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Full Length Research Paper

In vitro antigiardial activity of *Citrullus lanatus* Var. *citroides* extracts and cucurbitacins isolated compounds

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The present study was conducted to investigate the antigiardial activities of *Citrullus lanatus* var. *citroides* (wild watermelon) fruits, petroleum ether, ethyl acetate, butanol crude extracts as well as Cucurbitacin E and Cucurbitacin L 2-O-β-glucoside pure isolated compounds from *C. lanatus* var. *citroides*. Cucurbitacin E and Cucurbitacin L 2-O-β-glucoside were revealed to have strong potent antigiardial activity against *Giardia lamlia in vitro* with IC_{50}= 2 and 5 ng/ml after 5 days respectively. The ethyleacetate extract was the best among all examined extracts followed by petroleum ether and butanol with IC_{50} 0.1, 0.2 and 0.5 µg/ml respectively. The results suggest that all the crude extracts and isolated compounds were active against *G. lamblia,* hence *C. lanatus* var. *citroides* may be recommended as new source for the treatment of giardiasis.

Key words: *Citrullus lanatus* var. *citroides, Giardia lamlia,* Cucurbitacin E, Cucurbitacin L 2-O-β-glucoside.

INTRODUCTION

Wild melon (*Citrullus lanatus* var. *citroides* Cucurbitaceae) is low climbing, hairy and annual plants. In Sudan, it is found commonly on sandy or clay soils in savannah zone of central Sudan, Darfur, Kordofan, Red Sea and northwards to Khartoum (Loiy, 2009). The fruit is also diuretic, being effective in the treatment of dropsy and renal stones (Chiej, 1984). The rind of the fruit is prescribed in cases of alcoholic poisoning and diabetes (Duke and Ayensu, 1985). Cucurbitaceae plants are known to contain bioactive compounds such as cucurbitacin, triterpenes, sterols and alkaloids. Plants containing cucurbitacin were earlier recognized in folk medicine to have biological values. Scientific studies mainly refer to Middle East and Asia where cucurbit plants were actively used as herbal remedies. Cucurbit plants demonstrated anti-inflammatory, antitumor, liver protective and immunoregulatory activities (Ram, 1999; et al., 1982). Cucurbitacins are a group of highly oxygenated steroidal triterpenes characterized by the cucurbitane skeleton. Aglycons isolated from various species have been given the general name of cucurbitacin. Various letters in consecutive order of isolation follow this name, although in certain cases other names have been given (Lavie and Glotter, 1971). Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis (Upcrof et al., 2006); hence new antigiardiasis drugs are probably required. With the purpose of searching for new antigiardiasis agents, *C. lanatus* which was used traditionally for treatment of clinical signs associated with giardiasis and the plants was selected to evaluate the activity of their ethyl acetate, butanol and petroleum ether crude extracts against *Giardia lamblia* trophozoites *in vitro*.

MATERIALS AND METHODS

Plant materials and extraction

The plant used in this study was *C. latanus* var. *citriode* collected

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from AL- Musawarat, Northern Sudan, collected on February 2006. The taxonomic identification of this plant was carried out at Medicinal and Aromatic Plants Research Institute, National Center for Research by W.E.A/Alla. A voucher specimen was deposited at the herbarium of the institute. Fruits were cut into thin slices and dried at room temperature; seeds were separated and ground into a coarse powder. Fifty grams of the dried fruit were powdered and extracted using the method reported by Ciulie (1981). Dried fruit powdered extracted with petroleum ether, the filtrates were collected together and the residue was brought to dryness and extracted with chloroform, and lastly extracted with ethanol, which was modified to aqueous extract, the latter was extracted successively with equal volumes of two organic solvents of increasing polarity (ethyl acetate and butanol) (Harbone, 1984).

Isolation of compounds from *C. lanatus* var. *citroides* fruits pulps

Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography was performed on column (25 × 15 cm) packed with silica gel of particle size (0.04 to 0.06 mm) (60 to 120 mesh) and compacted with vacuum and pressing ten times. The crude extract of ethyl acetate (12 g) was subjected to (VLC) and the concentrated sample was applied on the wall of the column (Harbone, 1984). The elution used to fractionate ethyl acetate crude extract (12 g) was chloroform: methanol mixture of increasing polarity. Portions of 100 ml were collected, combined on the basis of TLC analysis using solvent system chloroform: methanol (9.5: 0.5, 9: 1 and 8: 2). Eight fractions were obtained, fraction five (5 g) was semi-pure, which was subjected to column chromatography, on a glass column (36 × 3.5 cm) packed with silica gel (115 g) of particle size (0.04 to 0.063 mm), and crystallized to give Compound (1) (Eth.c60) (300 mg). Fraction six (3 g) also was semi-pure, it was purified throw small column using the same eluent to give compound (2) (EA VLC 42-46) (400 mg).

Analytical techniques

Infra red (IR) spectroscopy

The IR spectra were recorded on Perkin-Elmer model 1650 FTIR spectrophotometer using 50 mg KBr and 1 mg of the isolated compounds. The following abbreviations were reported, S (strong), M (medium) and W (week) (Nakanishi and Solomon, 1977).

Nuclear magnetic resonance (NMR) spectra

^{1}H NMR spectra

These were recorded on NMR: Bruker Avance 400 spectrometer apparatus. Samples were dissolved in deuteriochloroform ($CDCl_3$) or deuterioacetone (CD_3COCD_3) using tetramethylsilane, as an internal standard (0.00 ppm). Data were presented in following order; chemical shift relative to tetramethylsilane, multiplicity and intensity as to the number of protons; coupling constant J; assignment (if appropriate). The following abbreviations were adopted; s (single); d (double); t (triplet); m (multiplet); dd (double of doublets) signal (Mohan, 2001).

^{13}C NMR spectra

These were recorded at 100 MHz instrument. Tetramethylsilane was used as a internal standard (0.00 ppm). Chemical shits were reported relative to tetramethlysilane, assignments were based on the multiplicities and chemical shifts. Multiplicities were determined from polarization transfer technique (DEPT) or from direct response to C-H couplings (APT) (Mohan, 2001).

Mass spectrometry

Electron impact mass spectra (EI- MS) were recorded on Finnigan MAT 31 mass spectrometer with a MATSPECO Data System. Peak matching and field desorption (FD-MS) experiments were performed (Sparkman, 2009).

Melting point

Melting point was measured using electrothermal melting point apparatus model No. 1A6304.

Parasite isolate

G. lamblia used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All positive samples were examined by wet mount preparation. Then the positive sample was transported to the laboratory in nutrient broth medium. Trophozoites of *G lamblia* were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ± 1°C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

In vitro susceptibility assays

In vitro susceptibility assays used the sub- culture method of Cedilla et al. (2002), which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *Entamoba histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* (Arguello *et al*., 2004). 5 mg from each extract and compound was dissolved in 50 µl of dimethyl sulfoxide (DMSO) at Eppendorf tube containing 950 µl D.W in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20°C for further analysis. Sterile 96-well microtite plate was used for different plant extracts, positive control and negative control.

Three out of 8 columns of microtitre plate wells (8 columns × 12 rows) were chosen for each extract, 40 µl (micro-liters) of an extract solution (5 mg/ml) were added to the first column wells C-1: On the other hand , Twenty µl of complete RPMI medium were added to the other wells the second column and third column (C-2 and C-3) . Serial dilutions of the extract were obtained by taking twenty µl of extract to the second column wells and taking 20 µl out of the complete solution in C-2 wells to C-3 wells and discarding 20 µl from the total solution of C-3 to the remaining 20 µl serial solutions in the successive columns. 80 µl of culture medium was complemented with parasite and added to all wells. The final volume in the wells was 100 µl.

In each test metronidazole (a trichomonocide) pure compound [(1-(2-hydroxyethl)-2-methyl-5 nitroimidazole], a was used as positive control in concentration 312.5 µg/ml, whereas untreated cells were used as a negative controls (culture medium plus trophozoites). For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was determined with haemocytometer three times for counting after 0, 24, 48, 72 and 96 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

$$\text{Mortality of parasite (\%)} = \frac{(\text{Control negative} - \text{tested sample})}{\text{Control negative}} \times 100$$

Table 1. IR spectral data of Cucurbitacin E (Compound 1).

Assignments	Wave length of (34-35.60) peaks(cm^{-1})
C-OH out-of-plane bend	14.26
C-H out-of-plane bend	1076,1028
C-H in-plane bend	1263.53
CH3 symmetric bend	371.89
CH3 asymmetric bend	1454.78

Only 100% inhibition of the parasite considered, when there was no motile parasite observed.

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program. Student t test was used to determine significant difference between control and plant extracts at level of $P < 0.05$.

RESULTS

Characterization of Compound (1)

Compound (1) was obtained as an amorphous brown powder from ethyl acetate extract. It has R_f value 0.47 in solvent system ($CHCl_3$: MeOH (85:15)). It developed brown colour with vanillin reagent. M. P. 1002.66. IR spectrum showed absorption at: 3434 cm^{-1} (OH) b, 2929 cm^{-1} (C-H), 1686 cm^{-1} (C=O) v. s, 1637 (C=C) m. s, 1076 (C-O) m. s. (Table 1). The ^{1}HNMR spectrum of Compound (1) displayed two singlet each of three protons integrations at δ 1.48 ppm two at 1.31 ppm, two at 1.04 ppm and one at 1.37 ppm indicated the presence of 7 methyl groups (Table 2). ^{1}H-NMR (400 MHz $CDCl_3$), 1.04 s (3 H), 1.87 d (1 H) J= 8 Hz. 2.05 (2 H) J= 16 Hz, 5.75 s (1 H), 4.08 d (1 H) J= 12 Hz, 2.6 s (3 H). 1.85 d (2 H) J= 8 Hz, 2.18 d (1 H) J=20 Hz, 1.3 s (3 H), 1.87 d (2 H) J= 8 Hz, 1.6 d (2 H) J=28 Hz, 3.23 m (1 H) 3.57 s (1 H), 1.90 s (1 H), 1.37 s (3 H), 3. 6 s (3 H), 7.02 s (3 H), 1.43 s (3 H), 2.25 s (3 H). ^{1}H- and ^{13}C NMR was presented in Table 2.

EI-MS m/z: 558.32 which was confirmed by HREI-MS to give a molecular formula $C_{32}H_{44}O_8$. The NMR data of this compound were identical to those of Cucurbitacin E as previously reported; ^{1}H NMR (Lavie et al., 1962) and ^{13}C NMR (Valde and Lavie 1983). Consequently, the structure of Compound (1) was identified as Cucurbitacin E (Figure 1). Cucurbitacins are obtained originally from Cucurbitaceae and are cytotoxic triterpenoid substances. Series of cucurbitacin cognates were identified and their pharmacological effects, such as anti-tumor, purgative, anti-inflammatory and antifertility activities have also been reported (Chen et al., 2005). It has been reported that Cucurbitacin E possesses anti-tumor activity and caused alterations in cell morphology by disrupting actin cytoskeleton (Duncan et al., 1996).

Characterization of Compound (2)

Compound (2) (VLC42-46) was obtained as an amorphous powder from ethyl acetate extract of the fruit pulp. It has the following characteristics: R_f value 0.47 in solvent system ($CHCl_3$: MeOH (85:15)), developed purple colour with vanillin reagents. It dissolved in acetone and MeOH. IR spectrum showed absorption at: 3402 cm^{-1} (OH), 2901 cm^{-1} (C-H), 1638.34 cm^{-1} (C=C) (Table 3). The ^{1}H and ^{13}C NMR spectra showed the presence of one sugar moiety, it was identified as a β-glucopyranosyl a terminal unit, with Compound (2). Enzymatic hydrolysis of Compound (2) with γ-amylase gave Cucurbtacin L, confirming that the β-glucopyranosyl unit is a sugar connected to a ß-glucopyranosyl unit at C-2. Furthermore, the ^{13}C NMR spectrum also revealed the attachment of a terminal sugar to C-2 of a β-glucopyranosyl unit due to the downfield shift of this atom (+9.4 ppm) and upfield shift of C-30 (-0.9 ppm) (Table 4). EI-MS m/z: 676.35 which was confirmed by HREI-MS to give a molecular formula of $C_{36}H_{52}O_{12}$. Consequently, the structure of Compound (2) was identified as Cucurbitacin L 2-O-β-glucopyranosyl (Figure 2). Cucurbitacin L 2-O-β-glucopyranosyl previously was isolated with two other nor-cucurbitacin glycosides from the root of specimen of *Wilbrandia* sp (Cucurbitaceae) (Maria et al., 1993). From the fruits of *Trichosanthes tricuspidata* (Cucurbitacaeae), 14 cucurbitane glycosides were isolated along with Cucurbitacin 2-O-β-glucopyranoside. Structural elucidations were based on chemical and spectroscopic analyses (Tripetch et al., 2002).

The activity of petroleum ether extract of *C. lanatus* showed increase in mortality with the increase in susceptibility period for all concentrations (125 to 500 ppm), however, after 120 h it gave 90% mortality for the highest concentration (500 ppm), while the other concentrations gave 82 and 87% mortalities for 125 t0 250 ppm respectively, on the other hand metrondizole (the reference control) shown 100% mortality at the same time (Figure 3). In contrast, no culture growth was observed after 96 h for 500 ppm concentration of *C. lanatus* in ethyl acetate extract. However, at other

Table 2. ^{13}C and ^{1}H-NMR spectral data for Cucurbitacin E (Compound 1).

Serial number	Carbon atom No.	Type	Δc (ppm)	δH (ppm)
1	30	CH3	18.6	1.04
2	18	CH3	19.2	1.04
3	32	CH3	21.4	2.35
4	21	CH3	25.3	1.37
5	28	CH3	27.1	1.31
6	29	CH3	27.1	1.31
7	26	CH3	27.5	1.48
8	27	CH3	27.5	2.48
9	7	CH2	24.4	2.05,1.96
10	1	CH2	35.3	1.85,1.60
11	10	CH2	48.3	2.18
12	9	C	48.4	-
13	13	C	50.0	-
14	14	C	50.4	-
15	4	C	48.6	-
16	17	CH	59.6	1.9
17	16	CH2	71.0	3.23
18	2	CH	71.3	4.08
19	25	C	80.3	-
20	20	C	81.5	-
21	12	CH2	48.3	1.86,1.60
22	24	CH	121.6	7.02
23	25	C	80.3	-
24	6	CH	122.8	5.75
25	5	C	140.5	-
26	31	C	170.2	-
27	11	C	213.2	-
28	3	C	213.4	-
29	23	C	204.5	5.68
30	22	CH	155.4	7.01
31	16	C	200.6	-

concentrations of 250 and 125 ppm, the cells were viable even after 120 h of incubation, the high concentrations of the extract showed mortality higher than the positive control (metrondizole) with mortality of 62, 81 , 85 and 100% after 24, 48, 96 h, respectively, while, metrondizole exerted 100% mortality just at 120 h (Figure 4). Figure 5, shows the activity of butanol extract of *C. lanatus*. The high dose of extract shows 90% mortality only after 120 h, however, all the concentrations showed mortality around 80% after 72 h, while metrondizole showed high mortality after 24 h. Figure 6 shows the activity of Cucurbitacin-E. The highest dose of compound gave 100% mortality after 96 h, this was almost similar to the result recorded with metrondizole, which exhibited about 92% mortality at the same time. As shown in Figure 7, the activity of Cucurbitacin L 2-o-β-glucoside compound, all the cells were alive at all concentrations (47.5,95 and 190 ppm) after 120 h, while in the control positive they gave 100% mortality at the same time. It relatively reported high mortality rates even in low concentration (7.5 ppm), while it exerted 64, 60, 75, 86% mortality after 24, 48, 72, 96 h, respectively.

DISCUSSION

G. lamblia is one of the most common intestinal pathogenic protozoan parasites (Newman et al., 2001). It is becoming increasingly important among HIV/AIDS patients. There are reports that some cases of acute and chronic diarrhea in AIDS patients may be associated with giardial infection (Merchant and Shroff, 1996). However, metronidazole, the common drug of choice, can cause mutagenicity in bacteria (Legator et al., 1975) and is carcinogenic in rodents (Rustia and Shubik, 1972). It also possesses undesirable side effects and treatment failures

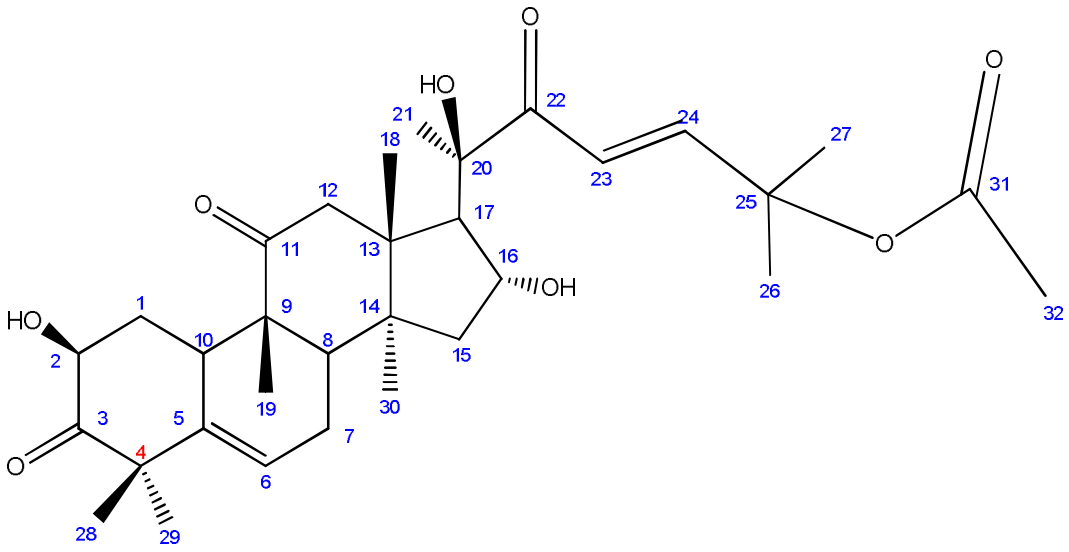

Figure 1. Cucurbitacin E compound (1).

Table 3. IR spectral data of Compound (2).

Wave length (cm⁻¹)*	Assignments	Wae length of (VLC42-46) peaks(cm⁻¹)
610	C-OH out-of-plane bend	613.64
970, 940	Ring stretching	939.44
1045, 1015	C-H out-of-plane bend	1040,1018
1140, 1120, 1090	C-C-C in-plane bend	1076.61
1180	C-H in-plane bend	1158.8
1260, 1240	C-H in-plane bend	1263.86
1360	CH 3 symmetric bend	1372.13
1440	CH 3 asymmetric bend	1460.29

Table 4. ^{13}C and ^{1}H-NMR spectral data Compound (2) (VLC42-46).

Serial number	Carbon atom No.	Type	Δc (ppm)	δH (ppm)
1	25	C	71.6	-
2	18	CH3	19.2	1.04
3	30	CH3	25.3	1.36
4	31	CH3	25.3	1.36
5	26	CH3	25.3	1.38
6	23	CH	121.6	4.28
7	5	C	137.8	-
8	13	C	49.3	-
9	3	C	199.3	-
10	9	C	49.3	-
11	6	CH	120.4	4.27
12	4	C	48.8	-
13	1	CH	150.0	5.45
14	2	C	145.4	-
15	17	C	51.2	-
16	7		CH2	24.6

Table 4. Contd.

17	28	CH3	22.5	1.56
18	8	CH	44.5	2.53
19	29	CH3	24.5	1.78
20	19	CH3	19.5	1.51
21	24	CH	155.7	5.73
22	20	C	79.9	-
23	27		CH3	25.3
24	10	CH	35.6	1.83
25	21	CH3	21.1	1.90
26	11	C	212.3	-
27	14	C	45.1	-
28	12	CH2	47.1	1.79
29	15	CH2	40.3	1.83
30	22	C	203.3	-
31	16	C	200.6	-

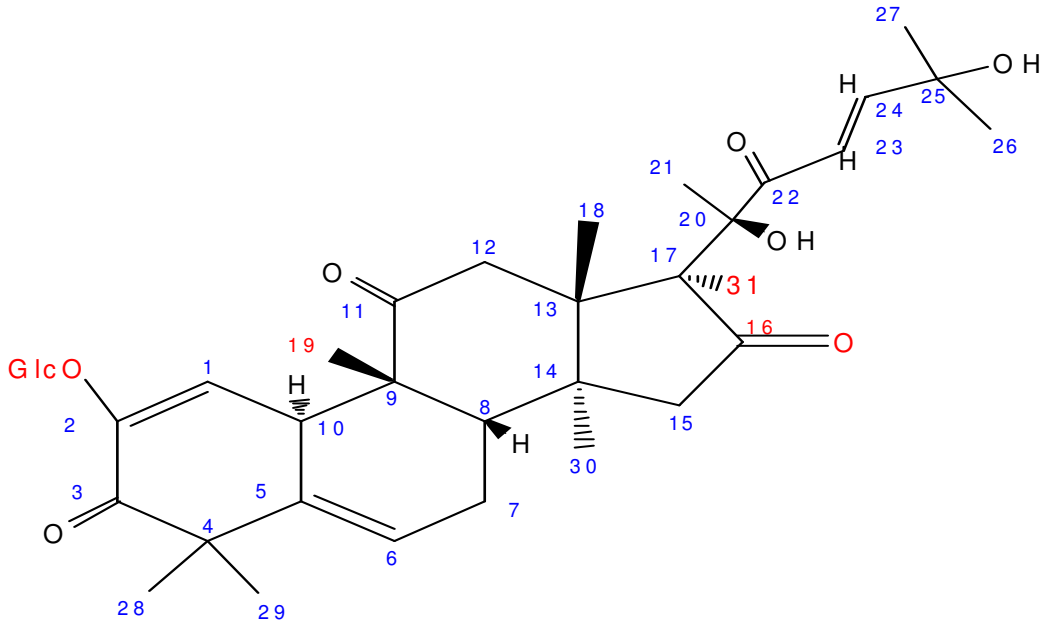

Figure 2. Cucurbitacin L 2-o-β-glucoside compound (2).

have been reported (Llibre et al., 1989). Our present *in vitro* investigation reveals promising results for the use ofthe plant for cultivated parasites to formulate such ingredient of the plant extract as the drug. However, the IC_{50} of petroleum-ether, ethylacetate and butanol extracts are 0.18, 0.15 and 0.45 ppm and their IC_{90} were found to be 432, 122 and 406 ppm respectively. While the IC_{50} and IC_{90} of Cucurbitacin-E and Cucurbitacin L 2-o-β-glucoside are 0.002, 0.005 and 15, 32 ppm respectively. Calzada et al. (2006), Moon et al. (2006), Barbosa et al. (2007) and Vidal et al. (2007) showed the effects of different plants, fractions and/or purified compounds against *G. lamblia* trophozoites. The IC_{50} values described in these studies range between 0.8 and 300 µg/ml, but the majority of plant extracts presented IC_{50} values above 100 µg/ml. Comparing it with the present data, we could conclude that the *C. lanatus* extracts and the isolated compounds could be considered as more effective than the majority of other plant extracts and their active principles. Moreover, our data agree with the recent suggestion that terpenoids rich in oxygen are of potent antigiardial activity (Machado et al., 2010). Hence

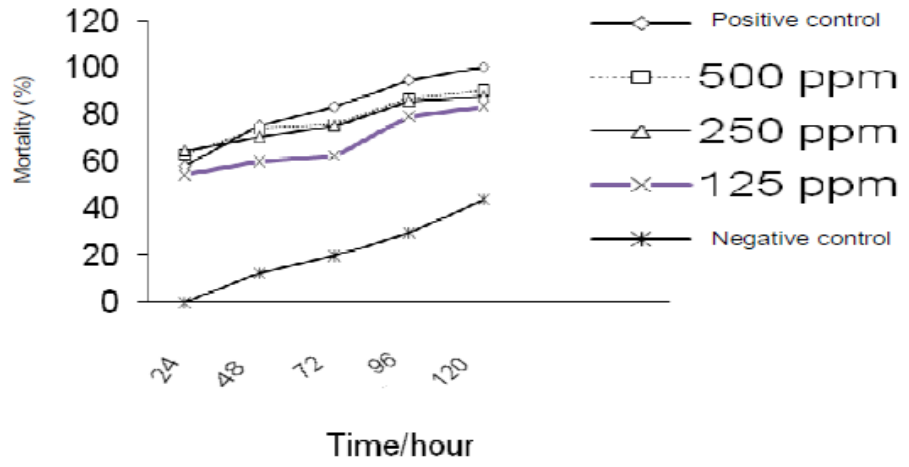

Figure 3. *In vitro* activity of *C. lanatus* petroleum ether extract against *G. lamblia.*

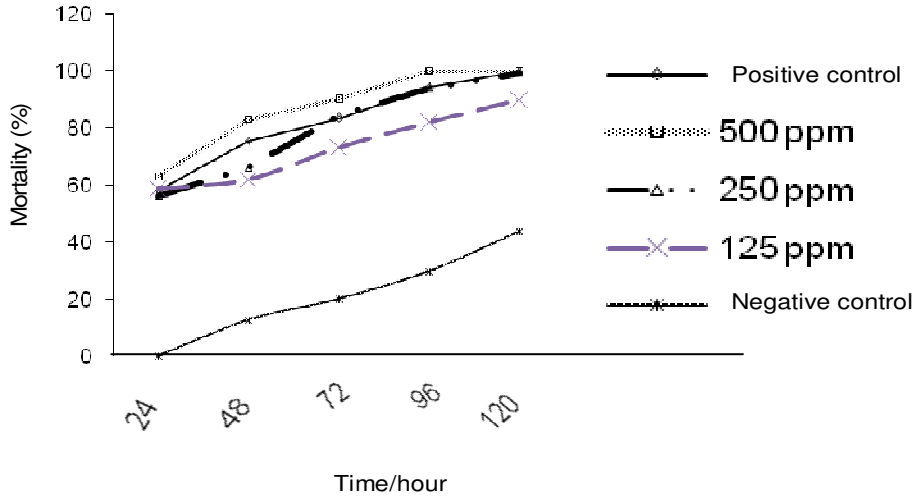

Figure 4. *In vitro* activity of *C. lanatus* ethyl acetate extract against *G. lambl.*

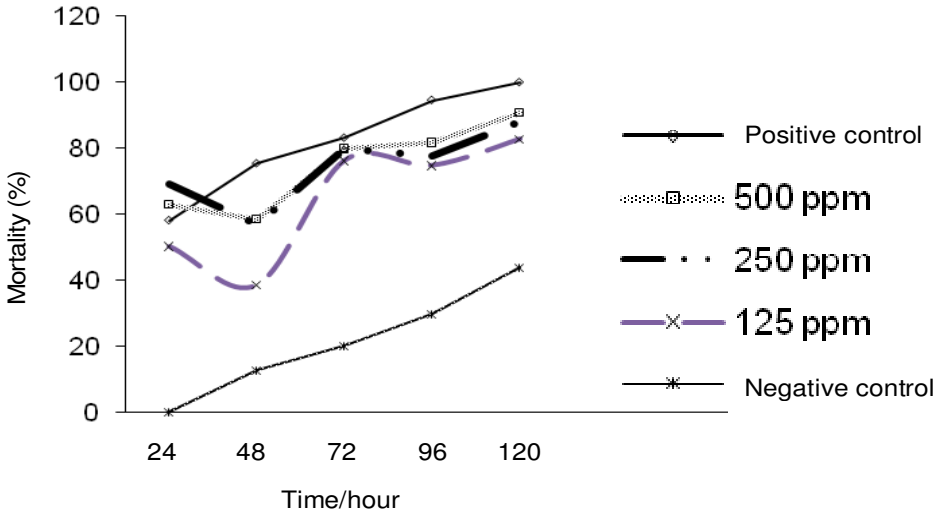

Figure 5. *In vitro* activity of *C.lanatus* butanol extract against *G. lamblia.*

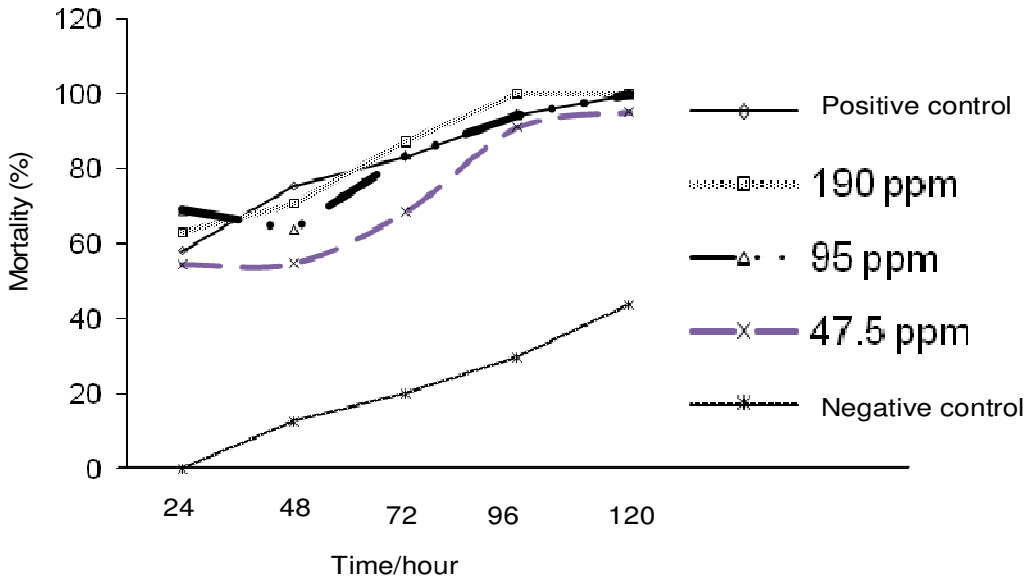

Figure 6. *In vitro* activity of Cucurbitacin-E against *G. lamblia.*

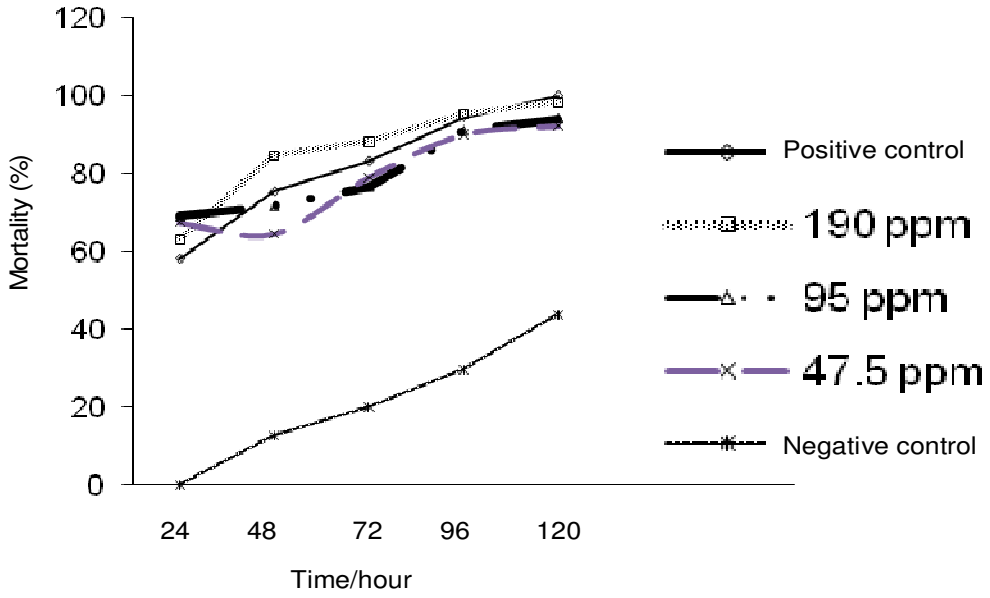

Figure 7. *In vitro* activity of Cucurbitacin L 2-o-β-glucoside against *G. lamblia.*

the present study highlights the efficiency of *C. lanatus* extracts and isolated compounds as alternative natural and chemical treatment for giardiasis.

Conclusion

This result enhances the ethno botanical uses of the plant as antidiarrheal in cases associated with giardiasis in central Sudan. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

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