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Fatty Acid Composition, Acetylcholinesterase and Bacterial Inhibition by *Inga cinnamomea* Pulp

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Abstract

Inga cinnamomea, a species from Fabaceae family, posses a convex-cylindrical fruit that has a white, slightly sweet, edible pulp, which is very appreciated in Brazil. The present study seeks to expand the knowledge about this fruit. The vegetable oil of the fruit's pulp, extracted in a Soxhlet extractor using hexane as solvent, was studied. Fatty acids were determined, after oil hydrolysis and methylation, using gas chromatography with flame ionization detector (GC-FID). Additionally, the oil was tested to determine its potential as acetylcholinesterase inhibitor using Eserine as positive control. Bactericidal potential of the oil was also determined. Both assays were accomplished using Elisa spectrophotometer. Eight major fatty acids were detected in the following concentrations: linoleic (ω6) (31.7%), palmitic (26.2%), linolenic (ω3) (13.6%), oleic (ω9) (12.5%), stearic (6.5%), palmitoleic (ω7) (2.0%), myristic (0.6%) and arachidic (0.6%) acids. The oil inhibitory activity towards acetylcholinesterase enzyme was 54.81%, being classified as a potent effect. Finally, the oil presented a modest inhibitory activity against the following bacterial strains: *Staphylococcus aureus* (24.68%), *Citrobacter freundii* (20.46%), *Listeria monocytogenes* (27.26%) and *Pseudomonas aeruginosa* (26.89%).

Keywords: *Inga cinnamomea*, fabaceae, oil, acetylcholinesterase, fatty acid

1. Introduction

Inga cinnamomea is a plant species from Fabaceae family, genus Mimosa. *Inga cinnamomea* is commonly known as ingá açu, ingá pracuuba, ingá chinelo and ingá guaçu in Amazonas (Brazil) (Peningtong, 1997). The importance of legumes in the world is due, mostly because of the content of proteins and minerals in their seeds, many of them for human and animal consumption (Smith et al., 2004; Heywood et al., 2007). It is also important their ability of enriching nutrient-poor soils by fixing nitrogen in symbioses with *Rhizobium* bacteria (Smith et al., 2004; Heywood et al., 2007; Kurppa et al., 2010; Remigi et al., 2016; Montandon et al., 2010; Grossman et al., 2006; Franco & Faria, 1997).

Particularly, the ingá trees are distributed throughout the world within the Bolivian Amazon, Peru, Equador, Colombia, extending within the Brazilian Amazon (Peningtong, 1997). In Mexico were reported the presence of

the following ingá species *I. alba*, *I. densiflora*, *I. multijuga*, *I. oerstediana*, *I. paterno*, *I. ruiziana*, *I.vera*, *I. bella*, *I. cabrerae*, *I. calcícola*, *I. cuspidata*, *I. chiapensis*, *I. dasycarpa*, *I. davidsei*, *I. dwyeri*, *I. ismaelis*, *I. lacustris*, *I. pseudoinvolucrata*, *I. sinacae*, *I. tenella* (Sousa, 1993). While in Brazil, the most common species are *I. cylindrica*, *I. laurina*, *I. marginata*, *I. edulis*, *I. vera*, and *I. cinnamomea* (Lorenzi, 2002). From them, only a few have been studied so far.

Between the species of ingá already studied, stand out the species *I. edulis* and *I. laurina*. It has been determined that the hydroalcoholic extract of *I. edulis* leaves has antinociceptive, anti-inflammatory, antiulcerogenic activity (Pompeu et al., 2012), as well as antioxidant, photoprotective and anti-inflammatory potential to the skin (Costa, 2015). The antioxidant activity of *I. edulis* leaves has been tested in several studies, showing high activity (Pompeu et al., 2012; Sousa et al., 2010; Sousa et al., 2007). It is also active in cancer prevention (Yang et al. 2001), has ability to inhibit specific enzymes, simulate some hormones or neurotransmitters, and eliminate free radicals (Havsteen, 2002). Additionally, from the fresh pulp of the *I. edulis* fruit, was extracted a peroxidase enzyme that has been used as a biosensor to quantify the antioxidant terc-butylhydroquinone (TBHQ) in food (Oliveira et al., 2014).

On the other hand, from the *I. laurina* seeds was extracted a Kunitz inhibitor called ILTI (*Inga laurina* Trypsin Inhibitor) (Macedo et al., 2007). It showed insecticidal activity against *Diatraea saccharalis*, *Heliothis virescens* (Ramos et al., 2012) and *Homalinotus coriaceus* (Macedo et al., 2011). This inhibitor also showed antimicrobial activity, especially against *Candida tropicalis* and *Candida buinensis* (Macedo et al., 2016). Besides, from the *I. laurina* seeds was extracted a new triterpenoid saponin named "ingasaponin", a component with immunological adjuvant activity as well as a significant hemolytic potential (Cruz et al., 2016). Therefore, ingá species present a great potential for several uses. In that sense, this work expects to contribute with the scientific knowledge on *I. cinnamomea* species, in order to point out new potentialities or significant properties to be further exploited.

Inga cinnamomea is yellow-green and convex-cylindrical fruit at maturity. In average, the seeds of *I. cinnamomea* measure 4 × 1.5 × 1.3 cm (Peningtong, 1997), have pleurogram (Smith et al., 2004), and are surrounded by a white pulp, slightly sweet, edible and very appreciated by the population (Galvão, 2005). *I. cinnamomea* pulp has been poorly studied, what motivates the accomplishment of this work. Nevertheless, it was reported that *I. cinnamomea*'s pulp is a source of minerals like Mg, Na, P, Mn, Fe, and Zn (Berto et al., 2015). Regarding the chemical composition of *I. cinnamomea*'s fruits pulp, it was reported the presence of volatile constituents such as methyl linoleate, chenopodiol, ethyl hexadecanoate and heptacosane (Silva et al., 2012). Also, several fatty acids have been reported in the pulp of this ingá, the more abundant being linoleic, palmitic, linolenic and oleic acids (Berto et al., 2015).

The objective of this study was to verify the fatty acid profile of the vegetable oil from the pulp of *Inga cinnamomea*, as well as to analyze the acetylcholinesterase and bacteria growth inhibition effect exerted by this oil.

2. Method

2.1 Identification of the Species and Obtaining the Fruit

The species was identified using a voucher specimen deposited in the Integrated Museum of Roraima (MIRR: 13654). Later the fruits were collected in Asa Branca neighborhood, Boa Vista, Roraima (lat: 2.81132; long: 60.720779).

Mature fruits with good physical appearance were collected and taken to the Environmental Chemistry Laboratory at the Federal University of Roraima, Boa Vista, Brazil.

2.2 Oil Extraction from Inga cinnamomea Pulp

Fruits were cleaned, and the pulp was separated from the peel and the seeds. Thereafter, the pulp was dried at 50 °C for 72 h. Dried pulp was ground, and the powder sieved with a 20-40 Mesh sieve. The vegetable oil obtained from the pulp was extracted using hexane as extraction solvent in a Soxhlet extractor for 3 h. It was done in triplicate and the oil was stored in amber bottles under nitrogen atmosphere and stored in a freezer (Fernández et al., 2016; Santos et al., 2015; Jorge & Luzia, 2012).

2.3 Hydrolysis and Methylation of Inga cinnamomea Vegetable Oil for Chromatographic Analysis

Hydrolysis was carried out using 12 mg of the oil sample in 100 μL of ethanol (95%)/potassium hydroxide 1 mol L^{-1} (5%) solution. After vortexing for 10s, the oil was hydrolyzed in a domestic microwave oven (Panasonic NN-ST254W) at 60% power (420 W) during 6 minutes. After cooling, were added 400 μL of hydrochloric acid 20%, NaCl (~20 mg) and 600 μL of ethyl acetate. After vortexing for further 10 sec and standing for 5 min, an

aliquot (300 μL) of the organic layer was removed, placed in microcentrifuge tubes and dried by evaporation, thus obtaining the free fatty acids (Christie, 1989). Subsequently, the free fatty acids were methylated with 100 μL BF_3/methanol (14%), BF_3 being the reaction catalyst. The mixture was heated for 10 minutes in at 60 °C water bath. The methylated fatty acids were extracted with 500 μL of hexane and analyzed by Gas Chromatography.

2.4 Determination of Fatty Acids by Gas Chromatography with Flame Ionization Detector (GC-FID)

The analyses were performed on a Gas Chromatograph HP7820A (Agilent) equipped with a flame ionization detector. Data Acquisition Program used was EZChrom Elite Compact (Agilent). It was used a Supelcoxax column [15 m × 0.2 mm × 0.2 μm (Supelco)] with temperature gradient: 120 °C, 0 min, 7 °C min^{-1} to 240 °C; Injector (1/50 split) at 250 °C and detector at 260 °C. Hydrogen was used as mobile phase at a rate of 3.0 mL min^{-1}. The volume of injection was 1 μL and peak identification was accomplished by comparison with methylated fatty acid standards Supelco37 Fame mix (Supelco cat no 47885-U) (Christie, 1989).

2.5 Acetylcholinesterase Inhibition Assay

A sample working solution (10 mg mL^{-1}) was prepared in DMSO. Then, 25 μL of this working solution was added to the wells of an Elisa plate. In addition, negative and positive controls were prepared. Eserine was used as positive control. In the first five wells of the positive control column, were added 25 μL of Eserine solution (10 mg mL^{-1} in Tris/HCl; pH 8.0 buffer), and 25 μL DMSO. To each well, were added 25 μL of acetylcholineiodide iodide (ATCI), 125 μL of DTNB solution (5',5-dithio-bis-(2-nitrobenzoate, Sigma) and 50 μL Tris/HCl 50 mM) with bovine serum albumin. The absorbance was measured at 405 nm every 1 min, 8 times (8 min in total). Then, 25 μL of the AChE electric eel solution (0.222 U mL^{-1}) in Tris/HCl were added to each well. The absorbance at 405 nm was measured 10 times (10 min in total) in a microplate reader (Ellman et al., 1961; Frank & Grupta, 2005).

2.6 Bacteria Inhibition Assay

For the bioassay of inhibitory microbial activity, in first place a pre-inoculum was prepared. Therefore, 3.0 mL of BHI (Brian heart infusion) culture medium was placed in test tubes and then the bacteria were transferred into these test tubes using a platinum loop. The tubes were incubated in an oven at 37 °C for 36 h. Using a micropipette, 200 μL of this pre-inoculum were transferred to test tubes containing sterile distilled water. The bacteria used were: *Listeria monocytogenes* (ATCC 15313), *Staphylococcus aureus* (ATCC29212), *Citrobacter freundii* (ATCC8090) and *Pseudomonas aeruginosa (*ATCC27853).

In order to obtain the inocula used in the bioassay, the aforementioned tubes were homogenized and then absorbances of the solutions were read in an Elisa spectrophotometer. The concentration of the inocula was adjusted at 600 nm, until reach a transmittance reading between 74-75%, corresponding to 10^8 cells mL^{-1} or 0.5 turbidity according to the McFarland standard turbidity scale. The pulp oil sample was solubilized in dimethylsulfoxide (DMSO) obtaining a solution with concentration of 12.5 mg mL^{-1}. Then, 40 μL of this solution were added to 960 μL of culture medium to prepare the working solution.

The bioassays were run in 96 micro wells in triplicate. In the well 1 (test) were added 100 μL of the working solution and 100 μL of standardized inoculum. In well 2 were added 100 μL of culture medium and 100 μL of standardized inoculum for growth control of the microorganism. In well 3 were added 100 μL of culture medium and 100 μL of sterile distilled water. The positive control was performed in well 4, replacing the working solution with the antibiotic ampicillin. The sterility control of the culture medium was made in well 5, containing 100 μL culture medium and 100 μL of sterile distilled water. The microplates were incubated in an incubator at 37 °C and, after 24 h, results reading was performed in an Elisa type reader (600 nm) (Santos et al., 2015).

3. Results and Discussion

3.1 Fatty Acids Determination and Quantification by Gas Chromatography Using Flame Ionization Detector (GC-FID) and Yield of Oil in I. cinnamomea Pulp

Extraction with hexane from *I. cinnamomea* pulp yielded 0.42% of vegetable oil. In contrast, Berto et al. (2015) reported a yield of 0.08%, a lower yield of vegetable oil compared to the result found in this research. This discrepancy is probably due to the differences in the methodology used in both researches, since a different solvent (Choroform) and a different extraction methodology was utilized. Analysis of the oil with GC-FID detected eight fatty acids, as shown in the chromatogram presented in Figure 1.

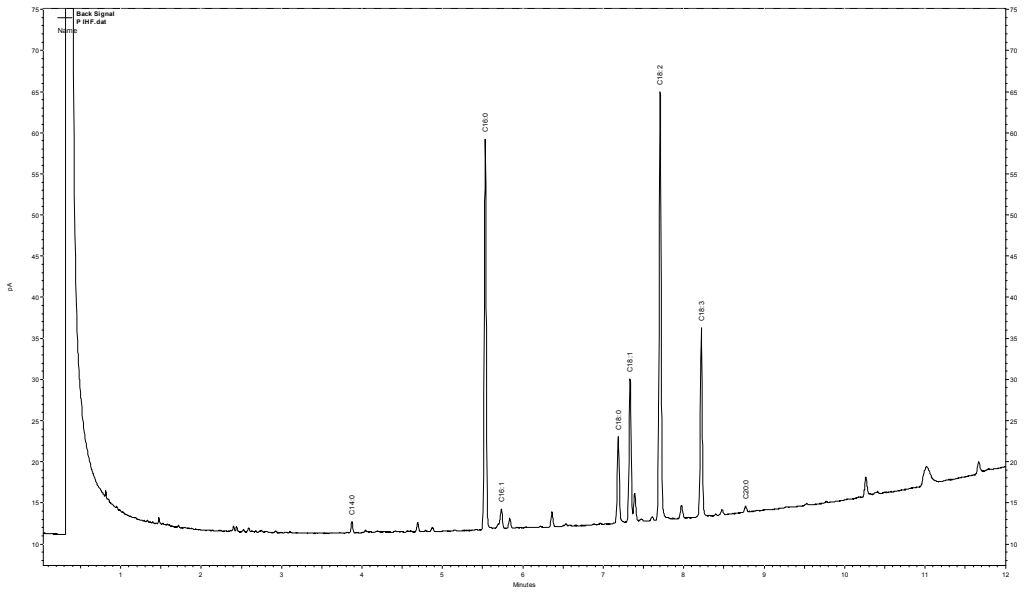

Figure 1. Fatty Acids Chromatogram of *I. cinnamomea cinnamomea* pulp

In Table 1 is presented a list of the fatty acids found, together with their respective concentration (%) compared to data reported in the literature.

Our results show that, in this oil, polyunsaturated fatty acids predominate, representing 45.3% of the total, whereas the saturated ones represent 33.9% and the monounsaturated form 14.5% of the total content. It is possible to note that the more abundant fatty acids in this oil are linoleic (ω6) (31.7%), palmitic (26.2%), linolenic (ω3) (13.6%) and oleic (ω9) (12.5%).

Linoleic (18:2 ω6) and linolenic acids (18: 3 ω3) are essential acids but cannot be synthesized by human body. These are required to maintain cell membranes under normal conditions (Food Ingredients Brazil, 2016; Strayer et al., 2016), brain functions and are necessary for transmission of nerve impulses, among many other functions (Food Ingredients Brazil, 2016). Lack of linolenic acid (ω3) has been associated with neurological abnormalities and poor growth (Strayer et al., 2016).

Omega 3 fatty acid can help improving concentration and memory. Omega 3 and 9 fatty acids are related to healthier triglyceride levels, and decreased levels of total cholesterol in blood (Food Ingredients Brazil, 2016).

The polyunsaturated and monounsaturated acids which predominate in this oil do not form fatty deposits that black the arteries, as occurs with saturated fatty acids (Food Ingredients Brazil, 2016). Other fatty acids found in the oil, at a lower concentration, were stearic (6.5%) and palmitoleic (ω7) (2.0%) acids, with myristic and arachidic acids both being present in still smaller quantities (0.6%).

Fatty acids found in this research correspond quite well with previous report found in the literature for *I. cinnamomea* (Table 1). However, some differences in concentrations of fatty acids were observed when comparing the results of this study with those reported by Berto et al. (2015). The differences were more noticeable for palmitoleic, oleic, linoleic and linolenic acids, but were never higher than 4%.

Since these fatty acids were quantified in plants grown in different states of Brazil, the differences in oil quantities may result in the different influences of environmental factors (Crowley & Fröhlich, 1998; Francis & Campbell, 2003).

In addition, the oils were extracted using different methodologies, including different extraction solvent, chloroform (Berto et al., 2015) vs hexane, which could also have influenced the final quantification of fatty acids.

Table 1. Fatty acids in vegetable oil from *I. cinnamomea* pulp compared to literature data

Fatty Acid	RT (min)	*I. cinnamomea* Current study	*I. cinnamomea* (Ingá açu) (Berto et al., 2015)	*Hymenaea courbaril* (Jatobá) (Dias et al., 2013)
		%	%	%
Myristic (C14:0)	3.881	0.6	0.7	0.2
Palmitic (C16:0)	5.558	26.2	27.2	25.1
Palmitoleic (ω7) (C16:1)	5.687	2.0	3.5	1.8
Stearic (C18:0)	7.236	6.5	5.7	3.1
Oleic (ω9) (C18:1)	7.432	12.5	8.9	46.1
Linoleic (ω6) (C18:2)	7.748	31.7	35.0	8.6
Linolenic (ω3) (C18:3)	8.223	13.6	10.6	14.5
Arachidic (C20:0)	8.924	0.6	0.4	0.6
Other	-	6.4	8.0	-
Total	-	100	100	100
Saturated Fatty Acids	-	33.9	34.0	29.0
Monounsaturated Fatty Acids	-	14.5	12.4	47.9
Polyunsaturated Fatty Acids	-	45.3	45.6	23.1

In Table 1, the results of the fatty acid content in the *I. cinnamomea* pulp are also compared with the results obtained for *H. courbaril* pulp, known as Jatobá, a plant that also belongs to the Fabaceae family. The fruit contains a floury, yellowish and smelly pulp, which is consumed as food in several countries (Flores & Benavides, 1990).

When comparing the results of the pulps of these two fruits belonging to the same family, a similar percentage of fatty acids was observed. A notorious difference was found concerning to oleic (ω9) and linoleic (ω6) acids. The concentration of oleic acid (ω9) in the *I. cinnamomea* pulp is 12.5% whereas for *H. courbaril* it is 46.1%, being more concentrated in the species *H. courbaril*. At the same time, the concentration of linoleic acid (ω6) in *I. cinnamomea* was 31.7% whereas for *H. courbaril* it was 8.6%. Also, *I. cinnamomea* has been found to have triple the amount of stearic acid than *H. courbaril*. Palmitic acid was one of the major constituents in both species and also was found in similar concentrations. Other fatty acids found in similar concentrations in both species were myristic, palmitoleic, linolenic and arachidic acids.

As result of the differences in fatty acids concentrations in these species, *I. cinnamomea* has a higher concentration of polyunsaturated acids, whereas *H. courbaril* has a higher percentage of monounsaturated acids.

3.2 Acetylcholinesterase Inhibition of Vegetable Oil from I. cinnamomea Pulp

Acetylcholine is a neurotransmitter involved in memory and association processes (Flores & Segura, 2003). Increasing the concentration of acetylcholine in the brain by modulating the activity of the enzyme acetylcholine esterase (AChE) is one of the most promising therapeutic strategies to improve cognitive function in patients with Alzheimer's disease (Pakaski & Kasa, 2003; Trevistan et al., 2003), since this enzyme terminates the neurotransmission mediated by acetylcholine, hydrolyzing it (Soreq & Seidman, 2001).

The vegetable oil of *I. cinnamomea* pulp inhibited acetylcholinesterase enzyme in 54.81%, as shown in Table 2.

Table 2. Acetylcholinesterase inhibition of vegetable oil from *I. cinnamomea* pulp

Sample	% Inhibition
I. cinnamomea Oil Pulp	54.81±0.79
Positive control: Eserine	92.93±0.10

This percentage of inhibition is considered potent, according to the classification given by Vinutha et al. (2007) in which any result greater than 50% is considered potent, values between 30-50% of inhibition are considered moderate, and below 30% are considered weak inhibitions. Therefore, vegetable oil from *I. cinnamomea* pulp has potential to be studied for therapeutic purposes to treat Alzheimer's disease.

3.3 Bacteria Inhibition of Vegetable Oil from I. cinnamomea Pulp

In Table 3 are presented the results of growth inhibition of bacteria *S. aureus*, *C. freundii*, *L. monocytogenes*, *P. aeruginosa* using the vegetable oil from *I. cinnamomea* pulp. As can be observed, inhibition of these bacteria

growth was poor compared to the positive control used (Ampicillin), nevertheless a certain inhibition was observed. The importance of inhibiting the growth of these bacteria relies in the severity of some diseases and infections that these bacterial species can cause.

Table 3. Bacteria inhibition of vegetable oil from *I. cinnamomea* pulp

Bacteria	% Inhibition *I. cinnamomea*	% Inhibition Ampicillin
S. aureus	24.68±1.98	94.64±0.62
C. freundii	20.46±1.88	94.51±0.55
L. monocytogenes	27.26±1.25	94.65±0.73
P. aeruginosa	26.89±0.12	94.64±0.74

Listeria monocytogenes produces a disease called Listeriosis. Some of the symptoms of this infection are diarrhea and fever. *Staphylococcus aureus* can produce enterotoxins that cause the symptoms of food poisoning, can cause infection in wounds, and are a common cause of post-surgical infections (Pelczar & Krieg, 2010). *Pseudomonas aeruginosa* also causes hospital infections. An initial localized infection may lead to invasion of the circulatory system. In a patient with cystic fibrosis, microorganisms often cause severe and fatal pneumonia (Pelczar & Krieg, 2010). Finally, *Citrobacter freundii* causes a variety of infections in the elderly, immunocompromised, and debilitated patients (Wang et al., 2000). Cases of meningitis caused by this bacterium have been reported (Tang et al., 1994).

4. Conclusion

The vegetable oil of *I. cinnamomea* pulp is constituted by eight major fatty acids, detected in the following concentrations: linoleic (ω6) (31.7%), palmitic (26.2%), linolenic (ω3) (13.6%), oleic (ω9) (12.5%), stearic, palmitoleic (ω7) (2.0%), myristic (0.6%) and arachidic (0.6%) acids. This oil has also a potent inhibitory activity (54.81%) of the enzyme acetylcholinesterase, a feature that can be exploited in the future for therapeutic use in Alzheimer's disease. Finally, a discrete bactericidal inhibition of this oil was determined on the bacteria *Staphylococcus aureus* (24.68%), *Citrobacter freundii* (20.46%), *Listeria monocytogenes* (27.26%) and *Pseudomonas aeruginosa* (26.89%).

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