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Phytochemical and Toxicity Studies of *Citrullus lanatus* var. *citroides* (Wild melon) on Brine Shrimps (lethality test)

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ABSTRACT

Background: *Citrullus lanatus* var. *citroides* (wild melon) belong to family Cucurbitaceae. It's wildly distributed in the savannah zone on sandy or clay soils of central Sudan. The fruit has been used as home remedy for alcoholic poisoning burns, swellings, rheumatism, gout and diabetes. It has dynamic pharmacological activities. **Objective**: The present study aimed to investigates toxicity of the different extracts as well as pure compounds isolated from *Citrullus lanatus* var. *citroides* (wild watermelon) on Brine Shrimps larvae. **Result:** The chloroform, ethyl acetate, butanol crude extracts and the isolated compounds were subjected to cytotoxicity activity tests. The butanol and ethyleacetate extracts were non-toxic, while chloroform extract showed high toxicity. Three compounds were isolated from the chloroform extract of the fruits. They were fully identified as β-Sitosterol, Cucurbitacin E and Cucurbitacin L 2-O-β-glucoside. The three isolated compounds showed significant toxicity, however cucurbitacin E exerted profound toxicity against brine shrimp larvae. **Conclusion**: The significant cytotoxicity of these compounds suggests the presence of antitumor compounds which could render *C. lanatus* var. *citroides* as new source of anticancer drugs.

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INTRODUCTION

Wild melon (*Citrullus lanatus* var. *citroides* Cucurbitaceae) is a low climbing, hairy and annual plant; evidence showed that it originated from the Kalahari Desert (Wasylikowa & van der Veen, 2004). In Sudan, the plant distributed in the sandy and clay soils of savannah zone of central region, Darfur, Kordofan, Red Sea and northwards to Khartoum (Hassan, Koko, Osman, Dahab, & Sirat, 2011).

The fruit known as a 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 & Ayensu, 1985). In Northern Sudan is often used for burns, swellings, rheumatism, gout and as a laxative (Hassan, Sirat, Yagi, Koko, & Abdelwahab, 2011). In central Sudan traditionally the fruit pulp is used as an antidiarrheal in cases associated with giardiasis. We previously investigated the *in vitro* antigiardial activity of *C. lanatus* var. *citroides* and isolated compounds, confirming the potent antigiardial activity of the crude extracts and isolated compounds (Hassan, Koko, *et al*., 2011).

Cucurbitaceous plants contain biologically active compounds such as cucurbitacin, triterpenes, sterols and alkaloids. Plants with high contents of cucurbitacins were early acknowledged in folk medicine to have therapeutic values. Scientific reports stated that cucurbit plants were used as herbal remedies in Middle East and Asia. Cucurbitacins proved to have extensive biological activities such as anti-inflammatory, antitumor, liver protective and immunoregulatory activities (Abdelwahab *et al*., 2011; Han, Ma, Chao, Chou, & Chung-Hua, 1979; Miro, 1995; Ram & Goel, 1999). Cucurbitacins are a class of highly oxygenated tetracyclic triterpenes, they are widely distributed in higher plants and they play a major role in protecting the plant from biological injury as heterologous chemical pheromones (Alghasham, 2013).

The pharmacological effect of bioactive compounds can be dose dependent; toxic at high

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dosage and therapeutic at lower dosage. Currently there is no *in vitro* or *in vivo* data on the toxicity of *Citrullus lanatus* var. *citroides*. *In vivo* screening method utilizing simple zoological organism such as brine shrimp is often employed in the discovery and monitoring of bioactive natural products. This *in vivo* lethality test has been used for bioassay-guide isolation of active cytotoxic and antitumor agents such astrilobacin from the bark of *Asimina triloba* and cis-annonacin from *Annona muricata* (Rieser *et al*., 1996; Zhao, Hui, Rupprecht, McLaughlin, & Wood, 1992).

MATERIAL AND METHODS

Plant materials and Extraction:

The fruits of *Citrullus lanatus* var. *citroides* were collected from AI- Musawarat, Northern Sudan, on February 2014. The taxonomic identification of this plant was carried by comparison with the herbarium specimens at Medicinal & Aromatic Plants Research Institute, National Center for Research by Wail El-Sadig. A voucher specimen was deposited at the herbarium of the institute. Fruits were chopped into thin slices and dried at room temperature; seeds were separated and grounded into coarse powder. Fifty grams of the dried fruit powder were macerated in petroleum ether, the filtrate was collected and the residue was brought to dryness followed by further extraction using chloroform and ethanol as sequential order. The ethanol extract was modified to aqueous form and extracted successively with equal volumes of two organic solvents of increasing polarity (ethyl acetate and butanol).

Toxicity test:

The toxicity test was applied by using brine shrimps, *Artemia salina* lethality test as described by Meyer (1982) (Meyer *et al*., 1982).

Brine shrimps preparation:

Instant Ocean salt (4g) was dissolved in distilled water (200 ml) and was filtered to form sea water, then transferred into a two compartment aquarium (16.0 x 9.5 x 9.5 cm) with depth 1.5 cm. One of the compartments was covered with dark paper and the other compartment was illuminated. Two spatulas of Brine Shrimp's eggs were sprinkled at the dark compartment. After 48 hours, the eggs were hatched and the brine shrimps larvae were moved towards the illuminated compartment.

Sample preparation:

Tested samples (20 mg) were dissolved in methanol (2 mL) or Dimethyle sulfoxide (for compounds hardly dissolved in methanol) to prepare a stock solution with concentration of 10000 ppm. Each sample solution was transferred into small vials by using micropipettes of 5, 50 and 500 µL to prepare series of concentrations (10 ppm, 100 ppm and 1000 ppm) in three replicates. All sample solutions were left over night at room temperature for evaporation process.

Brine shrimps transfer:

Two ml of sea water was added to each sample vials. Three controls, A1, A2 and A3 were prepared. A1 was a solution that consists of sea water (2 mL) and brine shrimps, while A2 consisted of sea water (5 mL), ten brine shrimps and drop of methanol, A3 was a solution of sea water (5 mL), ten brine shrimps and a drop of Dimethyle sulfoxide. Ten *nauplii* of brine shrimps were added to each sample by using a pipette. Then, the sea water was added until 5mL. The brine shrimps were left for 24 hours at room temperature. In each case three replicates of each concentration were assayed.

Calculation of toxicity:

The brine shrimps were considered dead if they failed to show any movement and sink at the bottom of the vial. The data were analyzed with Finney program to get the ED_{50} values.

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

Chloroform Extract:

Preliminary screening on brine shrimps larvae showed that the crude chloroform extract was the most toxic, thus it was subjected to fractionation and isolation. 20g was subjected to column chromatography, which was performed on a glass column (45x 5 cm) packed with silica gel (600 g) of particle size (0.04-0.063 mm). Elusion was carried out by petroleum ether: ethyleacetate mixture of increasing polarity. Fractions of 10 ml portions were collected; combined to 12 fractions on the basis of TLC analysis using petroleum ether: ethyl acetate or chloroform as solvent system and vanillin/ H_2SO_4 spray reagent. Fraction four (0.53 g) was applied on chromatotron.

Centrifugal thin layer chromatography (Chromatotron):

In this technique the compounds were separated on a spinning disc which carries a thin separating silica layer (2 mm) for 0.5 g sample. Elution with a solvent gives concentric bands of the components, which were spun off from the edge of the plate (rotor) together with the solvent. A collection system brings the elute into a single output tube. The elusion was carried out by petroleum ether: dichloromethane mixtures of increasing polarity, fractions of 10 ml were collected. Thin layer chromatography (TLC) aluminum sheets pre-coated with silica gel 60 F_{254} (0.2 mm thickness) was used to detect and monitor components present in the crude samples or fractions. The spots were visualized under UV light at 254 and 365 nm, incorporated with spraying reagents (vanillin-sulphuric acid, Dragendorff and

$FeCl_3$ in methanol). Vials exerting one spot were combined to give compound (1).

***Column chromatography (CC)*:**

Fraction six (1.4g) showed two major spots in TLC profile was applied to class column 36x3.5cm packed with silica gel (50 g) of particle size (0.04-0.063 mm) and eluted with n-hexane: dichloromethane and dichloromethane: methanol mixture of increasing polarity, Fractions of 10 ml portions were collected and the separation was monitored by TLC for combining vials of the same chromatograph to give compound 2 (500 mg) and compound 3(300mg)

Analytical Techniques:

The purified compounds were characterized by FT-IR spectrophotometer (FTIR-2000, Perkin Elmer, USA) using potassium bromide (KBr) disc method. Samples were mixed with oven dried IR grade KBr and ground to fine powder. A disc (12.7 mm diameter and around 1 mm thickness) was obtained using hydraulic press (capacity 15 tons max.) at 8 tons for about half minute. The spectrum was scanned at infrared region of 400–4000 cm^{-1}. Furthermore, the isolated compounds were analyzed by FT-NMR spectrometer (Bruker 500 MHz) in deuterated chloroform (CDCl3). The NMR peaks were labeled as singlet (s), doublet (d), triplet (t), and multiplet (m), chemical shifts were referenced with respect to solvent signals. The molecular weight of the compound was determined by liquid chromatography-mass spectrometer (LC-MS). The samples were prepared in HPLC grade methanol (1 mg/ml) and were filtered through 0.45 micron filter. Melting point was measured using electro-thermal melting point apparatus model No. 1A6304.

RESULTS AND DISCUSSION

Characterization of compound (1):

Compound 1 was purified as colourless crystals from chloroform extract of the fruit pulp. It has R_f value 0.5 in solvent system ($CHCl_3$: MeOH (17:3)), developed violet colour with ceric sulfate and vanillin reagents. M. P. 137-138°C.

According to the spectroscopic data (IR, NMR, EI- MS) and those reported by McCarthy (McCarthy *et al*., 2005), compound (1) was identified as β-Sitosterol (Figure 1)

Steroids are widely used in pharmacology as drugs and hormones owing to their anti-inflammatory, anti-cancer, diuretic, contraceptive and anti-androgenic properties (Marjorie, 1999).

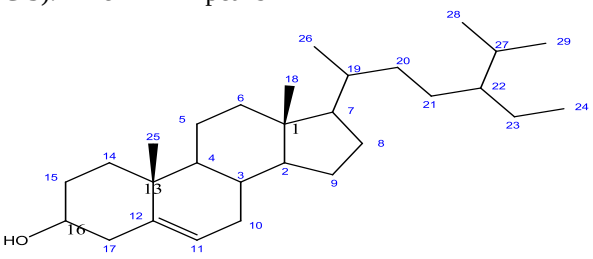

Fig. 1: β- Sitosterol (compound 1).

Compound (2) and (3) were obtained as an amorphous yellow powder from chloroform extract. They have R_f value 0.47 and 0.5 respectively in solvent system (Ch: MeOH (85:15)). They developed brown colour with vanillin reagent.

El-MS m/z: 558.32 of compound (2) was confirmed by HREI-MS to give a molecular formula $\mathbf{C_{32}H_{44}O_8}$. The NMR data were identical to those of Cucurbitacin E as previously reported; ^1H NMR (Momma *et al*., 2008) and ^{13}C NMR (Hassan, Koko, *et al*., 2011; Velde & Lavie, 1983) Consequently, the structure of compound (2) was identified as cucurbitacin E. (Figure2).

Likewise the structure of compound(3) was identified by spectroscopic methods (IR, NMR, El-MS and by comparison with the previous reports (Gry, Søborg, & Andersson, 2006) to be Cucurbitacin L 2-*O*-*β*-glucoside. (Figure 3)

Cucurbitacin L-2-*O*-*β*-glucopyranoside was isolated from the fruits of *Trichosanthes tricuspidata* (Cucurbitacaeae), along with fourteen cucurbitane glycosides. Structural elucidations were based on chemical and spectroscopic analyses (Kanchanapoom, Kasai, & Yamasaki, 2002).

Cucurbitacin L-2-*O*-*β*-Glucoside exhibited apoptogenesis in colon adenocarcinoma cells (HT-29). The mechanism by which CLG induce apoptosis to HT-29 was throw the inhibition of reactive nitrogen and oxygen species as well as triggering of caspase-3-regulated apoptosis (Abdelwahab *et al*., 2012).

Cucurbitacins are cytotoxic triterpenoid substances; they are very common in the family Cucurbitaceae. Series of cucurbitacin cognates were characterized and their biological effects, such as anti-tumor, purgative, anti-inflammatory, and antifertility activities have also been reported (Miro, 1995). In previous a study we demonstrated that cucurbitacin E isolated from C. *lanatus* var. *citroides* inhibits the production of NO in LPS/IFNγ-stimulated macro-phages. One of the possible mechanisms principally involved in the anti-

inflammatory effects of this natural compound seems to be the inhibition of RNS and COX (Abdelwahab *et al*., 2011). It has been reported that cucurbitacin E also demonstrates anti-tumor activity and can change the cell morphology by disrupting actin cytoskeleton (Duncan, Duncan, Alley, & Sausville, 1996). Our study coincides with those results since the isolated cucurbitacin E exerted very high toxicity against brine shrimps that render it as efficient anticancer.

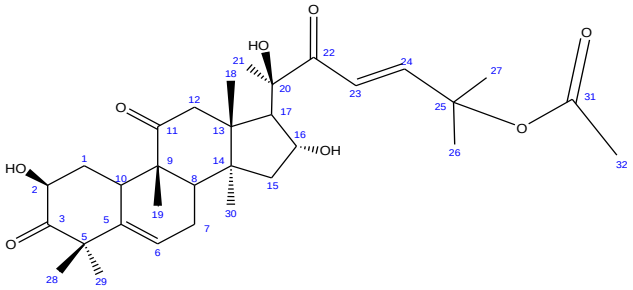

Fig. 2: Cucurbitacin E compound (2).

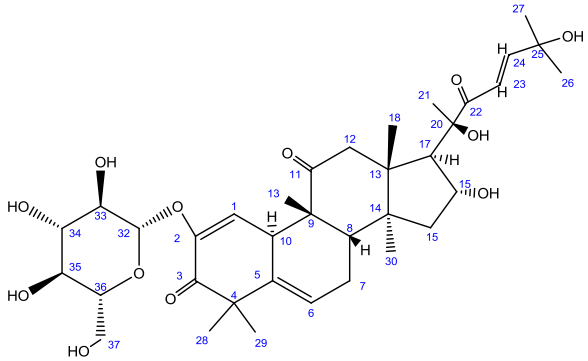

Fig. 3: compound (3) Cucurbitacin L 2-o-β-glucoside.

Toxicity test:

In this study we utilized the the brine shrimp nauplii assay to study the toxicity profile of *Citrullus lanatus* var. *citroides* extract and isolated principles. The brine shrimps lethality test is a rapid, inexpensive and simple toxicology screening test that have been used previously in a number of bioassay systems. Meyer and Mclaughling (Mclaughlin, 1991; Meyer *et al*., 1982) have further developed this method where–by natural products extracts, fractions, or pure compounds can be tested at initial concentrations of 10, 100, and 1000 ppm (mcg/ml) in vials containing 5ml of brine and 10 shrimp in each of three replicates. Survivors are counted after 24 hours. The data is processed with the aid of simple statistical software to determine the LC_{50} values. The authors found a good correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity (p=0.036 and kappa= 0.5), and they used the brine shrimp test as a prescreen for a panel of six human solid tumor cell lines. The authors have observed that ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50} values found in the brine shrimp test.

Therefore this method can be reliable, efficient and rapid screening tool for cytotoxicity studies on natural products

Results of toxicity activity presented in Table 1 and Fig. 4 showed that the butanol and ethyleacetate extracts of *C. lanatus* var. *citroides* fruit were virtually non-toxic on the shrimps. This indicated that polar preparations of *C. lanatus* var. *citroides* were safe. The chloroform extract showed relatively high toxicity with ED_{50} value 241.2970. The presence of alkaloids, cucurbitacins and other aglycones might be responsible for the observed brine shrimps lethality activity of the chloroform extract, thus chloroform extract was subjected to fractionation and isolation.

The extracted compound (2) cucurbitacin E showed very high toxicity followed by compound (1) β-Sitosterol and compound (3) Cucurbitacin L 2-o-β-glucoside with ED_{50} values 506.033, 122.409 and 99.999 respectively. This indicates the ability of compound (2) to induce programmed cell death to cancer cells in cell cultures, kill pests and exert a wide range of pharmacological effects (Hung, Chang, Lin, Ko, & Hsu, 2013). The growth inhibitory activity of cucurbitacin glucosides isolated from *Citrulus colocynthis* on human breast cancer cells was reported (Dakeng, Duangmano, Jiratchariyakul, Bögler, & Patmasiriwat, 2012). They showed that cucurbitacin B/E glucoside combination inhibited growth of ER+MCF-7 and ER¯MDA-MB-

231 human breast cancer cell lines suggesting that these compounds might have therapeutic value against breast cancer cells. Thus, the significant cytotoxicity of these compounds suggested the presence of antitumor compounds which could also render *C. lanatus* var. *citroides* under study as new source of anticancer drugs.

Table 1: Result of Brine Shrimp lethality test (BST) of crude extracts and isolated compounds from *C. lanatus* var. *citroides.*

Sample	Concentration	Average of dead brine shrimp	Average of survive brine shrimp	ED_{50}
compound (1) β-Sitosterol	10	0.0	10	122.4091
	100	4.33	5.666	
	1000	10	0.0	
Compound (2) Cucurbitacin E	10	0.0	10	506.033
	100	1.333	8.666	
	1000	7.0	3.0	
Compound(3) Cucurbitacin L 2-o-β-glucoside	10	0.0	10	99.9998
	100	4.6666	5.3333	
	1000	10	0.0	
Butanol extract	10	0.3333	9.6666	Not toxic
	100	0.6666	9.3333	
	1000	1.6666	8.3333	
Ethyleacetate Extract	10	0.3333	9.6666	Not toxic
	100	0.6666	9.3333	
	1000	10	0.0	
Chloroform Extract	10	0.0	10	241.2970
	100	2.3333	7.6666	
	1000	9.6666	0.3333	

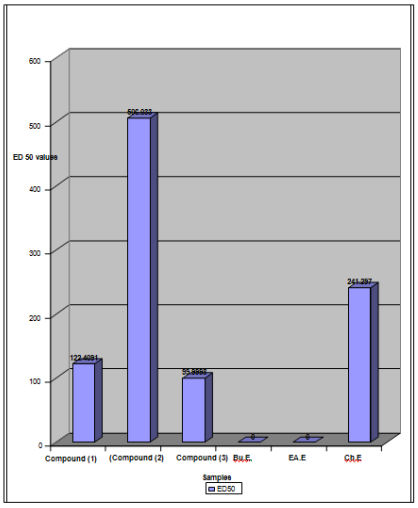

Fig. 4: Brine shrimp lethality test (BST) of crude extracts and isolated compounds from *C. lanatus* var. *citroides*

Conclusion:

This is the first study to explore the detailed phytochemistry and toxicity features of *Citrullus lanatus* var. *citroides in vitro*. Three compounds were isolated from the chloroform and ethyl acetate extracts and identified as β-Sitosterol, Cucurbitacin E and Cucurbitacin L 2-O-β-glucoside. Compound 2 (cucurbitacin E) showed significant toxicity whereas the others showed mild toxicity. In conclusion, *Citrullus lanatus* var. *citroides* displays promising biological activities and could potentially be a good botanical candidate for further investigations and development as potential anticancer agents.

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Conflict of Interests:

The authors declare no conflict of interests.

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