Labiatae) are among the common medicinal plants used by traditional healers to treat intestinal helminthiasis [10]. Our previous study has established the *in vivo* antischistosomal activity of *C. umbellatum* leaves aqueous extract [11]. The present study was then carried out to evaluate the *in vitro* activity of *C. umbellatum* leaves aqueous extract and derived fractions against *Schistosoma mansoni* adult worms.

2. MATERIALS AND METHODS

2.1 Plant Material and Component Analysis

2.1.1 Plant material

Clerodendrum umbellatum was collected in April 2012 at the locality of Mekok, near Sangmelima in the South region of Cameroon. The plant was identified at the National Herbarium of Yaoundé, Cameroon by comparison to the specimen 7405 SRF/Cam and conserved under the voucher N° 7405.

2.1.2 Extract preparation and fractionation

Leaves were dried in the shade, powdered and mixed with distilled water (100 g / L) for 24 hours of maceration at room temperature. The solution was filtered, frozen and then lyophilized to give the aqueous extract with an extraction yield of 17%. The aqueous extract (120 g) was fractionated by liquid – liquid partition with solvents of increasing polarity: *n*-hexane, ethyl acetate and methanol. Solvents were totally removed in a rotary evaporator (BÜCHI Rotavapor R-114) at maximum temperature of 40°C. With this process, we obtained 0.31 g of the *n*-hexane fraction, 3.22 g of the ethyl acetate fraction, 31.23 g of the methanol fraction and 66.59 g of the aqueous residue.

2.1.3 Phytochemical screening

The aqueous extract and fractions were subjected to qualitative chemical tests to identify phytochemical constituents. The screening of alkaloids, anthraquinones and cardiac glycosides was performed by the Mayer's test, the Bontrager's test and the Keller-Killiani's test respectively. Ferric chloride (FeCl₃) test was used for the identification of phenols and tannins, Fehling's test for reducing sugars, foam test for saponins and Liebermann-Burchard's test for steroids. The presence of flavonoids, lipids and terpenoids was performed using the ammonia test, the grease spot test and the Salkowski's test respectively [12].

2.2 Experimental Studies with Animals

2.2.1 Experimental animals and parasite maintenance

The experiments were performed in the Centre for Schistosomiasis and Parasitology of

Yaoundé. Eight weeks old BALB/c mice, weighing 20 - 25 g were used. They were housed in polypropylene cages in the animal house with natural conditions of light/dark cycle, temperature and aeration. Mice were fed with rodents' diet and water *ad libitum*. They were individually infected with 200 cercariae of *S. mansoni* (Cameroonian strain) using the method of tail and legs immersion. Cercariae were released from experimentally infected *Biomphalaria pfeifferi* snails. After 45 days of infection, adult worms were recovered under aseptic conditions by perfusion of the liver and mesenteric veins accordingly to the method described by Pellegrino and Siqueira [13]. Adult *S. mansoni* worms (male and female) recovered from infected animals were washed three times in a Glasgow Minimum Essential Medium (GMEM G-6148) (Sigma-Aldrich, St Louis, USA) supplemented with an antibiotic-antimycotic solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B) (Sigma-Aldrich, St Louis, USA) and gentamicin (40 µg/mL) (Atlanta Biologicals, Lawrenceville, USA).

2.2.2 *In vitro* studies with *Schistosoma mansoni* adult worms

To test the effect of *C. umbellatum* leaves aqueous extract and derived fractions on *S. mansoni* adult worms, the bioassay followed the standard operating procedures developed by Ramirez et al. [14]. In our experiments, 5 male and 5 female adult worms were transferred to each well of a 24-well culture plate containing 1800 µL of complete GMEM G-6148 culture medium [GMEM buffered to pH 7.5 containing 1M of HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), 40 µg/mL gentamicin, 50 µg/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL neomycin, 2 mM of L-glutamine and 5% heat-inactivated fetal bovine serum (Sigma Aldrich, St Louis, USA)]. In the literature, concentrations from 10 µg/mL to 50 mg/mL are generally used for *in vitro* screening of plants extracts or compounds for antischistosomal activity [6,9]. In this study, lyophilized aqueous extract of *C. umbellatum* was initially dissolved in distilled water, filtered through a 0.22 µm sterile syringe filter and diluted in complete GMEM culture medium to final concentrations of 125, 250, 500, 1000, 2000 and 4000 µg/mL. The methanol fraction and the aqueous residue were also dissolved in distilled water while the *n*-hexane and ethyl acetate fractions were dissolved in DMSO (Dimethyl sulfoxide). All

fractions were diluted in GMEM culture medium to final concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/mL. The final volume of the solution was 2 mL/well and the maximum concentration of DMSO in each well was 0.5% v/v. Negative control for aqueous extract, methanol fraction and aqueous residue was GMEM while GMEM containing 0.5% of DMSO was the negative control for the *n*-hexane and ethyl acetate fractions. Praziquantel was used as the positive control at the final concentration of 10 µg/mL. Quadruplicate measurements were carried out for each concentration and two independent experiments were performed for each sample. Culture plates were kept at 37°C in a 5% CO₂ incubator; afterwards, worms were monitored at 24h and 48h to evaluate their viability and motor activity by examination under an inverted microscope (Olympus CK 2, Tokyo, Japan). A visual evaluation of the worm motility was assessed at three levels: normal motility was marked by body and guts undulatory movements; reduction of motor activity was defined as the absence of worm motility apart from gut movements and occasional movement of head and tail of schistosome; parasite death was defined as the absence of motor activity during 2 minutes [14]. The median lethal concentration (LC₅₀) was calculated after 48h of incubation by using the Trimmed Spearman-Kärber (TSK) method [15], version 1.5 software downloaded from the US Environmental Protection Agency.

2.3 Cytotoxicity Assay

Cytotoxicity of the aqueous extract of *Clerodendrum umbellatum* and its more active fraction was investigated on C57/L mouse melanoma liver cells line (*Hepa 1-6*, ATCC CRL-1830) using WST-8-based assay. The cells were cultured in high-glucose Dulbecco's Minimal Essential Medium (DMEM) with pyruvate and L-glutamine (Gibco, Life Technologies, USA), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) heat inactivated (Serana, Australia) and 1% (v/v) penicillin/streptomycin (Gibco, Life Technologies, USA). The cells were grown in growth medium at 37°C in a 95% air, 5% CO₂-humidified incubator. Monolayer culture reaching a confluence between 80-90% where detached using trypsin solution (Sigma-Aldrich, Germany) and calibrated with a cell counter (Fast Read 102). Calibrated cell suspension was seeded into 96-well tissue culture microtiter plates at a density of 1×10^4 cells per well and incubated overnight at 37°C in a 5% CO₂ incubator for cell

adhesion. Following incubation, the medium was removed from the cells and replaced with fresh one follow by the addition of the extract / fraction at different concentrations (15.625, 31.25, 62.50, 125, 250, 500, 1000 µg/mL). Control wells with cells only were added and the plates were incubated for 24h in the same culture conditions. After incubation, cell viability was measured by mitochondrial activity in reducing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt to formazan using the Cell Counting kit-8 (WST-8, abcam, ab228554, UK) according to the manufacturer instructions. The assays were performed in triplicate. After 4 hours incubation, optical density was measured at 450 nm using Dynex MRX TC II (Dynex Technologies, USA) microplate reader. The results were expressed as a percentage growth of the control cells and IC₅₀ values were calculated using the Trimmed Spearman-Kärber (TSK) method [15].

2.4 Statistical Analysis

Results were expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test, performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, USA). The level of significance was set at $p < 0.05$.

3. RESULTS

3.1 Phytochemical Screening

The qualitative phytochemical analysis showed that alkaloids and tannins were present in *C. umbellatum* leaves aqueous extract and all its derived fractions. Lipids and steroids were present only in the *n*-hexane and ethyl acetate fractions. Anthraquinones were absent in both the aqueous extract and derived fractions. The aqueous extract and its methanol fraction have practically the same chemical constituents (Table 1).

3.2 Mortality of Schistosomes

The mortality rate of adult *S. mansoni* worms following exposure to different concentrations of *Clerodendrum umbellatum* aqueous extract and derived fractions is shown on Fig. 1. After 24 h of incubation, all parasites died after being exposed to the *n*-hexane and the methanol fractions at the highest concentration of 2000 µg/mL. No death was recorded neither in the aqueous extract nor

in the other fractions. After 48 h of incubation, we recorded a significant concentration-dependent increase of mortality ($P < .001$) of adult *S. mansoni* worms incubated with the aqueous extract and relative fractions. Mortality rates varied from 33.52 to 72.04% in the aqueous extract (250 to 4000 $\mu\text{g/mL}$). In the ethyl acetate fraction and aqueous residue, highest mortality rates of 51.48% and 55.91% were recorded at the highest concentration of 2000 $\mu\text{g/mL}$. For the methanol fraction, 87.50% of worms died at the concentration of 1000 $\mu\text{g/mL}$. Incubation of worms with aqueous extract at 125 $\mu\text{g/mL}$, the *n*-hexane fraction from 62.5 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$ and the aqueous residue at 62.5 $\mu\text{g/mL}$, did not promote any worm death. No death was observed in the negative control groups GMEM and GMEM + 0.5% DMSO. In the positive control group incubated with praziquantel (10 $\mu\text{g/mL}$), all parasites died within 24 h of incubation.

The evaluation of the median lethal concentration (LC_{50}) of *C. umbellatum* aqueous extract and fractions, using the Trimmed Spearman Karber method, disclosed the methanol fraction as the most active substance with a LC_{50} of 343.10 $\mu\text{g/mL}$ (266.76 – 441.28), followed by the

aqueous extract with a LC_{50} of 805.21 $\mu\text{g/mL}$ (Table 2).

3.3 Motor Activity of Schistosomes

The reduction of motor activity of surviving worms treated with *C. umbellatum* leaves aqueous extract and derived fractions was marked by the absence of worm motility apart from gut movements, a minimal motor activity marked by weak movement of the suckers and an occasional sway of the body (Fig. 2). In fact, after 24h of incubation, worms treated with *C. umbellatum* aqueous extract, ethyl acetate fraction, methanol fraction or the aqueous residue, at all concentrations, showed significant reduction ($P < .05$, $P < .01$ and $P < .001$) of their motor activity when compared to their negative control groups. This reduction was although not concentration-dependent. The worm motility was not affected in the negative control groups (GMEM medium and 0.5% DMSO + GMEM medium). The same observation was done in the *n*-hexane fraction group where adult *S. mansoni* worms showed normal motor activity marked by undulatory movements of the body and peristaltic waves along the body.

Table 1. Phytochemical screening of *Clerodendrum umbellatum* leaves aqueous extract and derived fractions

| Chemical constituents | Aqueous extract | <i>n</i> -Hexane fraction | Ethyl acetate fraction | Methanol fraction | Aqueous residue |
|-----------------------|-----------------|---------------------------|------------------------|-------------------|-----------------|
| Alkaloids | + | + | + | + | + |
| Phenols | + | - | - | + | + |
| Flavonoids | + | - | - | + | - |
| Saponins | + | - | + | + | - |
| Condensed tannins | + | - | - | + | - |
| Hydrolysable tannins | - | + | + | - | + |
| Terpenoids | + | - | - | + | + |
| Reducing sugars | + | + | - | - | - |
| Lipids | - | + | + | - | - |
| Steroids | - | + | + | - | - |
| Anthraquinones | - | - | - | - | - |
| Cardiac glycosides | + | - | - | + | + |

(+) = presence (-) = absence

Table 2. Median lethal concentration (LC_{50}) values of *Clerodendrum umbellatum* leaves aqueous extract and derived fractions after forty-eight (48) hours of incubation

| <i>Clerodendrum umbellatum</i> | LC_{50} ($\mu\text{g/mL}$) | 95% low limit ($\mu\text{g/mL}$) | 95% upper limit ($\mu\text{g/mL}$) |
|--------------------------------|---------------------------------------|------------------------------------|--------------------------------------|
| Aqueous extract | 805.21 | 579.85 | 1118.15 |
| <i>n</i> -Hexane fraction | 1414.21 | Not reliable | Not reliable |
| Ethyl acetate fraction | 1855.87 | 1010.05 | 3409.98 |
| Methanol fraction | 343.10 | 266.76 | 441.28 |
| Aqueous residue | 1723.33 | 1388.02 | 2139.63 |

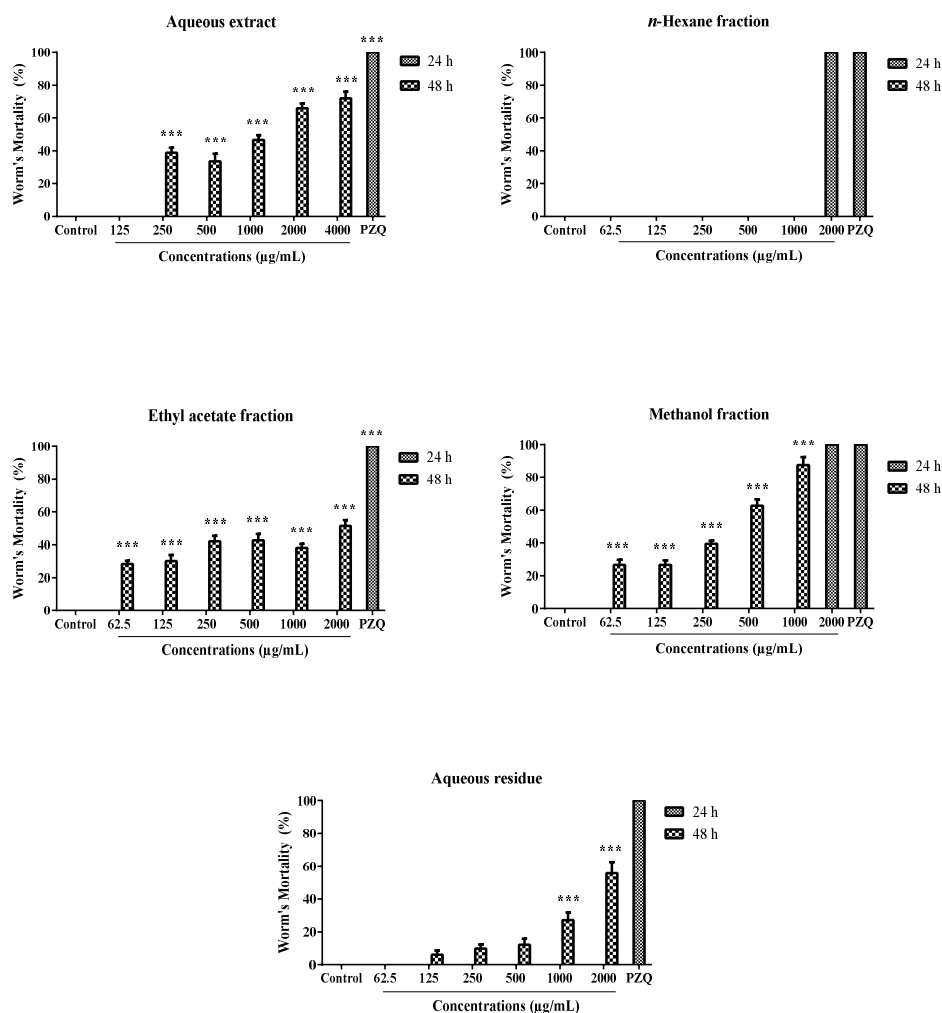


Fig. 1. In vitro effect of *Clerodendrum umbellatum* leaves aqueous extract and derived fractions on the mortality of *Schistosoma mansoni* adult worms after twenty-four (24) hours and forty-eight (48) hours of incubation

All values are expressed as mean ± SEM

***values are significantly different from controls (GMEM or GMEM + 0.5% DMSO) at $P < .001$
GMEM: Glasgow Minimum Essential Medium PZQ: praziquantel

After 48h of incubation, the reduction of motor activity reached 94.38% for all the worms exposed to 2000 µg/mL of *C. umbellatum* aqueous extract and was also significant ($P < .001$) at others concentrations excepted at 125 µg/mL. This period of incubation induced a significant reduction of motor activity by 58.38% in worms treated only with 1000 µg/mL of the *n*-hexane fraction. In the ethyl acetate group, the reduction of worm motor activity (41.07 to 74.11%) was significant ($P < .001$) at all concentrations and a 100% reduction was recorded for the methanol fraction at 1000

µg/mL. In the aqueous residue group, the reduction of motor activity progressed from 11.02 % to 70.52 % at the concentration of 2000 µg/mL. In the negative control groups, a slight reduction of the worm motility was recorded: 13.41% for the GMEM and 3.64% for the GMEM + 0.5% DMSO.

3.4 Cytotoxicity Assay

We assessed the cytotoxicity of *Clerodendrum umbellatum* aqueous extract and its methanol fraction which was the most active against *S.*

mansoni adult worms. The IC₅₀ for the mouse melanoma liver cells were 410.823 ± 32.93 µg/mL and 876.88 ± 18.14 µg/mL for the aqueous extract and the methanol fraction respectively.

4. DISCUSSION

Drug discovery is a complicated iterative process that is intimately linked to chemistry, but which is increasingly driven by biological sciences. A great deal of new drug discovery against

Schistosoma mansoni is dependent on *in vitro* and *in vivo* whole parasite screens [14]. The antischistosomal activity of *C. umbellatum* leaves aqueous extract was established in our previous study [11]. Since there is a need for searching new active compounds against *Schistosoma* species, the present study was carried out to evaluate the *in vitro* activity of *C. umbellatum* leaves aqueous extract and derived fractions at different concentrations against *Schistosoma mansoni* adult worms.

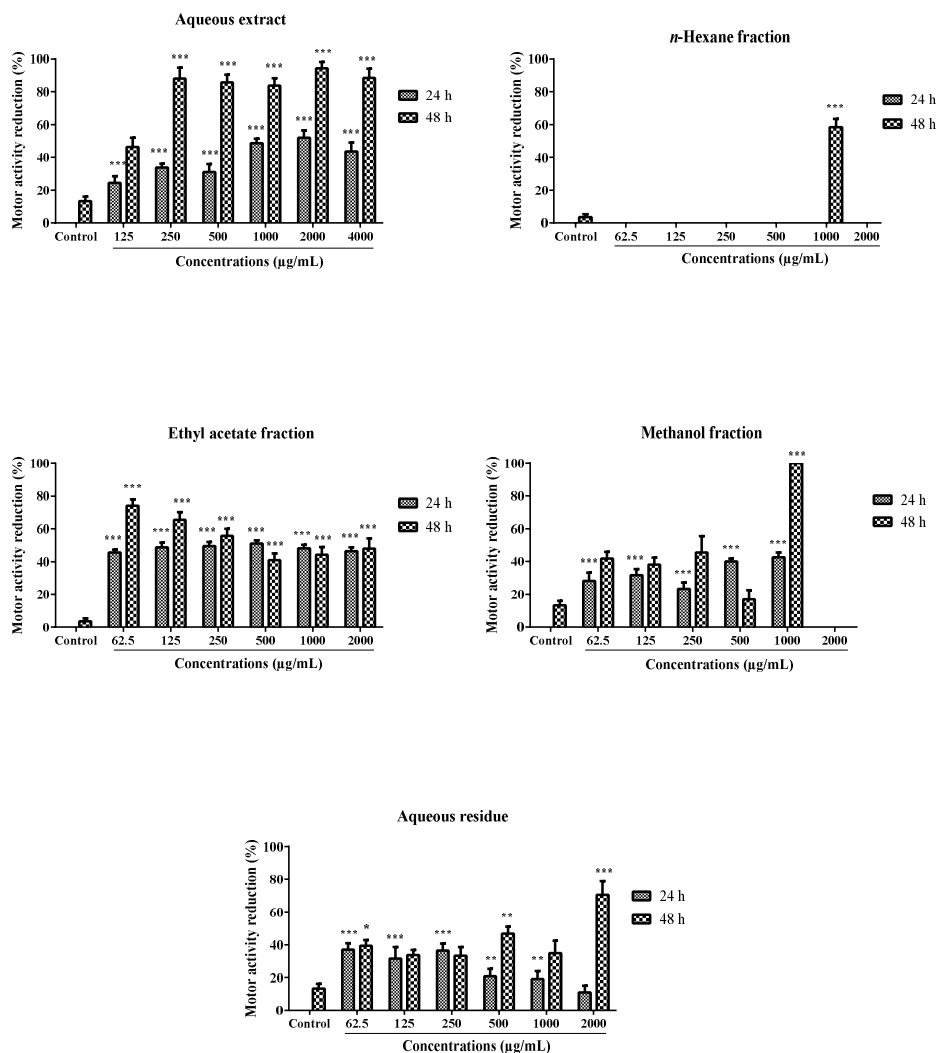


Fig. 2. *In vitro* effect of *Clerodendrum umbellatum* leaves aqueous extract and derived fractions on the motor activity of *Schistosoma mansoni* adult worms after twenty-four (24) hours and forty-eight (48) hours of incubation

All values are expressed as mean ± SEM

*, **, *** values are significantly different from controls (GMEM or GMEM + 0.5% DMSO) at P < .05, P < .01 and P < .001 respectively

GMEM: Glasgow Minimum Essential Medium

The first parameter evaluated was adult *Schistosoma mansoni* mortality. Our assay showed that the aqueous extract and various fractions were active against *S. mansoni*. Lethal concentration of 2000 µg/mL recorded with both the *n*-hexane and methanol fractions after 24h of exposure, was in the range of lethal concentrations of medicinal plant extracts and / or isolated compounds used against *Schistosoma mansoni* adult worms, such as curcumin isolated from *Curcuma longa* rhizome [16]; phloroglucinol derivatives from the rhizome of *Dryopteris* species [17]; *Sida pilosa* aqueous extract and derived fractions [18]. The activity of *C. umbellatum* extract and fractions on worm mortality was concentration-dependent in particular for the aqueous extract and the methanol fraction. This could be explained by the fact that the antischistosomal effect of their bioactive compounds increases with their concentrations. Concerning the median lethal concentration (LC₅₀), the methanol fraction was the most active one with LC₅₀ of 343.10 µg/mL, followed by the aqueous extract. This means that the fractionation of *C. umbellatum* leaves aqueous extract improved its *in vitro* schistosomicidal activity on adult worms. Jatsa et al. obtained similar result in their study in which the *n*-butanol fraction was more effective than the aqueous extract of *Sida pilosa* [18]. The methanol fraction also shown a low level of cytotoxicity on mouse melanoma liver cells with an IC₅₀ of 876.88 ± 18.14 µg/mL which is greater than its schistosomicidal effective concentration. On the contrary, the IC₅₀ of the aqueous extract on the growth of the mouse melanoma liver cells (410.823 ± 32.93 µg/mL) was inferior to its effective concentration on worm viability (805.21 µg/mL), thus revealing a certain level of cytotoxicity. The phytochemical screening revealed the presence of secondary metabolites such as alkaloids, flavonoids, phenols, tannins and terpenoids. The schistosomicidal activity of alkaloids has been proved by Miranda et al. [19]. Many alkaloids display their activity by causing disruption of the tegument of *S. mansoni* adult worms [20]. Since praziquantel is an isoquinoline pyrazine derivative, our tested substances could induce their schistosomicidal activity in the same way as praziquantel. In fact, it causes schistosome adult worm paralysis and death probably through the modification of the function of voltage operated Ca²⁺ channels (VOCC) whose play a critical role in regulating the levels of intracellular Ca²⁺ and are essential for a variety of parasite cellular events, including contraction, gene expression and

neurotransmitter release [21]. Terpenoids disclose a very broad range of biological activities including schistosomicidal activity. Some terpenoids are known to kill adult *S. mansoni* worms and cause complete separation of paired worms with tegumental disruption in worms [22].

Motor activity is an indicator of biological activity of schistosome species. Absence of motility apart from guts movement, feeble motor activity and reduction of peristaltic waves along schistosomes' body after incubation in *C. umbellatum* aqueous extract and derived fractions could be the consequence of the plant interference with the mechanism of contraction-relaxation of worm smooth muscles [23]. The reduction of worm motor activity was also reported after incubation of schistosomes with essential oil of *Ageratum conyzoides*, curcumin, phloroglucinol derivatives from *Dryopteris*, *Sida pilosa* aqueous extract and fractions and *Ozoroa pulcherrima* methanolic extract and fractions [9, 16-18, 24] and also with isolated compounds like alkyl-phenols, arctiin and primin [25-27]. Flavonoids are not able to kill the worms, but they can exhibit significant reduction in motor activity or pairing of the *S. mansoni* adult worms [20]. Flavonoids were identified as selective inhibitors of the *S. mansoni* NAD⁺ catabolizing enzyme (SmNACE), an important target located in the outer tegument of the adult worm and it is presumably involved in the parasite survival by manipulating the host's immune regulatory pathways [28]. It appears that the presence of phenols and flavonoids in *C. umbellatum* aqueous extract and methanol fraction could explain their remarkable activity against *S. mansoni* adult worms motor activity. In this study, the activity of *C. umbellatum* extract and fractions on the reduction of worms' motor activity was time-dependent. A possible explanation could be the increase of the number of voltage operated Ca²⁺ channels (VOCC) and SmNACE receptors recruited by the fixation of bioactive compounds during the incubation period.

5. CONCLUSION

This study indicated that *Clerodendrum umbellatum* aqueous extract and its derived fractions possess *in vitro* schistosomicidal activity against *Schistosoma mansoni* adult worms. The methanol fraction disclosed the lowest LC₅₀ and is therefore the most active fraction. It also shown low level of cytotoxicity on mouse melanoma liver cells. This fraction could be

considered as a promising source for schistosomicidal compounds. Further studies are needed to isolate compounds present in this fraction. There is also a need to investigate *in vivo* toxicity and antischistosomal activity of this fraction.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was presented and validated by the scientific committee of the laboratory of animal physiology, faculty of sciences, university of yaoundé i – cameroon, which follows the internationally accepted standard ethical guidelines for laboratory animal use and care described in the european parliament and council of the european union directive 2010/63/ue of 22th september 2010.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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