British Journal of Pharmaceutical Research

17(2): 1-8, 2017; Article no.BJPR.33381
ISSN: 2231-2919, NLM ID: 101631759

Antifilarial Activity of *Cucurbita pepo ovifera var ovifera* (Cucurbitaceae) on *Onchocerca ochengi* Adult Worms

Justin Kalmobé[1], Dieudonné Ndjonka[1*], Jacqueline Vildina Dikti[1] and Eva Liebau[2]

[1]Department of Biological Sciences, Faculty of Science, University of Ngaoundere, P.O.Box 454, Ngaoundere, Cameroon.
[2]Department of Molecular Physiology, Institute for Zoophysiology, Schlossplatz 8, 48143 Muenster, Germany.

Authors' contributions

This work was carried out in collaboration between all authors. Authors JK, DN and EL designed the study. Authors JK, DN and EL conducted the study. Authors JK, DN and JVD performed the statistical analyses and drafted the manuscript. All authors contributed substantially to the manuscript and approved its final version.

Article Information

DOI: 10.9734/BJPR/2017/33381
Editor(s):
(1) Nawal Kishore Dubey, Centre for Advanced Studies in Botany, Banaras Hindu University, India.
Reviewers:
(1) John Antwi Apenteng, Central University, Accra, Ghana.
(2) Hyacinth Highland, Biomedical Technology and Human Genetics, Gujarat University, India.
(3) Abdullahi M. Nuhu, College of Science and Technology (CST), Kaduna Polytechnic, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/19429

Original Research Article

Received 12th April 2017
Accepted 2nd June 2017
Published 9th June 2017

ABSTRACT

One of the strategies for developing novel pharmaceutical drugs is to use natural sources such as plants for therapeutic treatment. Plant extracts are a cocktail of compounds which act synergically and can improve treatment effectiveness, reduce therapeutic duration and resistance. The ethanolic extracts of leaves and seeds of *Cucurbita pepo ovifera var ovifera* from Sudano-Guinean and Sudano-Sahelian zones of Cameroon were evaluated on the cattle parasite nematode *Onchocerca ochengi*. Worms were incubated with different concentrations of the plant extracts in RPMI-1640 supplemented with streptomycin and gentamicin. Mortality at 37°C was monitored after 24, 48 and 72 h. Ivermectin was used as positive control and DMSO as negative. Plant extracts

**Corresponding author: E-mail: ndjonka_dede@yahoo.com;*

from the two ecological zones showed anthelminthic activities on *O. ochengi* after 72 h with LC_{50} varying from 20 to 1090 µg /mL. The highest antifilarial activity in Sudano-Guinean zone was obtained with leave extract of *C. pepo ovifera* (LC_{50} of 20 µg/mL), while highest antifilarial activity in Sudano-Sahelian zone was obtained with seed extracts of the plant with LC_{50} value of 17 µg/mL after 72 h. These results show that anthelmintic activity depends on the part of the plant and the ecological zones. Additionally, the plant is not toxic. These results on the ethanolic extracts of leaves and seeds of *C. pepo ovifera var ovifera* confirmed the use of this plant in traditional medicine in Cameroon to treat disease due to nematodes. The plants could be used as alternative anthelmintic to fight against Human and Bovine onchocerciasis.

Keywords: Cucurbita pepo ovifera var ovifera; Onchocerca ochengi; anthelmintic; Sudano-Guinean zone; Sudano-Sahelian zone.

1. INTRODUCTION

Onchocerciasis also called river blindness is a neglected tropical disease caused in human by the nematode *Onchocerca volvulus*. It is transmitted by blackflies of the genus *Simulium damnosum*. Pathologically, the disease is characterized by cutaneous manifestation such as nodules, skin-irritations and ocular syndrome [1]. Globally, about 86 million people are at risk of being infected, 37 million people are infected with 99% living in Africa, about 500,000 are blind [1-4]. In addition to its severe pathological effects, onchocerciasis causes disability, social stigmatization and forces the affected population to abandon the endemic areas, which usually have high agricultural potential. Thus a high burden of onchocerciasis in a country leads primarily to low productivity and consequently to a socio-economic problems [4]. Several control strategies have emerged; vector control, larvicide, nodulectomy which is the excision of nodules, and the use of anthelmintics. Actually, ivermectin is the main drug for treatment of onchocerciasis and filariasis in general. Ivermectin is toxic and cause serious side effects. However its efficacy is limited to microfilariae and requires continuous delivery for at least 14 years, which corresponds to the life span of the adult worm, to interrupt transmission [5]. The continuously use of ivermectin for onchocerciasis control has lead to *O. volvulus* resistance to ivermectin in some communities in Ghana and Sudan [6]. Therefore, there is an urgent need for a safe and effective antifilarial drug against onchocerciasis that will be able to kill macro- and microfilariae. Natural products such as medicinal plants have shown great potentials in treating infectious diseases in humans [7]. By generating billions of dollars in revenue, Herbal medicines are the most lucrative form of traditional medicine in some Asian and African countries [8].

The antifilarial activities of some medicinal plants from Cameroon have been reported on *Onchocerca ochengi* [7, 9-13]. Both parasites *O. volvulus* and *O. ochengi* are evolutionary closely related and are transmitted to their human and bovine host by the same blackfly vector *Simulium damnosum*, their microfilariae being equally sensitive to ivermectin [14]. *Cucurbita pepo ovifera var ovifera* (family: Cucurbitaceae) have been shown to express antibacterial, antidiabétic, anti-hypertensive and anti-inflammation activity [15]. Other separate studies have revealed that *C. ovifera* possess anti-parasitic and antitumoral activities [15-16], Seeds of this plant are reputed as anthelmintic against tape worms, trematodes, cestodes and nematodes. The kymographic studies revealed that the seed extracts act on the motility of the helminthes leading to temporary paralysis [17]. The effectiveness of the pesticide powder from pumpkin seeds of *Cucurbita moschata* L. on gastrointestinal helminths *Haemonchus contortus* has been demonstrated in the Democratic Republic of Congo by [18]. Different parts of *C. pepo ovifera var ovifera* depending on the ecological zones are used by traditional healers in Cameroon for the treatment of onchocerciasis. However, the anti-onchocercal activity of *C. pepo ovifera var ovifera* has not been evaluated. The present study was therefore, carried out to assess and to confirm the antifilarial activity of the leaves and seeds of *C. pepo ovifera var ovifera*, a plant used in the traditional treatment of onchocerciasis in two different ecological zones (Sudano-Guinean and Sudano-Sahelian zones) in Cameroon.

2. MATERIALS AND METHODS

2.1 Plant Material and Chemicals

All chemicals were purchased from Sigma (Deisenhofen, Germany). Leaves and seeds of *Cucurbita pepo ovifera var ovifera*

(Cucurbitaceae) were collected in Bini/Dang village (Sudano-Guinean zone: Adamawa Region) and Garey village (Sudano-Sahelian zone: Far North Region) in Cameroon, on the basis of ethnopharmacological data. Voucher specimens were identified by Dr Tchobsala, Department of Biological Sciences of the University of Ngaoundere. A voucher specimen was deposited in the National Herbarium of Yaounde (Voucher number: 1072HNC). The collected material was washed, air-dried and powdered before extraction.

2.2 Preparation of Plant Extracts

Plant extracts were prepared as described by [11]. Briefly, leaves and seeds of *Cucurbita pepo ovifera var ovifera* was dried at room temperature, weighed, ground finely and sieved on a 0.5 mm mesh screen. Ten grams of the powdered material were extracted with 100 ml of ethanol at 70°C for 48h, centrifuged (3500 ×g, 10 min) and filtered over filter papers No. 413 (VWR International, Darmstadt, Germany). The clear filtrate was concentrated by a rotary evaporator at a temperature not exceeding 40°C under reduced pressure, solidified at -20°C and lyophilized. The resulting powder was stored at 4°C. For further investigation each part of the dried plant extract was dissolved in dimethyl sulfoxide (DMSO) and RPMI to a final concentration of 100 mg/ml, centrifuged and aliquoted to determine their activity on *O. ochengi*.

2.3 Isolation of *O. ochengi* Adult Worms and Anti-filarial Screening of Plant Extracts

The isolation of *O. ochengi* adult worms was done as described by [11]. Briefly, fresh pieces of umbilical cattle skin with palpable nodules bought from local slaughterhouse were brought to the laboratory for dissection, washed, drained and sterilized with 70% ethanol. Nodules of *O. ochengi* were removed from the skin of cattle. *O. ochengi* adult worms were carefully scraped out of the nodules as single masses, isolated and washed three times in sterile phosphate-buffered saline (PBS). The adult female worms were isolated by digestion of the nodules with collagenase at 37°C and submerged in complete culture medium (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$, in 1 litre of water). Following the protocol of [11], adult worms (six individuals per 1 ml well) were incubated with different concentrations of the plant extract (0–2 mg/ml) in RPMI-1640 supplemented with penicillin/streptomycin (100 U/100 g/ml). Assays were incubated at 37°C in a CO_2 incubator and mortality was determined after 24 h, 48 h and 72 h.

2.4 Worm Mortality and LC_{50} Determinations

The number of dead and living worms was recorded under a binocular microscope. Worms which were fully straight or curved and immotile after shaking were considered to be dead. The percent of mortality was expressed as percentage of dead worms in relation to the number of living swimming worms. Plant extract together with the respective control groups were tested in three independent duplicate determinations. LC_{50} values were determinated (lethal concentration of the extract required to kill 50% of worms). Results are presented as mean values ± standard error of the mean (SEM). Ivermectin was dissolved in 10% dimethyl sulfoxide (DMSO). The drug was diluted with RPMI to a final concentration of 2.2 mg/ml (2.5 mM) used for preparation of the positive control groups and DMSO were used as negative control. The maximal final concentration of DMSO in test is 1%.

2.5 Phytochemical Tests

The concentrated residues from the ethanolic extracts were screened for secondary metabolites according to the method of [19].

2.6 Acute Toxicity in Wistar Rats

The test of toxicity was realized according to the recommendations and guidelines of the Organization of Cooperation and Economic Development [20] for chemicals' tests. Wistar rats used for the experiments were obtained from LANAVET (Laboratoire National Vétérinaire, Garoua, Cameroon). Animals were housed under ambient temperatures (25°C ± 3°C) and 12 h light/dark cycle. They were fed with standard rodent pellets and had unrestricted access to clean drinking water. The acute toxicity study of the ethanolic extracts of seeds and leaves of the plant was administrated in a single oral dose. Six females and six males were used for each dose. They were deprived of food, but not water 12-14 h prior to the administration of the test suspension. The doses vary generally between four values: 500; 1500; 3000 and 5000 mg/kg of body weight and was administrated by gavages

using a feeding needle. The control group received 1% DMSO at dose 0.2 mL/kg. They were observed for 24 h, the number of deaths was recorded and the LD_{50} determined. The appearance of toxic symptoms such as behavioural changes, locomotion, convulsions and mortality, were observed and recorded. Animals were observed constantly for the first 30 min after administration and thereafter every 4 h up to 24 h and subsequently once a day for up to 14 days [9,21]. There is no law yet regulating animal research in Cameroon [12]. All animal-related experimental procedures were approved by the regional delegation of Livestock, Fisheries and Animal industries (N°075/16/L/RA/DREPIA).

2.7 Statistical Analysis

Data obtained during the treatment on adults worms of *O. ochengi* after 24 h, 48 h and 72 h of incubation at 37°C have been introduced in Excel software 2007 before calculating the mean and LC_{50} ± SEM of three independent duplicate trials.

3. RESULTS

The objective of the present study was to test the activity of the ethanolic extract of leaves and seeds of *C. pepo ovifera var ovifera* from two ecological zones on the bovine filarial nematode *O. ochengi*, an established model for human onchocerciasis. The plant extracts inhibited the mortality of *O. ochengi* adult in a time and concentration-dependent.

The ethanolic crude extracts of leaves and seeds from plants of the two ecological zones showed an anthelmintic activity on macrofilariae of *Onchocerca ochengi* at 24, 48 and 72 h of incubation at 37°C with LC_{50} values between 17 µg/mL to 1390 µg/mL (Table 1).

Ethanolic extracts of leaves and seeds of *C. pepo ovifera var ovifera* from the Sudano-Guinean and the Sudano-Sahelian zones were tested for antifilarial activity using adults of *O. ochengi*. Ivermectin was used as positive control. No mortality was observed during the experimental period of two weeks in the negative control DMSO. The ethanolic crude extract of seeds of the tested plant from the Sudano-Sahelian zone and leaves from the Sudano-Guinean zone exhibited the lower LC_{50} of 17 and 20 µg/mL after 72 h on *O. ochengi* respectively (Fig. 1, Table 1), therefore cause a much higher mortality.

Table 1 and Fig. 1 show that the leaves but not the seeds of the *C. pepo ovifera var ovifera* from the Sudano-Guinean, exhibit macrofilaricidal activity at LC_{50} almost similar to the positive control ivermectin at the same exposure time (72 h).

Among the tested parts of the plant, leave crude extracts of *C. pepo ovifera var ovifera* from the two ecological zones (LC_{50} of 20 µg/mL, 35 µg/mL) and seeds of the Sudano-Sahelian zone (LC_{50} of 17 µg/mL) are the most potent extracts against *O. ochengi* compared to seeds of the same plant from the Sudano-Guinean zone (LC_{50} of 1090) which displayed a somewhat weakest, but still significant activity on *O. ochengi* after 72 h (Table 1).

Phytochemical analysis of the plants from both ecological zones reveal the presence of alkaloids, tannins, flavonoids, saponins, triterpenes, polyphenol and steroids in leaves, while only alkaloids and saponins were shown to be present in seeds from both ecological zones (Table 2).

Acute toxicity results at limit dose of 5000 mg/kg body weight when extracts of the tested plants from both ecological zones were given orally and the animals followed-up for 24 h, and after 14 days showed no adverse effects and no mortality (Table 3).

Table 1. LC_{50} values of the ethanolic extracts of leaves and seeds of *Cucurbita pepo ovifera var ovifera* from two ecological zones and positive control tested against adults of *Onchocerca ochengi* after 24 h, 48 h and 72 h exposure

Zones	Mean ± SEM LC_{50} (µg/mL)					
	Leaves			Seeds		
	24 h	48 h	72 h	24 h	48 h	72 h
Sudano-Guinean	90 ± 2.4	40 ± 1.6	20 ± 0.8	1390 ± 25	1290 ± 86	1090 ± 89
Sudano-Sahelian	110 ± 4.0	60 ± 1.7	35 ± 1.6	270 ± 12	92 ± 4.5	17 ± 1.3
Ivermectin	30 ± 1.0	19 ± 2.1	12 ± 1.2	30 ± 1.0	19 ± 2.1	12 ± 1.2

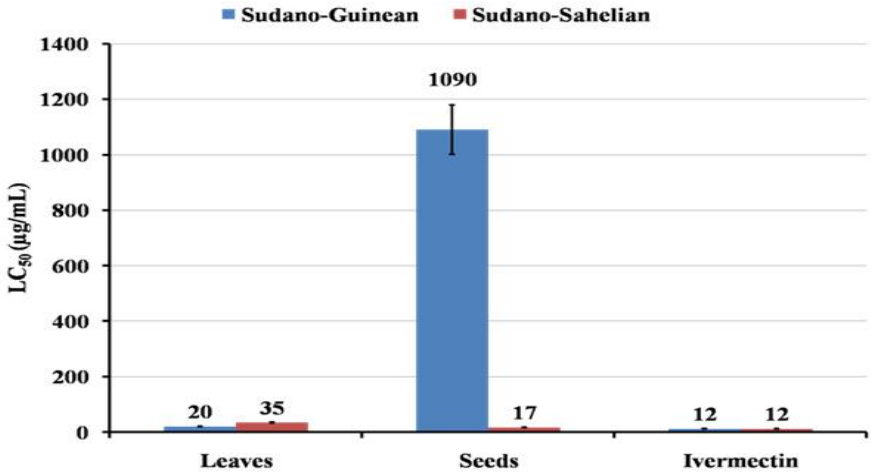

Fig. 1. Comparison of LC_{50} of *Onchocerca ochengi* exposed to different parts of the plant from the two ecological zones. Each point represents the mean of three independent duplicate studies with standard deviation of the LC_{50}

Table 2. Phytochemical screening of the ethanolic extracts of leaves and seeds of *Cucurbita pepo ovifera var ovifera* from two ecological zones. The phytochemical screening revealed the presence or absence of flavonoids, alkaloids, saponins, triterpens, polyphenols, steroids and tannins in the seeds and leaves of plants

Components	Sudano-Guinean		Sudano-Sahelian	
	Leaves	Seeds	Leaves	Seeds
Alkaloids	++	+	++	+
Tannins	+++	-	++	-
Flavonoids	+++	-	++	-
Saponins	+++	++	++	++
Triterpenes	+++	-	+++	-
Polyphenols	+++	-	+++	-
Steroids	+++	-	++	-

+=present; - =absent

Table 3. Percentage (%) mortality of male and female rats 72 h after administration of crude extracts of leaves and seeds of *Cucurbita pepo ovifera var ovifera* from two ecological zones

	Mortality rate of male and female rats (%)				
Crude extracts of the both zones	**Control**	**1500 mg/kg**	**3000 mg/kg**	**5000 mg/kg**	***O. ochengi* adults LC_{50} (µg/mL) ± SEM 72 h**
Leaves SG	-	0	0	0	20,0 ± 0,8
Seeds SG	-	0	0	0	1090,0 ± 89.0
Leaves SS	-	0	0	0	35 ± 1,7
Seeds SS	-	0	0	0	17 ± 1,3
Ivermectin	0	0	0	0	12,0 ±1,2
DMSO	0	-	-	-	-

SG: Sudano-Guinean zone; SS: Sudano-Sahelian zone

When compared with the control group treated with 1% DMSO dissolved in distilled water, and given at a dose of 0.2 mL/kg body weight), there was no significant difference in food and water intake. No change was observed in agility, physical appearance and behavior of the rats 24 h post-treatment.

4. DISCUSSION

In the present work, ethanolic extracts of *Cucurbita ovifera*, plant used in traditional medicine in Cameroon was tested for its anthelmintic activity. The bovine filarial nematode *O. ochengi* models were employed. In general,

the ethanolic extracts of plant have shown effective and strong anthelminthic activity on nematode parasite *O. ochengi* after 72 h incubation.

Recent reports have revealed that *C. pepo ovifera var ovifera* possess antibacterial, antidiabétic, anti-hypertensive, anti-inflammation, anti-parasitic and antitumoral activities [15,17]. Other separate studies have revealed that seeds of the plant are reputed in Ayurvedic system of medicine as an anthelmintic especially against tape worms. The aqueous, alcoholic and ethereal extracts of the seeds were tested *in vivo* and *in vitro* on trematodes, cestodes and nematodes [15,16,18]. Remarkably, this selected plant has never been tested on the bovine parasitic nematode *O. ochengi*. However, several parallel works have shown the anti-onchocerca activity of a number of plants such as *Khaya senegalensis, Anogeissus leiocarpus, Homalium africanum, Margaritaria discoidea, Craterispermum laurinum, Morinda lucida*, *Cyperus articulates, Tragia benthami* and *Piper umbellatum* [9-13] and close species to *C. pepo ovifera var ovifera* such as *Cucurbita moschata* for its inhibiting actions on the blossoming of eggs in *Haemonchus contortus* [18]. All those studies reported are not directly comparable to our results, due to differences in plant materials. However, recent studies using plants mentioned above have also demonstrated that the majority of these plants contains almost the same phytochemical products namely tannins, steroids, triterpenes, flavonoids, alkaloids, saponins, polyphenols [19]. The activity observed by *C. pepo ovifera var ovifera* on the filarial nematode *O. ochengi* might be attributed to the presence of these phytochemical products which might act synergically. Due to the presence of tannins in *C. pepo ovifera var ovifera*, mortality observed might be explained by the fact that tannins react directly with surface proteins of the parasite (*O. ochengi*) causing a physiological dysfunction in nematodes such as mobility and the absorption of nutrients, leading to the death of worms as observed by Katiki et al. [22]. It has been also demonstrated that tannins also interfere with the production of energy in helminth parasites by decoupling the oxidative phosphorylation [23]. Another possible anthelmintic effect of tannins is that they can bind to glycoproteins on the cuticle of the parasite and can cause death [23,24]. This might approve possible modes of action of *C. pepo ovifera var ovifera* because the majority of chemical families in these plants are tannins [25]. In addition to the putative anthelminthic activity of *C. pepo ovifera var ovifera*, it has been shown that secondary metabolites such as cucurbitine, cucurmosine, amino acids may directly act on worm [26,27]. The mechanism of action of these secondary metabolites is still unknown. Some authors speculate that these secondary metabolites might 1) disturb the integrity of the cuticle of the parasite; 2) bind to proteins normally used by the worms for their nutrition and reproduction functions; 3) disturb the operation of the genital tract of females as demonstrated in trichostrongles with consequences on their fruitfulness [18,24,28]. All these results confirm our findings with the selected plant extracts; reinforce the existing knowledge and the regular use of the plants by traditional healers for the treatment of helminth infections.

All symptoms including changes in awareness, mood, motor activity, posture activity and mortality were recorded and no changes were observed in behavior and mortality as well as in toxicity or death for all dose levels in the selected and treated animals. The LD_{50} of the ethanolic extracts was more than 5000 mg/kg and could not be determined. According to OECD, any test substance showing an LD_{50} of 1000 mg/kg after oral administration can be considered safe [20]. This result indicates that the ethanolic extracts under study, when given orally, could be considered relatively safe as previously observed by [21,29].

5. CONCLUSION

In summary, this work focused on the evaluation of the anthelmintic activity of the ethanolic extracts of *Cucurbita pepo ovifera var ovifera* (Cucurbitaceae) from two ecological zones of Cameroon on the nematodes *Onchocerca ochengi* the cattle parasites. It appears from the results that ethanolic extracts of all parts except seeds of the plant from the Sudano-Guinean zone displayed nematocidal effects on adult worms of *O. ochengi*. We can say that *C. pepo ovifera var ovifera* is an important medicinal plant. These results also allowed us to know that different parts except seeds from the Sudano-Guinean zone can be used in the treatment of onchocerciasis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Animal Ethical Committee of the Ngaoundere Regional Health Authority, Cameroon.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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