The seed oil of *Annona salzmannii* A. DC. was analyzed by GC-MS and 1H qNMR, revealing a mixture of unsaturated (80.5%) and saturated (18.7%) fatty acids. Linoleic (45.3%) and oleic (33.5%) acid were the major unsaturated fatty acids identified, while palmitic acid (14.3%) was the major saturated fatty acid. The larvicidal effects of *A. salzmannii* seed oil were evaluated against third-instar larvae of *Aedes aegypti* (Linn.). The oil exhibited moderate larvicidal activity, with a LC50 of 569.77 ppm (95% CI = 408.11 to 825.88 ppm). However, when the cytotoxic effects of the oil were evaluated, no expressive antiproliferative effects were observed in tumor cell lines B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia), HL-60 (human promyelocytic leukemia), and non-tumor cell line PBMC (peripheral blood mononuclear cells), with IC50 values > 50 μg·mL⁻¹. This is the first study to evaluate the chemical composition, larvicidal and cytotoxic activity of *A. salzmannii* seed oil.

**Keywords:** *Annona salzmannii*. Annonaceae. Larvicidal activity. Cytotoxic activity. Seed oil.

**INTRODUCTION**

The family Annonaceae is extremely diverse, having a pantropical distribution comprised of approximately 2500 species distributed throughout 135 genera. In the neotropics, the family is represented by 40 genera composed of approximately 900 species distributed across the Amazonia and Guianas (Chatrou, Rainer, Maas, 2004). Species of the family Annonaceae are extremely common in Brazil, mainly in Bahia, Minas Gerais, São Paulo, Pernambuco, and Paraíba (Sobrinho, 2010; São José *et al.*, 2014). Due to its economic importance, the genus *Annona* is significant, with emphasis being placed on species with market potential, including *A. muricata* L. (“graviola”), *A. squamosa* L. (“pinha” or “fruta-do-conde”), and *A. atemoya* (a hybrid of *A. squamosa* and *A. cherimola*) (Pereira *et al.*, 2011; São José *et al.*, 2014). These species produce exotic and savory fruits having nutritional value and bioactive properties, which in turn bring health benefits (Jiang, Bryce, Horrobin, 1998; Luzia, Jorge, 2012; São José *et al.*, 2014). These characteristics have led to great acceptance of these fruits in the world market. Additionally, some species have been reported in the literature as having pharmacological importance in the production of phytochemical (São José *et al.*, 2014) and...
insecticidal properties for the control of *Aedes aegypti* (Ae. aegypti) (Linn.) (Diptera: Culicidae) (Dill, Pereira, Costa 2012; Costa et al., 2013c; Grzybowski et al., 2013).

**Zika**, Dengue, and Chikungunya are diseases transmitted to humans by the mosquito *Ae. aegypti* L. After viral infection, these diseases cause flu-like symptoms that may progress into more serious conditions (WHO, 2015). The Zika virus has caught the attention of world authorities as it can cause microcephaly up to eight months after viral clearance and be sexually transmitted. Although the disease itself is not vectored in non-tropical regions, it has become a global concern (Rasmussen, 2016) due to the virus being commonly found in locations having high tourist traffic. Since there are no specific treatments or vaccines for Zika and Chikungunya, the only method for controlling and/or preventing viral transmission is to control the mosquito vector through environmental management and chemical methods.

Larvicides target larvae in breeding sites before they grow into unmanageable adult targets. Larviciding is considered the most appropriate method for *Ae. aegypti* L. control due to its method of action occurring during the early stages of larval development in a contained environment. Although there are several larvicides available for mosquito control, only six products for *Ae. aegypti* L. control in drinking water have been approved by the WHO: organophosphate temephos, *Bacillus thuringiensis israelensis* (BTI) protein, chitin synthesis inhibitors diflubenzuron and novaluron, and juvenile hormone analogs methoprene and pyriproxyfen (WHO, 2006). The application of larvicides and insecticides, such as malathion, as space sprays have resulted in mosquito resistance in subtropical and tropical regions of the world. Additionally, non-selective chemical usage has caused damage to non-target organisms, other than the transmitting vector, and the environment (Songa, 2016). As a result, pesticide resistance has persuaded researchers to find new methods to control *Ae. aegypti* L. proliferation involving syntheses of hit molecules and formulation, implementation, and efficacy evaluations of new plant extract-based products in the field.

Seeds of Annonaceae are an interesting source of vegetable oils that are currently discarded. These oils are mostly composed of essential fatty acids (EFAs), such as oleic acid (OA), stearic acid (SA), and linoleic acid (LA). These EFAs cannot be biosynthesized by humans and then must be obtained from the diet. Moreover, several studies have reported the cytotoxic effects of EFAs on different types of tumor cell lines (Meterissian et al., 1995; Du Toit, Van Aswegen, Du Plessis, 1996; Comba et al., 2010; Moon, Batirel, Mantzoros, 2014; Soto-Gusman et al., 2013; Zajdel, Wilezock, Tarkowski, 2015). Therefore, the search for new sources of EFAs is essential, and the seeds of edible fruits are an interesting option for consideration in this context as well as serving as potential sources of fatty oils for production of edible oils and condiments (Ferreira et al., 2006; Silva et al., 2015; Issaoui, Delgado, 2019; Ramadan, 2019). Additionally, species of the family Annonaceae have been shown to be promising sources of alternative fuels for conventional diesel engines and biodiesel production (Reyes-Trejo et al., 2014; Senthil, Silambarasan, 2015).

*Anonna salzmannii* A. DC. (popularly known as “araticum-da-mata” or “araticum-apê”) is a 6–20 m-tall tree found in northeast Brazil, mainly in Pernambuco, Paraiba, and Bahia (Pereira et al., 2011; São José et al., 2014). Their leaves, bark, roots, and seeds of *A. salzmannii* are used in traditional medicine to treat human illnesses, such as diabetes, tumors, ulcers, and inflammation (Corrêa, 1984). Previous studies on the chemical composition, antioxidant, antitumor, trypanocidal, antimicrobial, and larvicidal activity of essential oils from the leaves (Costa et al., 2011; Costa et al., 2013a), antitumor activity of extracts from the leaves and bark (Ribeiro et al., 2012), and antimicrobial and antioxidant activity of alkaloids from the bark (Paulo et al., 1992; Costa et al., 2013b) regarding *A. salzmannii* can be found in the literature.

The present study aimed to evaluate the chemical composition of *A. salzmannii* seed oil by means of gas chromatography-mass spectrometry (GC-MS) and 1H quantitative nuclear magnetic resonance (qNMR) spectroscopy and investigate its larvicidal activity against *Ae. aegypti* L. larvae, as well. Furthermore, cytotoxic effects were investigated against tumor cell lines B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia), HL-60 (human promyelocytic leukemia), and non-tumor cell line PBMC (peripheral blood mononuclear cells) in order to improve the chemical and biological knowledge of the Brazilian Annonaceae species.

**MATERIAL AND METHODS**

**Chemicals and Standards**

Methanol and deuterated chloroform were obtained from Sigma-Aldrich (St. Louis, MO). A reference standard mix of fatty acid methyl esters (FAMEs) in methylene chloride (grain fatty acid methyl ester mix 99–100%, #47801) was purchased from Supelco (Bellefonte, PA). All
other chemicals and solvents were of analytical grade and obtained from Vetec Química Fina Ltda. (Duque de Caxias, RJ, Brazil) or Tedia Brazil (Rio de Janeiro, RJ, Brazil).

**Plant material**

Fruits of *Annona salzmannii* A. DC. were collected from Mata do Crasto, Santa Luzia do Itanhy [coordinates: 11º24'24''S, 37º25'86''W], Sergipe, Brazil in November 2012 (SISGEN #ACAAA36). The material was identified by Prof. Dr. Ana Paula Nascimento Prata, a taxonomist in the Department of Biology at the Federal University of Sergipe. A voucher specimen (#25603) was deposited in the Herbarium of the Federal University of Sergipe (ASE/UFS), Sergipe, Brazil. This work was performed according to special authorization for access to genetic resources in Brazil #010240/2013-6, issued by CNPq/MCTI.

**Seed oil extraction**

Seeds of *A. salzmannii* were removed from the mature fruits, washed with water to remove pulp, and dried in a circulating air oven at 45 ºC for five days. They were then milled using a cutting mill. The seeds (752.0 g) were submitted to exhaustive extraction with *n*-hexane (four times, one liter each) for 24 h at room temperature (≈ 24 ºC). The resultant crude extract was submitted to solvent removal with aid of a low-pressure evaporator (40–50 ºC) and then dried under a continuous stream of N₂ gas.

**GC-MS and ¹H qNMR seed oil analyses**

Aliquots of the seed oil (100 µL) were dissolved in CHCl₃-MeOH (1:1 v/v) solution and dried under a gentle stream of N₂ gas. Methanalysis was then carried out with methanol-methanolic HCl (1 mol·L⁻¹) at 100 ºC for 2 h to obtain FAMES. The resulting FAMES were extracted by partition between *n*-hexane (1 mL) and distilled water (0.5 mL). The organic phase (top phase) was collected and evaporated under a stream of N₂. The derivatives were then directly injected into the GC-MS chromatograph. The analyses were carried out on a Varian 3800 GC system with a Saturn 2000 RMS detector (Agilent Technologies, Santa Clara, CA) equipped with a 30 m × 0.25 mm i.d. low-bleed/MS DB-225 capillary column. The temperature ramp used for the FAME analyses was: injector 250 ºC; initial oven 50 ºC, hold for 2 min, then heat to 210 ºC (40 ºC per min, then hold for 45 min). Electron ionization (EI) spectra were obtained at 70 eV and 200 ºC. The injection volume was 5 µL at mg/200 µL with a split ratio of 1:5. Post-run analyses were performed on a Saturn Workstation 5.1. software (Sassaki et al., 2008). FAMES were identified through comparison with retention times of standards (National Institute of Standards and Technology, 1992; National Institute of Standards and Technology, 1998).

Quantitative ¹H NMR (qNMR) spectra were acquired in CDCl₃, at 303 K on a Bruker AVANCE III 600 NMR spectrometer. Relative quantification of the fatty acids was determined from the ¹H qNMR spectra according to Barison et al. (2010).

**Larvicidal assay**

The larvicidal assay was performed using *Ae. aegypti* L. third-instar larvae (Rockefeller strain) (Santos et al., 2010). Concentration ranges were determined based on a previous concentration-response curve of 20 larvae. A 20,000 ppm stock solution was prepared using the seed oil (20 mg·mL⁻¹), Tween 80 (10% v/v), dimethyl sulfoxide (DMSO) (30% v/v), and dechlorinated water (60% v/v). The stock solution was used to make 20 mL water solutions ranging from 10 to 1000 ppm. Twenty larvae were collected with a Pasteur pipette and placed in a 25 mL graduated cylinder. Dechlorinated water was added to a volume of 20 mL, and the solution was then transferred to disposable cups containing variable volumes of stock solution. A mortality count was conducted after 24 h of treatment. Controls were prepared at the highest concentration used in the larvicidal assay [Tween 80 (0.75%) and DMSO (2.25%) in dechlorinated water]. Each solution concentration and control were performed in triplicate. As a positive control, commonly used organophosphate insecticide temephos was used at concentrations of 0.015–0.135 ppm. Probit analysis (Finney, Stevens, 1948) was conducted on the mortality data, collected after 24 h exposure to different concentrations of testing solutions, to establish lethal concentration for 50% mortality (LC₅₀) and 95% confidence interval (CI) values for each sample and temephos. For the cases where deaths occurred during the control experiment, mortality data were corrected using Abbott’s formula: (% Death = [1 (test/control)] x 100).

**Cell lines**

Tumor cell lines B16-F10 (mouse melanoma) and HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia) and HL-60...
(human promyelocytic leukemia), and non-tumor cell line PBMCs (peripheral blood mononuclear cells) were donated by Hospital A.C. Camargo, São Paulo, SP, Brazil. Cells were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 2 mM glutamine, and 50 mg·mL$^{-1}$ gentamicin. Adherent cells were harvested through treatment with 0.25% trypsin-EDTA solution (Gibco-BRL, Gaithersburg, MD). All cell lines were cultivated in cell culture flasks at 37 ºC under 5% CO$_2$ and were subcultured every 3–4 days to maintain exponential growth. Cytotoxicity experiments were conducted using cells in the exponential growth phase. All cell lines were tested for mycoplasma using a LookOut mycoplasma qPCR detection kit (Sigma-Aldrich Corp., St. Louis, MO) and were free of contamination.

Human lymphocyte cells were obtained by primary culture. Heparinized blood samples (from healthy, non-smoking donors who had not taken any drugs for at least 15 days before sampling) were collected and peripheral blood mononuclear cells (PBMC) were isolated using a standard Ficoll density gradient protocol (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Björkgatan, Upsalla, Sweden). The PBMC were washed and resuspended at a concentration of 0.3 × 10$^6$ cells/mL in RPMI-1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and 50 μg·mL$^{-1}$ gentamicin at 37 ºC under 5% CO$_2$. Additionally, concanavalin A (ConA; Sigma-Aldrich Corp., St Louis, MO) was used as a mitogen to trigger cell division of T-lymphocytes. ConA (10 μg·mL$^{-1}$) was added at the start of the culture, and the cells were treated with seed oil after 24 h.

Cytotoxicity assay

Cell viability was quantified by alamarBlue assay (Ahmed, Gogal, Walsh, 1994). For all experiments, cells were cultured in 96-well plates (0.7 × 10$^5$ cells/mL for adherent or 0.3 × 10$^5$ cells/mL for suspended cells in 100 mL of medium). After 24 h, seed oil (0.19–50 μg·mL$^{-1}$) dissolved in DMSO (LGC Biotechnology, São Paulo, SP, Brazil) was added to each well for incubation for 72 h. Doxorubicin (doxorubicin hydrochloride, purity 99.0%; Sigma-Aldrich Corp., St. Louis, MO) was used as a positive control. The negative control was composed of the solution (0.5% DMSO) used for the sample dilutions. Four hours (24 h for the PBMC) before ending incubation, 20 mL of alamarBlue stock solution (0.312 mg·mL$^{-1}$) (resazurin; Sigma-Aldrich Corp., St. Louis, MO) was added to each well. Absorbance measurements were obtained on a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA), and drug effects were quantified as a percentage of the control absorbance at 570 and 600 nm. Data are presented as the mean ± standard deviation (SD) of two independent experiments performed in triplicate. All statistical analyses were carried out on a GraphPad Prism v6 software (GraphPad Software Inc.).

Results and Discussion

The extraction of A. salzmannii seed resulted in a yellow crude oil which comprises 25.2% of that of the dry weight plant material. Quantitative information on the chemical components of the seed oil was assessed though GC-MS and 1H qNMR. Additionally, the larvicidal and cytotoxic effects of the seed oil were investigated.

GC-MS analyses of the seed oil revealed a fraction rich in methyl esters, containing a high percentage of unsaturated fatty acids (80.5%), including linoleic (LA) and oleic (OA) acids (45.3% and 33.5%, respectively), and a low concentration of palmitoleic acid (1.7%). Regarding saturated fatty acids (18.7%), palmitic acid (14.3%) was the most abundant, followed by stearic acid (4.4%), while linolenic and myristic acids were not detected (Table I).

The 1H qNMR spectra from A. salzmannii seed oil revealed the presence of signals related to triacylglycerols containing OA, LA moieties, and saturated fatty acids (Figure 1). Therefore, the relative amounts of each fatty acid esterified to a glycerol moiety were determined, revealing that the oil is composed of OA (35.1%), LA (49.3%), and saturated fatty acids (13.5%).

The findings observed in this study are in accordance to those for other Annona species, including A. cornifolia (Lima et al., 2011), A. squamosa (Ansari, Afaq, Ahmad, 1985), A. crassiflora (Luzia, Jorge, 2013), A. diversifolia (Reyes-Trejo et al., 2014), and A. muricata (Castro et al., 1984), where the unsaturated fatty acids content was higher than that of saturated fatty acids, ranging from 71.4 to 78.4%. In previous studies, the saturated fatty acids content varied from 21.6 to 33.3%. Comparatively, the seeds of A. salzmannii appear to have a higher amount of unsaturated fatty acids and lower amount of saturated fatty acids than those of other Annona species. Linolenic acid, for example, is either not present or present in low concentrations (<1% of the total fatty acid content) in other species (Barison et al., 2010).
TABLE I – Fatty acids analysis by GC-MS of *Annona salzmannii* seed oil

| Peak | Namea | Rt (min)b | EI-MS (m/z)c |
|------|-------|-----------|-------------|
| 1    | Palmitic acid (16:0) | 6.454 | 55, 73.9, 101, 143, 227.1, 270, 271.1 |
| 2    | Palmitoleic acid (16:1) | 6.565 | 55, 69, 83, 97 |
| 3    | Stearic acid (18:0) | 7.777 | 55, 73.9, 75, 87, 101, 143.1, 199.1, 255.2, 298 |
| 4    | Oleic acid (18:1n-6) | 7.922 | 55, 67.1, 81, 82, 97, 123, 264, 296.8 |
| 5    | Linoleic acid (18:2) | 8.252 | 67.1, 81, 95.1, 108.9 |

aName of the fatty acid, bRetention time, cEletron Ionization-Mass Spectrometry

FIGURE 1 – ¹H qNMR spectrum of *Annona salzmannii* seed oil.

Table II shows the percent mortality of *A. salzmannii* seed oil against third-instar *Ae. aegypti* L. At the highest concentration, 1500 ppm, the seed oil caused 76.6% mortality, while lower concentrations resulted in lower mortality. *A. salzmannii* seed oil exhibited moderate larvicidal activity (LC₅₀ = 569.7 ppm, CI = 408.1 to 825.8 ppm) against *Ae. aegypti*, while the positive control, a WHO-approved larvicide, temephos, exhibited a LC₅₀ = 0.003 ppm (0.0030 to 0.0039 ppm). No mortality was observed for the negative controls.

In the literature, there are few reports of insecticide assays using *Annona* seeds. The larvicidal effects of *Annona muricata* seed-ethanol extract-water suspension against fourth-instar *Ae. aegypti* L. larvae have been...
shown to produce 100% mortality at 0.5 mg·mL\(^{-1}\) after 24 h exposure (Bobadilla, Sisnigas, Zavaleta, 2005). However, no difference in the mortality rate was observed between acetogenins polar (ethanol) and non-polar (petroleum ether) extracts of A. muricata seeds evaluated against third- and fourth-instar larvae, showing 100% mortality at 0.18 mg·mL\(^{-1}\) for both (Morales, Gonzáles, Aragón, 2004). In addition, bioassays of methanol and hexane extracts of A. coriacea seeds against Ae. aegypti L. larvae at concentrations < 0.1 mg·mL\(^{-1}\) resulted in 100% mortality at all concentrations tested (LC\(_{50}\) = 0.007 mg·mL\(^{-1}\)) (Costa et al., 2013c).

| Concentration (ppm) | Average % Mortality |
|---------------------|----------------------|
| Annona salzmannii seed oil |                      |
| 10                  | 6.6                  |
| 100                 | 13.3                 |
| 450                 | 35.0                 |
| 1000                | 63.3                 |
| 1500                | 76.6                 |

| Positive control (temephos) |                      |
|-----------------------------|----------------------|
| 0.001                       | 3.3                  |
| 0.003                       | 33.3                 |
| 0.005                       | 75.0                 |
| 0.007                       | 88.3                 |
| 0.011                       | 98.3                 |
| Negative control            | 0                    |

TABLE II – Percentage mortality of AeDES aEGypti mosquito larvae in the control and experimental group after exposition to Annona salzmannii seed oil for 24 h

Literature results have also shown differences in extracts and fractions of A. crassiflora seeds against third-instar Ae. aegypti L. larvae. Crude extracts of A. crassiflora showed higher mortality rates than those of the fractions. Extracts of hexane (LC\(_{50}\) = 0.507 mg·mL\(^{-1}\)) and dichloromethane (LC\(_{50}\) = 0.185 mg·mL\(^{-1}\)), as well as hexane fractions (LC\(_{50}\) = 0.433 mg·mL\(^{-1}\)), showed mortality rates > 90% at 1.0 mg·mL\(^{-1}\) (Costa et al., 2013c).

Therefore, it has been demonstrated that the bioactivity of species of the family Annonaceae on Ae. aegypti L. larvae can vary significantly depending on species, as well as the extraction solvents and concentrations used.

To investigate mammalian toxicity, the cytotoxic effects of A. salzmannii seed oil were evaluated against tumor cell lines B16-F10, HepG2, K562, HL-60, and non-tumor cell line PBMC. The cytotoxicity assay of the seed oil from A. salzmannii was initially performed at a concentration of 50 µg·mL\(^{-1}\) against BF6-F10 and HepG2 tumor cell lines. The seed oil was active once it caused 84.19 ± 8.18 and 72.96 ± 12.65 cell growth inhibition of BF6-F10 and HepG2, respectively. Furthermore, the oil was tested in the B16-F10, HepG2, K562, and HL-60 tumor cell lines and PBMC normal cell line to determine 50% inhibitory concentrations (IC\(_{50}\)).

According to the criteria adopted by the American National Cancer Institute and our cytotoxic screening assay, a crude extract oil showing an IC\(_{50}\) value < 30 µg·mL\(^{-1}\) in tumor cell lines is considered promising for anticancer drug development (Suffness, Pezzuto, 1990). The oil from A. salzmannii seeds did not show any expressive antiproliferative effects, with IC\(_{50}\) values > 50 µg·mL\(^{-1}\) in the tumor cell lines tested. Although the seed oil has a high content of unsaturated fatty acids and a well-known antineoplastic activity (Fauser et al., 2011), it did not show cytotoxic effects against the tumor and non-tumor cell lines.

Different cytotoxic effects have been reported for some species of Annona. FAMEs from the seed oil of A. cornifolia have shown interesting cytotatic and cytocidal effects against some tumor cell lines (Lima et al., 2012). This oil also had a high percentage of unsaturated (71.4%) versus saturated (22.7%) fatty acids (Lima et al., 2011). The seed oil of A. squamosa has been shown to suppress H22 solid tumor development, which might be attributed to the presence of unsaturated fatty acids and acetogenins. Additionally, this oil showed selective cytotoxic activity against HepG2 cell lines (Chen et al., 2016).

In the literature, the cytotoxic effects of fatty acids have been individually evaluated in different tumor cell lines with differing results (Meterissian et al., 1995; Du Toit, Aswegen, Du Plessis, 1996; Soto-Guzman et al., 2013; Moon, Batriel, Mantzoros, 2014; Wang et al., 2014). In this context, when evaluating the effects of fatty acids on tumor cell lines, the fatty acid type, degree of
saturation/desaturation, method of delivery to cancer cells or host, and tumor/cell type must be considered (Jiang, Brice, Horrobin, 1998). Another consideration is that the chemical compositions of seed oils may contain other metabolites besides triacylglycerides, such as acetogenins, which are highly cytotoxic.

This report presents the first study on the chemical composition, larvicidal effects, and cytotoxicity of *A. salzmannii* seed oil. The oil was found to have a high percentage of unsaturated (80.5%) and saturated (18.7%) fatty acids (i.e. fatty acid esterified to a glycerol moiety), which make it an interesting source of EFAs. Furthermore, the oil exhibited moderate larvicidal toxicity against *Ae. aegypti* larvae and did not show cytotoxic effects in tumor and non-tumor mammalian cell lines.

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