CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608- 56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864037

Chemical Composition, Bioactivities and Kinematic Viscosity of *Cucurbita argyrosperma* Seed Oil

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The aims of this study were to perform a detailed analysis of the chemical composition by gas chromatography, cinematic viscosity in function of temperature by viscometer Schott AVS 350, and biological tests of *abóbora* (*Cucurbita argyrosperma*) seed oil, collected in Mucajai city, Roraima state, Brazil. Thus, the *abóbora* seed samples were manually submitted to the oven at 50 °C for 72 hours, and crushed and sieved between 20-40 Mesh. The seed oil extraction was carried out in a Soxhlet apparatus with hexane for 3 hours, providing an oil yield of 29.8%. In the results were identified a total of 8 fatty acids by gas chromatography; of these 33.8% are saturated fatty acids and 65.7% are unsaturated fatty acids: palmitic (21.5%), stearic (11.6%), oleic (37.0%), linoleic (28.1%) and linolenic (0.3%). In the biological test, the oil was more active against *Staphylococcus aureus* (29.01%), *Listeria monocytogenes* (34.56%), *Pseudomonas aeruginosa (23.86%)* and *Citrobacter freundii* (32.74%), which features a characteristic low therapeutic index for these bacteria. The kinematic viscosity of the oil reduces in function of temperature (298.15 to 353.15 K) in an exponential curve, demonstrating to refer to a Newtonian fluid.

1. Introduction

The pumpkins belong to the genus *Cucurbita* and to the Cucurbitaceae family, endemic of the Americas and widely used for economic purposes. The outstanding species of pumpkins are *Cucurbita argyrosperma*, *C. ficifolia*, *C. maxima*, *C. moschata* and *C. pepo* (Heiden et al., 2007). The species *C. argyrosperma* is also known as *C. mixta*, which is a species native to Mexico and Central America. It is observed proximity of *C. argyrosperma* with *C. moschata* (Merrick et al., 1990). There are three cultivated varieties belonging to the *C. argyrosperma* species, of which Argyrosperma and Stenosperma were selected in the production of seeds. The third variety, Callicarpa, is used in the production of fruits and seeds, because it adapts easily (Merrick et al., 1990). In Brazil there are still no records in the scientific literature regarding the cultivation of *C. argyrosperma* (Barbieri et al., 2008). The fruits of *Cucurbita* vary in size, color, shape and weight. They have a firm and rigid shell, dense pulp and a central cavity that holds the seeds. The fruit has many seeds, which in turn has a high content of vitamins and proteins (Sousa et al., 2012). These pumpkins seeds, according to folk medicine, are used as vermifuge, due to the anthelmintic action (Cruz et al., 2006), as well as, it assists in the maintenance processes of epithelial tissue, maintenance of growth, reproduction, immunological mechanisms and antioxidant action, due to the presence of zinc, calcium and vitamins A and E (Bueno, Czepielewski,

Please cite this article as: Estevam Ribeiro P.R., Souza V.D.S., Goncalves Reis De Melo A.C., Magalhaes Neto A.T., Carvalho Dos Santos R., Takahashi J.A., Ferraz V.P., Melo Filho A.A., 2018, Chemical composition, bioactivities and cinematic vicosity of cucurbita argyrosperma seed oil, Chemical Engineering Transactions, 64, 217-222 DOI: 10.3303/CET1864037

2007). They present anti-inflammatory and vermifuge action (Sant'anna, 2005; Suzuki, 2008) and studies on prostate cancer were also evaluated (Carbin et al.,1990).
The objective of this work was to measure the kinematic viscosity of the seed oil of *C. argyrosperma* seeds as a function of temperature, as well as to identify and quantify the chemical composition of seed oil by gas chromatography, to identify the main functional groups by Infrared, to determine biological properties against bacteria and fungus and inhibition of acetylcholinesterase.

2. Material and Methods

2.1 Sample preparation

The pumpkin (*C. argyrosperma*) seeds were manually obtained, dried in an air circulating oven at 50 °C for 72 hours, then ground and sieved between 20-40 mesh to obtain a homogenized powder. This powder was extracted with hexane in Soxhlet extractor for 3 h. The oil obtained was stored in amber flask under nitrogen atmosphere for further analysis. The solvent was removed by rotoevaporator (Santos et al., 2015).

2.2 Oil analysis by GC-FID

An aliquot (10 mg) of *C. argyrosperma* oil was transferred to a 2 mL cryotube, which contained 100 µL of a mixture made of ethanol (95%) and KOH 1 mol L^{-1} (5%). After vortexing for 10 s, esters in the oil were hydrolyzed in a microwave oven (Panasonic Piccolo) at 80 W (power 2) for 5 min. After cooling and neutralization with 400 µL of hydrochloric acid 20%, 20 mg NaCl and 600 µL of ethyl acetate were added. Afterwards, free fatty acids were obtained by using a protocol adapted from the literature (Christie, 1989). Thus, after vortexing for 10 s and rest for 5 min, aliquots (300 µL) of the ethyl acetate layer was taken, placed in microcentrifuge tubes and dried by evaporation. Free fatty acids were methylated using 100 µL of BF_3/methanol (14%) and the reaction mixture was heated for 10 min in a water-bath at 60 °C. After dilution with 400 µL methanol, fatty acid methyl esters were analyzed by Gas Chromatography equipped with flame ionization detector (GC-FID).
Free fatty acids were resolved by GC-FID using HP7820A (Agilent). An Innowax column (HP) 15 m × 0.25 mm × 0.20 µm was used and the following temperature gradient: 100 °C min^{-1} and 0.7 °C min^{-1} up to 240 °C; injector (1/30 split) to 250 and 260 °C detector. Hydrogen was used as carrier gas (3 mL min^{-1}) and injection volume was 1 µL. The data acquisition program used was EZChrom Elite Compact (Agilent). The peaks were identified using FAME Mix C14-C22, CRM18917 Supelco fatty acid methyl esters standard.

2.3 Determination of functional groups by Infrared spectroscopy

The sample was analyzed in the Infrared region using the IRPrestige-21 Shimadzu Spectrophotometer from the Post-Graduation Program in Chemistry of the Federal University of Roraima, using KBr pellets. For this analysis the following conditions were listed: measurement mode: absorbance; number of scans: 16; resolution: 4 cm^{-1}; wavelength range: 4000-400 cm^{-1}.

2.4 Kinematic viscosity

For the determination of the viscosity an AVS 350 viscometer with thermostatized bath CT52 and capillary with diameter of 1.01 mm, constant k equal to 0.09148 mm^2 s^{-1}, was used. The volume used was approximately 8 mL and the temperature varied between 303-363 K for *C. argyrosperma* oil in order to verify its behavior. The kinematic viscosity of *C. argyrosperma* seed oil was determined according to the instruction manual of the equipment. Equation 1 was used to determine the kinematic viscosity:

$$\mu = k.t \quad (1)$$

Where: µ = kinematic viscosity (mm^2 s^{-1}); k = capillary constant; t = time in seconds that the oil runs the space between the first and second marking of the capillary.

2.5 Acetilcolinesterase inhibition assay

Aliquots of a working solution (25 µL) (sample in DMSO 10 mg mL^{-1}) were added to microplate wells and positive and negative controls were also prepared. To the first five wells of a column (positive control) 25 µL of an eserine solution prepared at 10 mg mL^{-1} (31 mM; 2.7 mM in the whole reaction mixture 275 µL) in Tris/HCl at pH 8.0) was added. Then, 25 µL of acetylthiocholine iodide 15 mM; the reaction mixture, 125 µL of 5',5-dithio-bis (2-nitrobenzoate) (3 mM) and 50 µL of Tris/HCl (50 mM, pH 8) containing 0.1% (m/v) bovine serum albumin was added to each well. Absorbance was measured at 405 nm every 1 min for 8 times. Then 25 µL (0.226 U mL^{-1}) of Electric eel AChE (type VI-S) in Tris/HCl was added to each well. Absorbance was measured at 405 nm by 10 times (Frank and Gupta, 2005; Ellman et al., 1961).

2.6 Antimicrobial assay

Staphylococcus aureus, *Listeria monocytogenes, Pseudomonas aeruginosa, Listeria monocytogenes* (34.56%), *Citrobacter freundii* and *Candida albicans* were used in the assay following the procedures for inhibitory activity described below. Concentrations assayed were 12.5 mg mL^{-1} (Zacchino and Gupta, 2007). Samples were weighed and dissolved in DMSO to 12.5 mg mL^{-1}. Forty µL of this solution were added to a flask containing 960 µL of BHI (Brain Heart Infusion) broth (working solution). A pre-inoculum was prepared in which the bacteria and the yeast, stored under refrigeration, were transferred with a platinum loop to test tubes containing 3 mL of freshly made BHI broth. The tubes were incubated at 37 °C for 18 hours. Then, the pre-inoculum (500 µL) was transferred to tubes containing 4.5 mL of sterile distilled water. The tubes were homogenized and the concentration adjusted to 0.5 of McFarland turbidity standard (10^8 CFU mL^{-1}), thereby obtaining the inocula used in the bioassays. Assays were performed in 96-microwell plates in duplicate. One hundred µL of BHI broth was added to each well. In the first well 100 µL of working solution was also added. The solution was homogenized and 100 µL transferred to the next well and so on until the last well, from where 100 µL was discarded. Then, 100 µL of microorganism inocula was added to wells. Eight different concentrations of each sample were tested. A positive control devoid of the working solution allowed us to examine microorganism growth. A negative control, which lacked the inoculum permitted us to discount the color coming from the working solution. A control plate containing 100 µL of BHI culture medium and 100 µL of sterile distilled water were added to the experiment as a control of BHI broth sterility. Microorganism growth was measured in ELISA plate reader (492 nm) immediately after ending the experiment (0 h). They were incubated at 37 °C and read again after 24 h of experiments, ending the test.

3. Results and Discussion

3.1 Yield of *C. argyrosperma* oil and its lipid composition by GC-FID

The vegetable oil of *C. argyrosperma* obtained by Soxhlet method showed a greenish coloration and yield of 29.8% of oil; compared to the literature, the yield of *Cucurbita* ranges from 20 to 50%. Sousa (2012) reported a yield of 47%. Regarding to the lipid composition of the above-mentioned *C. argyrosperma* oil, it is possible to observe the oil chromatogram (Figure 1 and Table 1) showing eight saturated and unsaturated fatty acids.

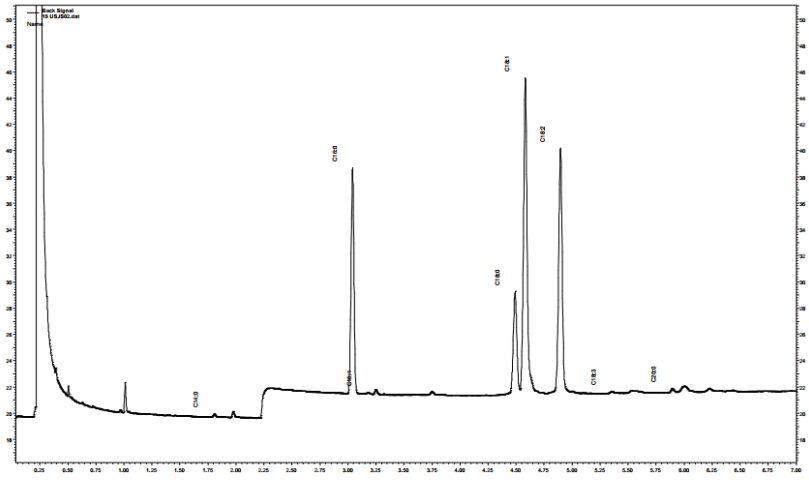

Figure 1: Chromatogram obtained by GC-FID of the oil of the seeds of C. argyrosperma

Table 1: Fatty acid profile of the oil from seeds of C. argyrosperma

C14:0	C16:0	C16:1	C18:0	C18:1 (ω-9)	C18:2 (ω-6)	C18:3 (ω-3)	C20:0	Outros
Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Eicosanoic acid	
0.3%	21.5%	0.3%	11.6%	37.0%	28.1%	0.3%	0.4%	0.5%

The main saturated fatty acids were palmitic acid (21.5%) and stearic acid (11.6%), while the majority unsaturated fatty acids are oleic (37.0%) and linoleic (28.1%) acids. These fatty acids have great importance

in human health, such as for prevention or reduction of cancer, autoimmune diseases, antirheumatic, anti-inflammatory, antidiabetic, coronary atherosclerosis, among many others (Sales-Campos et al., 2013; Lou-Bonafonte et al., 2012; Carrillo; Cavia; Alonso-Torre, 2012; Pauwels, 2011; Bermudez et al. 2011; Vassiliou et al., 2009; Sales; Oliviero; Spinella, 2009; Colomer; Menéndez, 2008; Menendez; Lupu, 2006).

3.2 Identification of functional groups by IR

In order to prove the lipid composition of the oil from seeds of *C. argyrosperma*, the non-destructive spectroscopic technique of Infrared (IR) was used, which can help to identify the main functional groups of fatty acids. For this reason, it was possible to attribute the characteristic band absorptions of triacylglycerides as a strong and intense band at approximately 1750 cm^{-1}, referring to the carbonyl group (C=O) of carboxylic acids, as well as an absorption at 3005 cm^{-1} (CH stretch). The absorptions between 2925 and 2850 cm^{-1} refer to the axial deformation of C-H bond (sp^3-s), in which the strong absorption at 2925 cm^{-1} refers to the methyl group (CH_3), followed by an absorption of mean intensity at 2850 cm^{-1}, attributed to the methylene groups CH_2. A band at 1450 cm^{-1} is also attributed to a symmetrical angular strain of the methylene group in the plane. At 1150 cm^{-1} a characteristic axial intense deformation band of the functional group (C-O-) is observed and, finally, a absorption at 725 cm^{-1}, attributed to out-of-plane deformation of the (CH) groups (Castilho-Almeida et al., 2012; Lerma-García et al., 2010).

3.3 Kinematic viscosity

The determination of the kinematic viscosity was verified by multiplying the capillary constant versus the time of oil drop. For the best fit of the exponential curve, Andrade equation was applied, where Pi and Pf are estimated parameters depending on the nature of the sample, μ = kinematic viscosity in $mm^2\ s^{-1}$. Figure 2 shows the exponential curve of the kinematic viscosity of *C. argyrosperma* oil in relation to temperature.

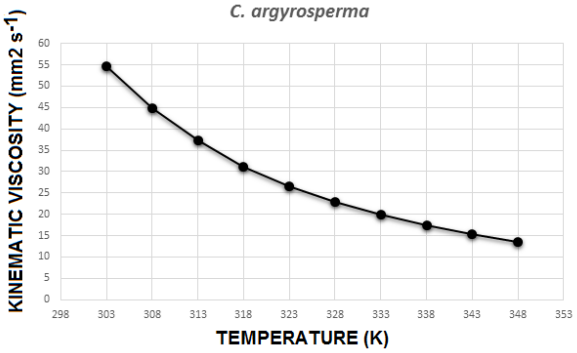

Figure 2: Kinematic viscosity of C. argyrosperma oil in relation to temperature

3.4 Antiacetylcholinesterase assay

Seed oil from *C. argyrosperma* inhibited 13.26% of the acetylcholinesterase enzyme. According to Vinutha et al. (2007) inhibition values below 30% are considered a weakly inhibiting oil, while values between 30 and 50% means moderate and above 50% potent inhibitors. Trevisan and Macedo (2003) verified that from 58 Brazilian plants, 11 species showed to be potent inhibitors and eight were moderate inhibitors of the enzyme. Acetylcholinesterase is of great importance in the human body, since this enzyme is responsible for the transmission of impulses in cholinergic synapses and thus hydrolyze the neurotransmitter acetylcholine in acetate and choline (Čolović et al., 2013). Change in this pathway can generate neurodegenerative diseases, from which the most common is Alzheimer's disease (AD), which is increasing alarmingly in the world. According to the World Health Organization (WHO, 2012) the Alzheimer's disease will affect more than 115 million people by 2050.

3.5 Antimicrobial assay

Many of the elderly patients or those bearing some diseases such as cancer, diabetes, among others, may have low immunity, allowing fungus such as *C. albicans* to take advantage of the situation causing candidemia (Giolo and Svidzinski, 2010).The bacteria listed in Table 2 are also of great concern in human health and may therefore cause great harm to the health of individuals as well as to the economy. Some known diseases caused by *S. aureus* are skin infections and pneumonia (Sung et al., 2008). *L. monocytogenes* is an opportunistic pathogen and causes listeriosis. In severe cases it can lead to death (Cruz et al., 2008). As for *C. freundii*, this remains one of the causes of neonatal bacterial meningitis (Badger et al., 1999). And finally, *P.*

aeruginosa, which is the main pathogen causing pulmonary cystic fibrosis (Davies, 2002). Table 2 shows the results of inhibitions of *C. argyrosperma* oil on Gram (+) bacteria: *S. aureus*; *C. freundii* and Gram (-) bacteria: *L. monocytogenes*; *P. aeruginosa* and also against the fungus *C. albicans*.

Table 2: Bioactivity on bacteria and fungus

Bacteria	**% Inhibition**		
	C. argyrosperma oil	Ampicilin	
S. aureus	29.01 ± 3.73	94.64 ± 0.62	
C. freundii	32.74 ± 2.27	94.51 ± 0.55	
L. monocytogenes	34.56 ± 2.80	94.65 ± 0.73	
P. aeruginosa	23.86 ± 2.22	94.64 ± 0.74	
Fungus	**% inhibition**		
	C. argyrosperma oil	Miconazole	Nystatin
Candida albicans	0.00	94.80 ± 0.71	92.78 ± 0.50

Inhibition of the bacteria was above 50% and there was no inhibition of the fungus, but it is worth mentioning that vegetable oil is actually a crude extract obtained from a vegetable source and applied directly on the microorganisms, therefore different from ampicillin, miconazole and nystatin, which are pure compounds known for the antimicrobial biological function. According to the World Health Organization (WHO, 2014) there is concern about resistance development due to the use of nonprescription antibiotics, being necessary to search for new drugs.

4. Conclusions

The vegetable oil of *C. argyrosperma* from Roraima, Brazil, presented yield similar to those found in the literature. The seeds oil presents a profile of fatty acids favorable to human health, since it has a high concentration of omega 9 in its composition, followed by omega 6 and 3. It is noteworthy that the consumption of oils rich in omega 9 presents many benefits to humans, as for example in the prevention or reduction of diseases like cancer, diabetes, autoimmune, among others. Therefore, it was observed an excellent potentiality of the seed oil studied. The result of this research around pumpkin oil proved potentially weak on the inhibition of acetylcholinesterase, bacteria and no inhibition on the fungus. The kinetic viscosity decreases with increasing temperature, thus presenting a behavior of a Newtonian liquid.

Acknowledgments

UFRR and CNPQ for their financial support.

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