Pumpkin seeds (Cucurbita moschata - Jacarezinho cultivar): characterization of the oil extracted by solvent and supercritical fluid and study of anti-parasitary activity

Sementes de abóbora (Cucurbita moschata - cultivar Jacarezinho): caracterização do óleo extraído por solvente e fluido supercrítico e estudo da atividade antiparasitária

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ABSTRACT
The presence of many biologically active components makes pumpkins extremely attractive to the phytochemical manufacturing industry. Studies have demonstrated that the oil extracted from the seeds has different biological activities. This study aimed to determine the fatty acid composition and total tocopherol content of the pumpkin seed oil (*Cucurbita moschata* – Jacarezinho cultivar) extracted by supercritical carbon dioxide (Sc-CO_2) and hexane/isopropanol. The fatty acids composition and content of total tocopherols were determined by GC, GC/FID, HPLC, respectively. We also evaluated *in vitro* schistosomicidal activities of the crude oil, which have been described with anti-parasitic activities. Sc-CO_2 extracted pumpkin seed oil, with a maximum yield of 24.3 ± 0.4%, much higher than hexane/isopropanol extraction (8.3 ± 2.7%). Was not observe differences between the non-polar compounds present in the oil extracted by both methods. In the seed oil, unsaturated acids are dominant (oleic and linoleic). The results indicate that the oil has an excellent quality, with high contents of unsaturated fatty acids (73% of total fatty acids) and total tocopherols (14 $mg100g^{-1}$ of oil). Although popularly reported as anti-parasitic activities caused no mortality, tegumental alterations or significant decrease in motor activity in all adult parasites. Sc-CO_2 was able to extract pumpkin seed oil with a much higher yield than extraction with hexane/isopropanol and no differences were observe between the non-polar compounds present in the oil extracted by both methods. The oil is popularly used as an antiparasitic agent but in this study did not show antiparasitary actividies.

Keywords: green chemistry, extracts, application, biological activity.

RESUMO
A presença de componentes biologicamente ativos tornam as abóboras atraentes para a indústria farmacêutica e de alimentos. Estudos demonstram que o óleo extraído de suas sementes possui diferentes atividades biológicas. Este trabalho teve como objetivo determinar a composição de ácidos graxos e o teor de tocoferol total do óleo de semente de abóbora (Cucurbita moschata - cultivar Jacarezinho) extraído por fluido supercrítico (Sc-CO_2) e por solventes hexano/isopropanol. A composição de ácidos graxos e o conteúdo de tocoferóis totais foram determinados por GC, GC/FID, HPLC, respectivamente. Também foram avaliadas as atividades esquistossomicidas *in vitro* do óleo cru já descrito pela literatura com potencial

atividade antiparasitária. O óleo de semente de abóbora extraído com Sc-CO_2, apresentou um rendimento máximo de 24,3 ± 0,4% o qual foi muito superior à extração com hexano/isopropanol (8,3 ± 2,7%). Não foram observadas diferenças entre os compostos apolares presentes no óleo extraído por ambos os métodos. No óleo da semente, os ácidos insaturados dominantes são oleico e linoleico. Os resultados indicaram que o óleo é de excelente qualidade, com alto teor de ácidos graxos insaturados (73% dos ácidos graxos totais) e tocoferóis totais (14 $mg100g^{-1}$ de óleo). Embora seja popularmente relatado que o óleo possui atividade antiparasitária, neste estudo não foram observados mortalidade, alterações tegumentais ou diminuição significativa da atividade motora em todos os parasitas adultos.

Palavras-chave: química verde, extratos, aplicação, atividade biológica.

1 INTRODUCTION

Native to tropical regions, pumpkin belongs to the family *Cucurbitaceae*, genus *Cucurbita*. This family is formed by 120 genus that include more than 800 species. In Brazil there are about 30 genus and 200 species. Regarding economic and food importance, *Cucurbita moschata, maxima* and *pepo*, are three of the most widely cultivated pumpkin species[1,2]. The cultivation of pumpkins (*Curcubita moschata; Jacarezinho cultivar*) usually occurs in small farms and commercial crops purposes. Pumpkins are often cooked and used as an ingredient in pies, soups, candy and bakery preparations[1]. Its use for animal feed is common due to productivity of plants and fruit durability[3].

In Latin America, some regions of Africa and Europe pumpkin seeds are commonly used as food additive, toasted and salted[2] or in the form of extracted oil for seasoning of salads due to its characteristic flavor and aroma[4]. The seeds, usually discarded during preparation of pumpkins, are a rich source of vitamins A, B and E, linoleic acid, oleic acid, zinc, selenium, carbohydrates and phytosterols. Oil is the seed's main component (which in many instances accounts for 50% of the total content), so pumpkin seeds are regarded as a valuable source of fat[5,6]. Their content, however, differs among the pumpkin varieties, and were found to be dependent on climate and cultivation conditions. It is therefore advisable to assess the composition of the fatty fraction in the pumpkin seed oil before determining the range of potential uses of a new variety. The presence of many biologically active components makes pumpkins extremely attractive to the phytochemical manufacturing industry[4]. Studies have demonstrated that the oil extracted from the seeds of *Cucurbita pepo* inhibited prostate-induced hyperplasia of the prostate in rats. In folk medicine a varied use is reported and scientifically anti-diabetic, anti-hypertensive, anti-tumoral, immunomodulatory, anti-bacterial, hypolipidemic, anti-inflammatory and anti-parasitic actions were described[3,7].

About anti-parasite activities, the plants of the *Cucurbitaceae* family contain some phytochemicals that show anti-helminthic activity, making *Curcubita moschata* an alternative drug[8]. Intestinal parasitosis is a severe public health problem, concentrated in the poorest countries and related to high morbidity. Some parasitic diseases, such as ascariasis, trichiuriasis and hookworm infection, are considered neglected diseases. In addition to morbidity, these diseases often cause organic deficits, compromising the physical and intellectual development and limiting the adults' production capacity. Thus, parasitosis produces, in its more severe forms, a large number of sicknesses, which generate financial costs for families and government with medical and hospital care[7,9,10]. In addition to attacking humans, parasites also affect animals, causing great damage to the agricultural economy[8,11].

Several extraction techniques have been used to recover lipids from pumpkin seeds, such as organic solvents extraction with the mixture hexane/isopropanol, cold pressing and supercritical carbon dioxide (Sc-CO_2) extraction, among others. All of them have been described as effective to extract oil from pumpkin seeds, although Sc-CO_2 extraction is particularly convenient due to the possibility to obtain solvent-free extracts. Furthermore, the mild critical properties of CO_2 allow using moderate temperature and pressure that do not affect the biological activities of the extract[12]. Besides, CO_2 is also safe, nontoxic and environmentally friendly. The objective of this work was to characterize the composition and content of fatty acids and tocopherols in oil extracted by Sc-CO_2 and hexane/isopropanol techniques from pumpkin seeds (*Cucurbita moschata* – *Jacarezinho* cultivar) cultivated in Brazil. Microstructural analysis of pumpkin seeds was performed before and after the extraction methods. Due to the scarcity of conclusive studies reporting the parasitic activity of pumpkin seed oil, we also evaluated the *in vitro* schistosomicidal activities of the crude oil, which have been popularly described as anti-parasitic activities.

2 EXPERIMENTAL

2.1 SAMPLE PREPARATION

Pumpkins were provided by a local producer (Campo do Meio - Farm, Candeias, MG – Brazil) and randomly selected for the collection of seeds (Curcubita moschata – Jacarezinho cultivar). All the selected seeds had intact kernels and no visible damages. The seeds were washed with water and then dried in a forced convection oven at 45 °C for 24h. The dried seeds were then milled and stored in a hermetic container, in the dark, at -18 °C, until use. Extraction of oil for chromatographic analysis was carried out by two procedures: supercritical fluid extraction and Hara and Radin lipid extraction[13].

2.2 SUPERCRITICAL EXTRACTION

The extractions were performed in a supercritical fluid extraction unit in the Laboratory of High Pressure in Food Engineering (LAPEA), located in the University of Campinas, São Paulo-Brazil. Figure 1 shows a diagram of the extraction unit used in this work.

In order to establish the solvent to raw matter (S/F) mass ratio to be used in Sc-CO_2, extraction, a kinetic experiment was conducted, collecting the extracted oil in different times (40 , 80 and 150 minutes). For that, 3 g of the dried and milled material were introduced in a 50 mL extraction vessel, using glass wool as a filtering material and glass spheres to complete the vessel volume. CO_2 with 99% purity was used at 35 MPa and 40 °C, at a flow rate of 10.5 g/min. Triplicate of the extractions were performed[14].

Global Yield was calculated as shown in Eq.1.

$$X_0 = 100 * \frac{Moil}{F} \qquad \text{Eq.1}$$

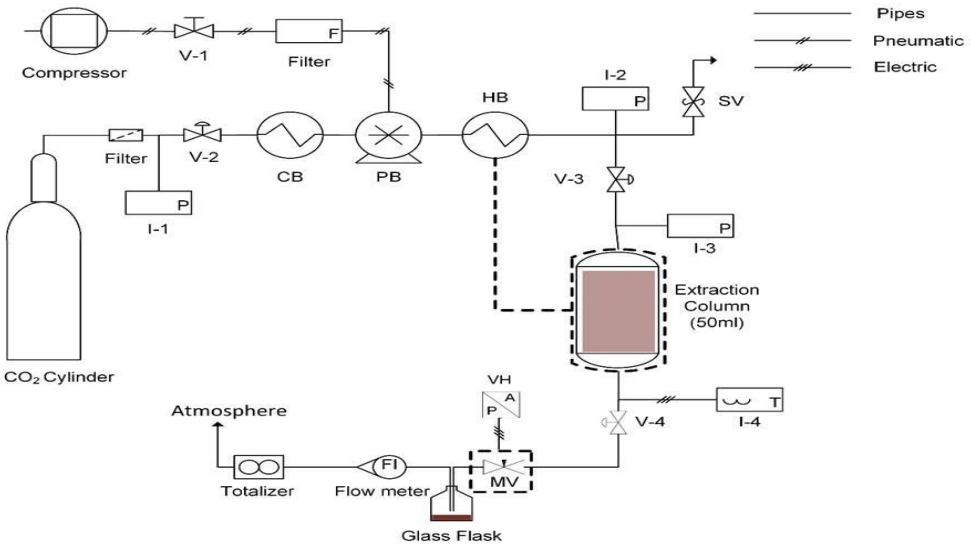
Where:

M_{oil} = mass of extracted oil;

F= mass of dry seeds;

X_0= global yield.

Figure 1: Flow diagram of the supercritical extraction unit; V-1, V-2, V-3, and V-4 - Control valves; SV – Safety valve; MV – Micrometer valve; I-1, I2, and I-3 – Pressure indicators; I-4 – Temperature indicator; CB – Cooling bath; PB – Pneumatic booster; HB - Heating bath; VH – Micrometer valve temperature controller.



2.3 LIPID EXTRACTION BY HEXANE/ISOPROPANOL

Lipid extraction was carried out employing three parts of hexane to two parts of isopropanol as extraction solution (ES). Approximately 500 mg of the dried and milled material was weighed in a flask, where 3.75 mL of ES and 2.50 mL of anhydrous sodium sulfate (0.5 mol/L, Na_2SO_4) solution were added. The mixture was sonicated for 10 minutes and vortexed for 1 minute. Then, the flask was left in an ice bath until the phase separation. The organic phase was transferred to a flask and the aqueous phase was two times washed with 2.5 mL of ES. Volume was made up to 10 mL with hexane. The extract was separated in two pre-weighted flat-bottom flasks and the organic phase was evaporated in a rotary evaporator at 40 °C until only the oil phase remained. After that, each flask was weighed again to obtain the extracted oil mass. The extracted material was re-suspended with hexane and transferred to a 10 mL volumetric flask[13].

2.4 OIL CHARACTERIZATION CG - FID

2.4.1 Hydrolysis and methylation of oils

Approximately 12 mg of the oil sample was dissolved in 100 µL of a 1mol/L (5%) solution of ethanol (95%)/potassium hydroxide in a 2 mL cryogen tube. After vortexing for 10s, the oil was hydrolyzed in a domestic microwave oven (Panasonic Piccolo) at power of 80W (Power 3) for 5 minutes. After cooling, 400 µL of hydrochloric acid (20%), one NaCl spatula tip (~20 mg) and 600 µL of ethyl acetate were added. After vortexing for 10 s and rest for 5 min, an aliquot of 300 µL of the organic layer was removed, placed in microcentrifuge tubes and dried by evaporation, thereby obtaining the free fatty acids (WW Christie, Gas Chromatography and Lipids, 1989, Pergamon Press). Subsequently, the free fatty acids were methylated with 100 µL of BF_3/methanol (14%) by heating for 10 minutes in a 60 °C water bath. The methylated fatty acids were extracted with 500 µL of hexane and analyzed[15].

2.4.2 Free fat acids extraction from supercritical sample

The supercritical fluid sample (~ 50 mg) was subjected to liquid-liquid extraction with 500 µL of 2.8% ammonium hydroxide and 1000 µL of ethyl acetate. After vortexing 30 s and standing for 10 min, 250 µL of the aqueous layer (containing free fatty acids) was taken. After acidifying with HCl (20%), the free fatty acids were extracted into 500 µL of ethyl acetate. Subsequently, the free fatty acids were methylated with 100 µL of BF_3/methanol (14%) by heating for 10 minutes in a 60 °C water bath extracted with 50 µL of hexane and analyzed by Gas Chromatography.

2.4.3 Analyses

The methylated samples and those extracted by the supercritical method were analyzed in an HP7820A Gas Chromatograph (Agilent) equipped with a flame ionization detector. Data Acquisition Program (EZChrom Elite Compact (Agilent) was used. The BP20 15 m x 0.22 mm x 0.25 μm column (SGE) with temperature gradient: 120 °C, 0min, 7 °C/min to 240 °C; injector (split of 1/50) at 250 °C and detector at 260 °C was used. The hydrogen as entrainment gas (3.0 mL/min) and injection volume of 1 μL was performed. Peak identification was done by comparison with methylated fatty acid standards (Supelco 37 Fame mix - Supelco cat nº 47885-U).

2.5 TOCOPHEROL CONTENT

About 50 mg of pumpkin seed oil was weighed into a 1.5 mL eppendorf. 400μL of isopropanol was then added to the eppendorf. The mixture was placed on an ultrasound bath for 5 minutes. Subsequently, it was centrifuged for 4 minutes. The supernatant was transferred to a vial. For the analytical curve, α, β, and γ-tocopherols were used. Chromatographic analyzes were performed in a Shimadzu liquid chromatography coupled with: two LC-20AD pumps, DGU-20A degasser, SIL-20A self-sampler, CBM-20A communicator, SPD-20A UV-Vis detector and CTO-20AC column oven. Elution occurred on a Kinetex column (100 x 3 mm, C18, 2.6 μm). As mobile phases a solution of methanol and water 97:3 v/v(Phase A) and a solution of isopropanol and hexane 5:4 v/v (Phase B) were employed. The components of the oils were gradient separated. The scheduling of the phases was as follows: 100% A from 0.01 to 7 minutes; 50% A in 15 minutes; 0% A in 20 minutes. The wavelength employed in the quantification was 292 nm, the injection volume was 6 μL and the flow rate used in the column was 0.4 mL/min.

2.6 MICROSTRUCTURAL ANALYSES OF THE GROUND SEEDS

The structures of crushed seeds samples previously and after application of extraction techniques were analyzed by scanning electron microscopy (Hitachi TM 3000 Scanning Microscopy-SEM). The samples were fixed on double-sided carbon tape, mounted on the stub and inserted into the microscope. To ensure the reproducibility of the results, a large number of images was obtained in order to select the most representative.

2.7. SCHISTOSOMICIDAL ASSAYS

2.7.1 In vitro studies of adult schistosomes

Schistosoma mansoni (BH strain Belo Horizonte, Brazil) worms are maintained in *Biomphalariaglabrata* snails as intermediate hosts and *Mesocricetusauratus* hamsters as definitive host at the Adolfo Lutz Institute (São Paulo, Brazil), according to standard procedures previously described[16]. The Committee for Ethics in Animal Care of Institute Adolf Lutz (CEUA, 11.794/08) authorized all experiments accordingly to principles for laboratory animal use and care.

All *in vitro* assays were performed according to standard procedures previously described[16]. Briefly, adult worms were washed in RPMI 1640 medium kept at pH 7.5 with HEPES 20 mM, supplemented with 10% fetal bovine serum and containing 200 µg/mL streptomycin and 200 IU/mL penicillin. Adult worms pairs (male and female) were incubated in a 24-well culture plate, containing the same medium supplemented with 10% heat-inactivated calf serum at 37 ºC in a 5% CO_2 atmosphere. Samples of pumpkin seeds oil (400 µg) were dissolved in dimethyl sulfoxide (DMSO; 0.5 % final concentration), and the final volume in each well was 2 mL (200 µg/mL). The control worms were assayed in RPMI 1640 as negative control group and PZQ (5µM) as positive control group. The effects of samples were assessed under an inverted microscope (Nikon, Melville, NY) with an emphasis on changes in tegumental changes and mortality[16].

2.7.2 Statistical analysis

Statistical tests were performed with Graphpad Prism software. Significant differences were determined by a one-way analysis of variance (ANOVA) and by applying Tukey's test for multiple comparisons with the level of significance set at $p< 0.05$.

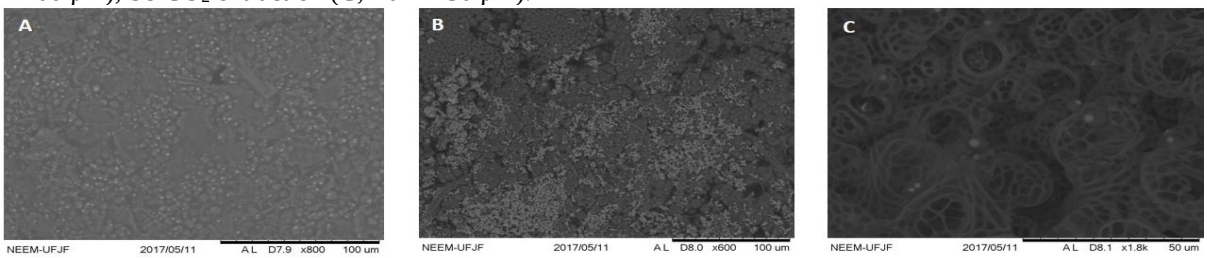
3 RESULTS AND DISCUSSION

3.1 EXTRACTION

Sc-CO_2 was able to extract pumpkin seed oil with a maximum yield of 24.3 ± 0.4% (150 minutes), close to the 30.4% obtained for another pumpkin species[17]. Comparing to hexane/isopropanol extraction (8.3 ± 2.7%/45minutes - total extraction time), Sc-CO_2 extracted about three times of non-polar compounds present in the seeds in one third of the time, demonstrating its great affinity for hydrophobic compounds. Furthermore, although the Sc-CO_2 extraction time is longer, the oil obtained does not present contamination by solvent residues[14].

The images (SEM) of the crushed seeds samples unextracted (A); after oil extraction by solvent (B); and Sc-CO_2 extraction (C) are shown in Figure 2. In Figure 2A it is possible to observe changes in the structure with some deformation when compared with the images after solvent extraction (Figure 2B) and application of Sc-CO_2 (Figure 2C). The most relevant structure observed in Figure 2A is similar to oil droplets dispersed in all surface, which is notably different from those observed in Figures 2B and 2C. After Sc-CO_2 extraction, all dispersed material was removed and only lignin-cellulosic structures were observed. This shows the efficiency of this extraction when compared to extraction methods that use chemical solvents.

Figure 2**:** SEM images of crushed seeds unextracted (A; Barr = 100 µm); after oil extraction by solvent (B; Barr = 100 µm); Sc-CO_2 extraction (C; Barr = 50 µm).



Sc-CO_2 extraction has been applied to recover lipids from several seeds, like passion fruit[14], pumpkin[17], coriander[18], rosehip[19] and papaya[20]. In most cases the temperature was between 40 °C and 80 °C, with pressures between 10 and 40 MPa. In this ranges of variables, the best extraction yield is usually achieved at the condition that results in the highest Sc-CO_2 density, which corresponds to the highest pressure and the lowest temperature [21].

3.2 FATTY ACIDS CHARACTERIZATION AND TOCOPHEROL CONTENT

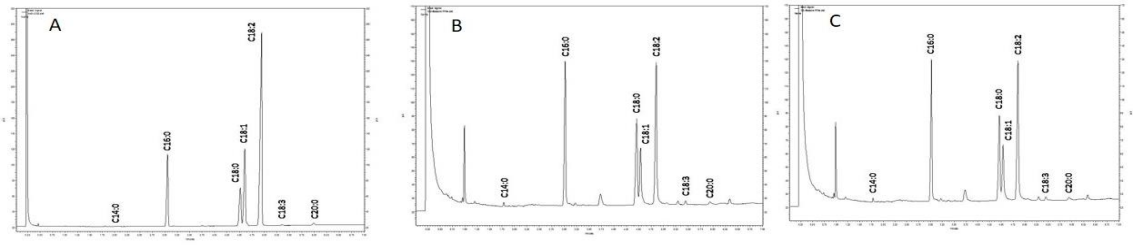
The fatty acid compositions of the oil extracted by Sc-CO_2 and hexane/isopropanol are given in Table 1. The fatty acid composition of the seed oilshows that the four dominant fatty acids found in *Cucurbita moschata: Jacarezinho* cultivar were: palmitic (15.2%), stearic (11.2%), oleic (19.35%) and linoleic (52.25%). The content of these four main fatty acids is approximately 98% of the total fatty acid composition of the oil. Although the total amount of oil extracted was quite different in both methods, the composition in terms of fatty acids remained basically the same. The chromatograms are shown in Figures 3 (A, B and C).

Table 1. Fatty acids characterization **(PERCENTAGE)**

FATTY ACIDS	RETENTION TIME	Sc-CO_2 extraction (%)	Solvent extraction (%)	***FFA (%)
C14:0	1.979	0.1	0.2	0.6
C16:0	3.036	15.0	15.4	27.0
C18:0	4.495	10.8	11.6	0.4
C18:1	4.587	19.6	19.1	20.1
C18:2	4.923	53.4	51.1	14.3
C18:3	5.337	0.2	0.3	32.2
C20:0	5.965	0.8	0.8	0.6
Others		0.1	1.4	0.6

***FFA = Free Fat Acids from Sc-CO_2 extract

Figure 3**:** A - chromatogram supercritical oil; B - chromatogram solvent extract; C - free fatty acids from Sc-CO_2 extract.



The oil contains an appreciable amount of unsaturated fatty acids (71.85%) and is a rich source of linoleic acid. In the present study the composition of fatty acids differed according to both pumpkin variety and species, according to literature (Table 2). The same observation has been made[22] in their result for twelve pumpkin species, among *Curcubita maxima* and *pepo.* The data reported in the literature makes clear that fractions differ considerably in quantity from one raw material to another[15,23]. The content of certain fatty acids depend on the origin and specific cultivars. The ratio of fatty acids reported in Table 2 was different when compared with literature[22]. In the study of the *Curcubita pepo* specie cultivated in three different lands in Eritrea and found similar percentages of the fatty acids: palmitic C16:0 (11.2%); stearic C18:0 (8.0%), oleic C18:1 (28.2%) and linoleic C18:2 (43.0%)[24,25].

Table 2. Comparison of the fatty acid composition of the seeds from different cultivars between our study and the results obtained from literature (percentage)

	Fatty acid					Total Tocopherol
Sample	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**(mg/100g)**
*Cucurbita moschata**						
Jacarezinho	15.0	10.8	19.6	53.4	0.20	14.0
*Cucurbita Maxima***						
Amazonka**	13.1	6.1	35.9	42.6	0.25	16.3
Ambar**	13.3	6.13	28.3	49.7	0.45	22.5
Bambino**	13.1	6.13	26.1	52.1	0.47	29.0
*Curcubita Pepo***						
Danka**	12.3	6.99	36.2	42.0	0.36	22.4
Junona**	12.8	6.60	31.3	46.9	0.36	40.4

Miranda**	12.7	6.21	31.7	47.0	0.35	42.9

* Sc-CO_2 extraction; **Data from literature[22]

Tocopherols are important biological antioxidants that prevent oxidation of body lipids, including polyunsaturated fatty acids and lipid components of cells and organelle membranes. Concerning the total tocopherol analysis, the technique used here does not distinguish the isomers of tocopherol. The total concentration of tocopherol was of 14mg/100g by Sc-CO_2 extraction (Table 2) and was not possible to quantify in the oil extracted by hexane/isopropanol.

3.3 IN VITRO SCHISTOSOMICIDAL EFFECTS ON ADULT SCHISTOSOMES

Although pumpkin seed oil is popularly used as an anti-parasitic agent[8,9,10, 11], in this experiment it did not cause caused mortality, tegumental alterations or significant decrease in motor activity in all adult parasites after 24h of incubation when tested at (200 µg/mL) (Table 3). On the other hand, PZQ (1.56 µg/mL), used as positive control, reduced the motility and caused the death of all the parasites after 24h of incubation. Also, in control groups, when adult worms were maintained in the RPMI medium containing 0.5% DMSO, their appearance were similar to those maintained in the same medium without DMSO even after 24h of incubation. During this period, all parasites revealed normal motor activity with natural peristalsis of the worm body and peristalsis of the gut (Table 3). In a patent[26] reported the application of tocopherol in preparation of a drug for treating schistosomiasis (patent CN105078963 A). These authors shave showed that tocopherol has *in vitro* schistosomicidal activity, inhibiting the development of the reproductive organ of schistosome, and then, the eggs laid by schistosome in a host are reduced[27]. Also, *In vivo* experiments showed that the administration of tocopherol is able to reduce the number of testicles and cause testicular atrophy in male worms, while in female worms it was observed immature ovary, uterus and vitellarium, causing a significant reduction in eggs laid. However, in this work, the amount of tocopherol presented in the oil was not enough to cause any anti-parasitic activity[28, 29].

Table 3: In vitro effects of crude extract against adult worms of S. mansoni.

Groups	**Incubation period (h)**	**Dead worms (%)[a]**	**Decrease of motor activity (%)[a]**		**Worms with tegumental alteration (%)[a]**	
			Slight	**Significant**	**Slight**	**Significant**
Control [b]	24	0	0	0	0	0
	48	0	0	0	0	0
0.5 % DMSO	24	0	0	0	0	0
	48	0	0	0	0	0
PZQ[c]	24	100	0	100	0	100
	48	100	0	100	0	100

Oil	24	0	0	0	0	0
200 µg/mL	48	0	0	0	0	0

[a]Percentages relative to 20 worms investigated. [b]RPMI 1640. [c]Tested at concentration of 5 µM or 1.56 µg/mL.

4 CONCLUSIONS

Pumpkin seed oil of the Jacarezinho's cultivar belonging to the species Cucurbita moschata is characterized by a higher content of unsaturated fatty acids when compared to cultivars of the species Cucurbita pepo and maxima. In seed oil, unsaturated oleic and linoleic acids are dominant. Sc-CO_2 was able to extract pumpkin seed oil with a much higher yield than extraction with hexane/isopropanol. No differences were observed between the non-polar compounds present in the oil extracted by the Sc-CO_2 and hexane/isopropanol methods, demonstrating that Sc-CO_2 has a great affinity for hydrophobic compounds. The results indicated that the oil has an excellent quality, with a high content of unsaturated fatty acids and tocopherol presence. Although the oil is popularly used as an antiparasitic agent, in this study the oil did not cause mortality in tegumentary alterations or significant decrease in motor activity in all adult parasites after 24h of incubation.

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