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GC-MS analysis of esterified fatty acids obtained from leaves and seeds of *Triplaris gardneriana* Wedd.

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***Triplaris gardneriana* Wedd. belongs to the family Polygonaceae. In Brazil, the plant is known as "Pajéu". The plant has a traditional use in folk medicine for the treatment of some human diseases. In this study, it was realized for first time, the extraction and characterization by gas chromatography–mass spectrometry (GC-MS) of the fixed oils from the leaves and seeds of *T. gardneriana* collected at different times and it was evaluated the antioxidant and antibacterial activities. The esters were identified by comparing the mass spectra obtained with those of the equipment database. The antioxidant activity was evaluated by the methods of radical scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) and co-oxidation of β-carotene against linoleic acid. The antibacterial effect was evaluated by the microdilution broth method. In the fixed oil of the leaves were identified 22 compounds, totaling 61.53%. Methyl palmitate (15.14%) and methyl oleate (14.35%) were the majoritary compounds. For the sample of the seeds fixed oil, 13 compounds were identified, representing 97.81%. Among these, 10-Octadecenoic acid, methyl ester (45.53%) was identified as majoritary constituent. The oils do not show antioxidant activity, but showed moderate antibacterial activity. The fatty acid composition of the fixed oils showed differences, noting a greater variety of constituents in the leaf oil. The presence of these compounds in the studied plant is important phytochemically because it contributes to the chemical and pharmacological knowledge of this specie.**

Key words: Polygonaceae, *Triplaris gardneriana*, fixed oils, esters, Caatinga, antioxidant activity, antibacterial activity.

INTRODUCTION

The Polygonaceae family comprises 51 genera and about 1100 species, widely distributed in tropical and temperate regions. The plants belonging to this family are known to produce a large number of biologically important molecules such as alkaloids, benzenoids, carotenoids, coumarins, depsídeos, stilbenes,

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phenylpropanoids, flavonoids, lactones, lignans, quinoid, tannins, terpenoids, and xanthones. In Brazil, seven genera are of spontaneous occurrence, among them is *Triplaris*, which comprises approximately 20 species in South and Central America (Oliveira et al., 2008).

Phytochemical studies on species of genus *Triplaris* have identified the presence triterpenes, amide, phenylpropanoid glycoside, benzenoid, and flavonols simple and glycoside (Oliveira et al., 2008; Hussein et al., 2005; Macedo et al., 2015). In the folk medicine, the species are used in the treatment of malaria diarrhea, dysentery, stomach pain, enteritis, fever, sores, inflammation of the throat, skin lesions induced by leishmaniasis, linfatites, measles, cough, intestinal worms and is also used as an energetic exciting and hallucinogen (Oliveira et al., 2008). Plants of this genus showed activities such as antioxidant, cytotoxicity against the human cancer cell lines, anticholinesterase, anti-HIV, larvicidal, antimicrobial, leishmanicidal, immunomodulatory, agglutinating, antiplasmodial, antimalarial, anti-inflammatory, stimulating smooth muscle and molluscicidal activity (Schertz et al., 1960; Tan et al., 1991; Deharo et al., 2004; Estevez et al., 2007; Camones et al., 2010).

Triplaris gardneriana Wedd. is a tree that belongs to the family Polygonaceae and occurs only in the biome Caatinga and Pantanal. In brazilian northeast this specie is known as "Pajéu" and occurs in the riparian forest of the Rio São Francisco. It is used in folk medicine to treat bleeding hemorrhoids, cancer, gastritis, ulcers, cough, pain, heartburn, flu, rheumatism, bronchitis, leucorrhoea, gonorrhea and inflammation of internal organs (Cartaxo et al., 2010; Pereira et al., 2014). However, few studies of biological activities are reported for specie, only, moluscicide, stimulating smooth muscle and toxicity activities (Souza and Rouquayrol, 1974; Barros et al., 1970). Recent studies of seed extracts of *T. gardneriana* showed antibacterial, antioxidant and anticholinesterase activities (Farias et al., 2013) and extracts of the leaves showed antioxidant and photoprotective activities (Macedo et al., 2015).

Phytochemical study with extracts from the wood of this specie revealed the presence of aliphatic hydrocarbon, sitosterol, ferulatos (Braz Filho and Rodrigues, 1974) and betulinic acid. Another study on this plant, identified the volatile constituents of the essential oil of fruits, the identification of the constituents was performed by gas chromatography-mass spectrometry (GC-MS) (Carneiro et al., 2010). Recently, flavonol glycosides were identified in extracts of leaves using liquid chromatography-mass spectrometry (LC-MS) (Macedo et al., 2015).

However, no study by GC-MS identified the constituents present in the fixed oil of the specie. The fixed oils are mixtures of lipidic substances insoluble in water and soluble in nonpolar organic substances, are constituted, mainly, by saturated and unsaturated fatty acids (Matos et al., 2015). These oils have immense value for cosmetic application by the properties emollient and researches of biological acctivity reveal insecticidal potential, anti-inflammatory, laxative, antiedematogenic, fungicide and larvicidal for these compounds (Souza et al., 2006; Pereira et al., 2008; Saraiva et al., 2011; Matos et al., 2015). In this way, fixed oils characterization becomes a complementary on phytochemical characterization of the species under study.

The chemical composition of plants, as those found in fixed oils, can be influenced by many factors, and among them we can mention genetics and heredity in terms of secondary metabolites, the morphogenetic variability and ontogenetic, which is the content of the difference active substances in the different parts of the plant and during the stages of development, in addition to environmental influences, such as weather, temperature and other factors (Pereira et al., 2005; Emara and Shalaby, 2011).

The aim of this study was to investigate the phytoconstituents present in the fixed oil by GC-MS from the leaves and seeds of *T. gardneriana* collected at different times and to evaluate the antioxidant and antibacterial activities.

MATERIALS AND METHODS

Plant material

The leaves were collected in the city of Santa Maria of Boa Vista in the state of Pernambuco, Brazil in July 2013, located at 349 m elevation (08°47'59, 00 S, 039°50'42, 40 W). The botanical identity of the plant was confirmed by specialist Diogo de Oliveira Gallo. A voucher specimen of the plant was deposited in the Herbarium of the Federal University of San Francisco Valley (HVASF) under registration 21221. The seeds were obtained in the Seed Laboratory of the Reference Center for the Recuperation of the Degraded Areas of the Caatinga, registered under the LAS number 818.

Extraction

The extraction of fixed oils of the leaves and seeds of *T. gardneriana* was performed through the extraction in a Soxhlet apparatus. Aliquots of 37 g of leaves and 59 g of seed powder were placed, separately inside the apparatus containing 250 ml of hexane and heated electric blanket for two hours. Then, the mixture of oil/solvent was evaporated in a rotary evaporator to yield the crude fixed oils. The oils were weighed and their percentages were calculated based on the dry weight of the botanic material.

Saponification

The methodology used with some adaptations, was described by Matos et al. (1992). For esterification, the oils were saponified by being refluxed in 50 ml of methanol containing 1.0 g of KOH, for 30 minutes. Methanol was distilled to reduce the volume and then the volume was completed to 50 ml of water. The unsaponifiable alkaline solution was extracted with ethyl ether mixture.

Methylation of saponified fraction

The aqueous alkaline solutions were acidified to pH 2 with 10%

hydrochloric acid and the fatty acids extracted with ethyl ether. Subsequently, water was removed with anhydrous sodium sulfate and the ether distilled off. The methylation of the fatty acids was carried out by refluxing the samples for 2 min with 3 drops of concentrated hydrochloric acid in 5 ml of anhydrous methanol. The methyl esters were extracted with hexane after the addition of 10 ml of water and then the same was removed of solution with anhydrous sodium sulfate, followed by filtration.

GC-MS analysis

The substances present in the fixed oil of *T. gardneriana* were investigated on a Shimadzu QP-2010 -MS. The following conditions were used: ZB-5MS column Phenomenex Zebron (30 m x 0.25 mm x 0.25 mm); helium (99.999%) carrier gas at a constant flow of 1.1 ml/min; 1µl injection volume; injector split ratio of 1:40; injector temperature 240°C; electron impact mode at 70 eV; ion-source temperature 280°C. The oven temperature was programmed at 100°C (isothermal for 5 min), with an increase of 10°C/min to 250°C and 10°C/min to 280C. A mixture of linear hydrocarbons (C_9H_{20}–$C_{40}H_{82}$) was injected under the same experimental conditions as samples, and identification of the constituents was performed by comparing the mass spectra obtained with those of the equipments database (Wiley 7 lib and Nist 08 lib).

Evaluation of antioxidant activity

DPPH free radical scavenging assay

The free radical scavenging activity was measured using 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\cdot}$) (Mensor et al., 2001; Almeida et al., 2011). The absorbance values were measured at 518 nm and converted into the percentage Antioxidant Activity (AA) using the following equation:

$$\% AA = [(AC - AA) / AC] \times 100$$

Where AC is absorbance of the control and AA is sample absorbance. Ethanol (1.0 ml), with solutions of the fixed oils (2.5 ml), was used as a blank. DPPH solution (1.0 ml), with ethanol (2.5 ml), was used as a negative control. The positive controls (ascorbic acid, BHA and BHT) were used as standard solutions. The values were expressed as mean ± standard deviation (SD). Assays were carried in triplicate.

Inhibition of auto oxidation of β-carotene

The β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion (Wannes et al., 2010). Ascorbic acid, BHT and BHA standards and the fixed oils were used as positive control. In the negative control, the fixed oils were substituted with an equal volume of ethanol. The absorbance was measured immediately at 470 nm. The antioxidant activity (AA%) was evaluated in terms of bleaching of β-carotene using the following formula:

$$AA\% = [1 - (A_0 - A_t) / (A_0^0 - A^0_t)] \times 100$$

Where A_0 is the initial absorbance and A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance and A_t^0 is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

Evaluation of antibacterial activity

The antibacterial effect of the fixed oils was evaluated by microdilution broth method (CLSI, 2006). In the evaluation of antibacterial activity of the following standard strains from the American Type Culture Collection (ATCC) the following were tested: *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and *S. aureus* resistant methicillin (MRSA).

The fixed oils of the seeds was diluted in 10 ml of ethanol 95% and the fixed oils of the leaves was diluted in pure DMSO (dimethyl sulfoxide) and methanol (1:1) to obtain the solution at concentration of 25 mg/ml. In the preparation of the inoculum, colonies obtained in Mueller-Hinton agar were used for the preparation of bacterial suspension in a 0.085% saline solution with turbidity equivalent to the tube 0,5 of the MacFarland scale. The suspension was inoculated, 100 µl in tubes containing 9.9 ml of Mueller-Hinton broth. For determination of minimum bactericidal concentration (MBC), 200 µl of Mueller-Hinton broth were distributed into 96-well microplates. Then, 200 µl of solution of samples were added to the first well and, after homogenization, transferred to the second and so forth; the following final concentrations were achieved: 12500; 6250; 3125; 1562.5; 781.2; 390.6; 195.3 and 97.6 µg/ml. Then, 10 µl of bacterial suspension were placed in 96-wells of microplate containing the sample dilution, the material was incubated at 37 °C for 24 hours. Using a replicator, aliquots were removed from the microplates after the incubation and plated on Petri plates containing Mueller-Hinton agar, followed by incubation for 24 h at 37°C. MBC was defined as the lowest concentration of the extract able to cause the death of bacteria. Thus, we considered only the results of the minimum bactericidal concentration (MBC). Tests were carried out in triplicate.

RESULTS AND DISCUSSION

After of the extraction, the yield of the fixed oils obtained from the leaves and seeds were 1.5 and 0.8%, respectively. The total ion chromatograms (TIC) are showed in Figure 1 and 2. In the Tables 1 and 2, a list of the identified compounds and their quantification in the fixed oils are presented according to their retention times and total peak area (%) of each of methylated fatty acids. The product obtained from the transesterification is showed in GC-MS analysis total ion chromatogram (TIC) in Figure 1, in which one observes the presence of 38 peaks in the chromatogram of the leaves and 15 peaks in the chromatogram seeds (Figure 2), whose comparison with the corresponding spectra of the device library allowed the identification of 22 constituents of the fixed oil of leaves totaling 61.53% (Table 1) and 13 constituents of the fixed oil from the seeds representing 97.81% (Table 2).

The chemical composition of the fixed oil of the leaves (Table 1), with fatty acids, hydrocarbons, steroids and a triterpene, was more diverse with respect to the fixed oil of the seeds (Table 2). Comparing the chemical composition of the fixed oils, it is observed that nine esterified fatty acids methyl palmitate, palmitic acid, methyl linoleate, methyl stearate, methyl 9,11-octadecadienoate, methyl behenate, methyl cerotate, *cis*-

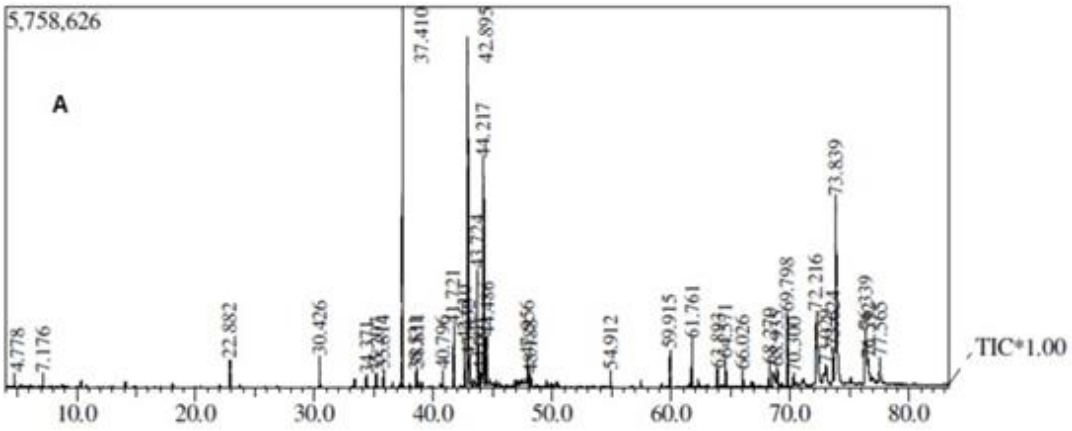

Figure 1. TIC of chemical constituents of the fixed oil from the leaves of *T. gardneriana.*

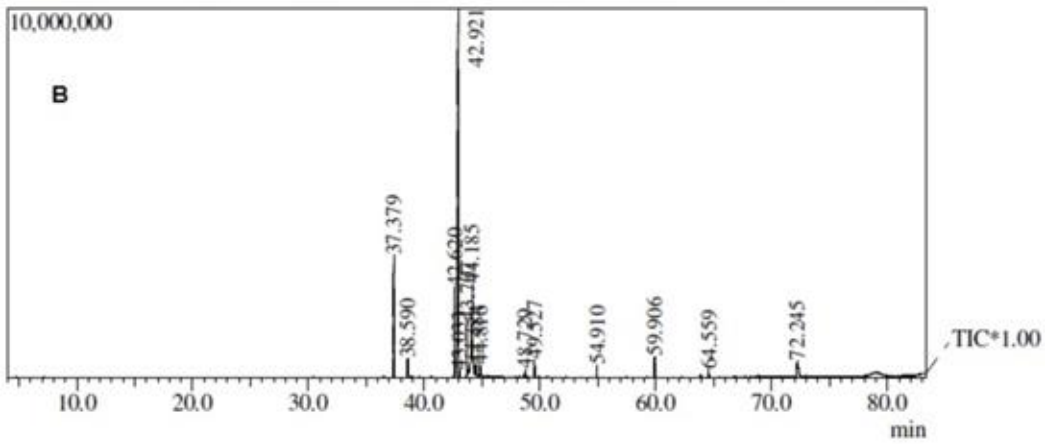

Figure 2. TIC of chemical constituents of the fixed oil from the seeds of *T. gardneriana.*

Vaccenic acid and methyl lignocerate are present both in the leaf and seed oil. The β-sitosterol was also observed in both samples of fixed oil. It was found that the content of unsaturated fatty acids in the fixed oil of leaves was only 18.77%, while 71.1% of the methyl esters identified in the seeds oil were derived from unsaturated fatty acids.

The results obtained from the identification of compounds present in fixed oils demonstrated difference in secondary metabolites as function of the different parts of the plant and the stages of development, in addition to environmental influences. The major chemical constituents of the leaves oil were methyl palmitate (15.14%), methyl oleate (14.35%). In the oil of the seeds was 10-Octadecenoic acid, methyl ester also known as methyl 10-octadecenoate (45.53%). It is found that methyl palmitate and β-sitosterol appear principally in both fixed oils analyzed. The mass spectra of methyl palmitate are shown in Figure 3 and methyl octadec-10-enoate in Figure 4.

The major compounds from the oil of the leaves and seeds, palmitate methyl (15.14%) and methyl 10-octadecenoate (45.53%), respectively, were identified in the essential oil of the fruits of *T. gardneriana* (Carneiro et al., 2010). In the fruit oil, the constituents methyl palmitate (21.67%) and methyl 10-octadecenoate (21.72%) were also the majority. It is notable that the fixed oil from the leaves showed the double of amount of methyl 10-octadecenoate compared with the oil obtained from the fruits.

The analysis presented a remarkable difference in chemical composition between fixed oils, since the leaf oil presented greater variation, and the concentration of saturated fatty acids was higher than unsaturated. There are several factors that can influence their presence, and among them we can mention the difference in the content of substances in different parts of the plant during the stages of its development and can also be related to

Table 1. Chemical constituents of the fixed oil from the leaves of *T. gardneriana.*

Peak	RT (min)	Compound	(%) GC-MS
1	4.778	NI	0.24
2	7.176	Undecane	0.30
3	22.882	Methyl laurate (C12:0)	0.89
4	30.426	Methyl myristate (C14:0)	1.05
5	34.371	NI	0.39
6	35.207	1-Octadecyne	0.39
7	35.207	NI	0.52
8	37.410	Methyl palmitate (C16:0)	15.14
9	38.531	Palmitic acid (C16:0)	0.44
10	38.611	NI	0.58
11	40.796	NI	0.53
12	41.721	NI	2.16
13	42.610	Methyl linoleate (C18:2)	1.59
14	42.895	Methyl oleate (C18:1)	14.35
15	43.032	Methyl Octadec-13-enoate (C18:1)	0.87
16	43.724	Methyl stearate (C18:0)	4.35
17	44.001	cis-Vaccenic acid	0.26
18	44.217	NI	10.31
19	44.486	Methyl 9,11-octadecadienoate (C18:2)	1.70
20	47.956	Methyl linolenate (C18:3)	1.00
21	48.188	NI	0.31
22	54.912	Methyl behenate (C22:0)	0.54
23	59.915	Methyl lignocerate (C24:0)	1.30
24	61.761	Squalene	1.87
25	63.893	Tetratetracontane	0.63
26	64.571	Methyl Cerotate (C26:0)	1.05
27	66.026	NI	0.42
28	68.270	NI	0.78
29	68.935	Methyl octacosanoate (28:0)	0.61
30	69.798	NI	3.02
31	70.300	NI	0.38
32	72.216	β-Sitosterol	6.80
33	73.029	Methyl melissicate (30:0)	0.29
34	73.624	NI	1.54
35	73.839	NI	13.96
36	76.339	β - friedelanol	6.10
37	76.592	NI	2.03
38	77.565	NI	1.30
Total	**-**	**-**	**61.53**

RT= Retention time; NI = not identified.

seasonal issues, as the plant source used in each experiment was acquired in different times and places (Nazifi et al., 2008; Emara and Shalaby, 2011).

The methyl oleate known as oleic acid, one constituent of the leaf fixed oil is an unsaturated long chain of fatty acid with 18 carbons in its structure. It is known as omega 9 essential, which participates in the metabolism in the synthesis of hormones. The methyl linoleate known as linoleic acid is a component of the two fixed oils analyzed. It is considered an omega 6, which is essential for normal cell function (Lehninger et al., 2011). Among these characteristics, oleic and linoleic acid also have the ability to reduce cholesterol levels in the blood (Erdogan et al*.,* 2014).

The fixed oils showed no antioxidant activity in any of the tested methods (Table 3). The negative outcome of

Table 2. Chemical constituents of the fixed oil from the seeds of *T. gardneriana*.

Peak	RT (min)	Compound	(%) GC-MS
1	37.379	Methyl palmitate (C16:0)	10.98
2	38.590	Palmitic acid (C16:0)	1.74
3	42.620	Methyl linoleate (C18:2)	8.57
4	42.921	Methyl Octadec-10-enoate (C18:1)	45.53
5	43.033	Methyl elaidate (C18:1)	0.58
6	43.707	Methyl stearate (C18:0)	4.66
7	44.185	cis-Vaccenic acid	15.57
8	44.484	Methyl 9,11-octadecadienoate (C18:2)	0.81
9	44.816	Stearic acid (C18:0)	1.45
10	48.729	NI	0.62
11	49.527	NI	1.57
12	54.910	Methyl behenate (C22:0)	0.99
13	59.906	Methyl lignocerate (C24:0)	1.86
14	64.559	Methyl Cerotate (C26:0)	0.53
15	72.245	β-Sitosterol	4.54
Total	-	-	**97.81**

RT = Retention time; NI = not identified.

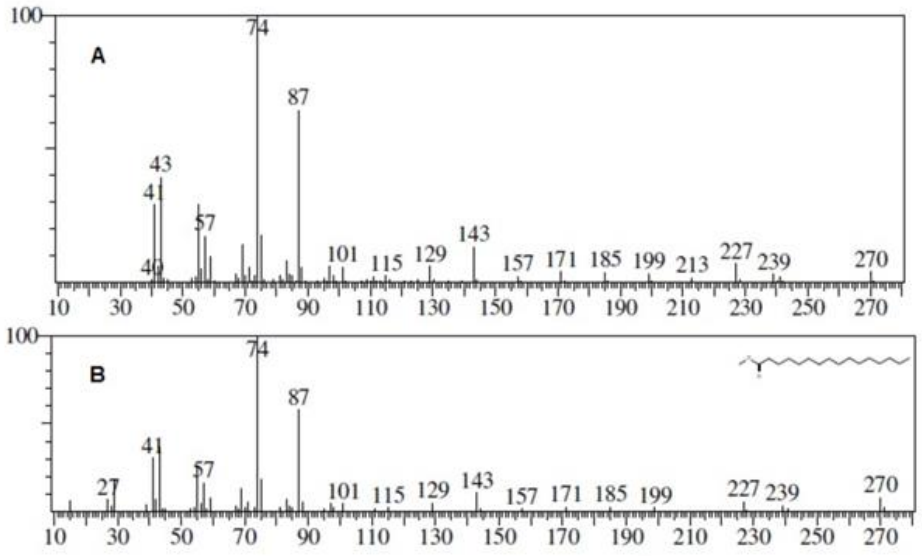

Figure 3. (A) Mass spectrum of compound methyl palmitate (15.14%) present mainly in the leaf oil. (B) Mass spectrum obtained from the library NIST08 and WILEY7.

antioxidant activity is justified in considering that lipophilic constituents are not good antioxidants. Furthermore, the presence of linoleic acid in both fixed oils, with a higher concentration in the seed oil, may have caused the oxidation of β-carotene, since free radicals formed during peroxidation of linoleic acid oxidizes the β-carotene (Alves et al., 2010).

According to the results of the antibacterial activity (Table 4), fixed oils from the seeds and leaves showed the same activity against *S. aureus, S. epidermidis,* and *S. aureus* resistant methicillin (1562.50 µg/ml). The oil of the seeds showed moderate inhibition against *E. faecalis, K. pneumoniae* (781.35 µg/ml) and the oil of the leaf was also effective against *E. faecalis* (781.35 µg/ml), with weak inhibition against *S. enterica* (3125.00 µg/ml).

According to the results, the difference in the chemical

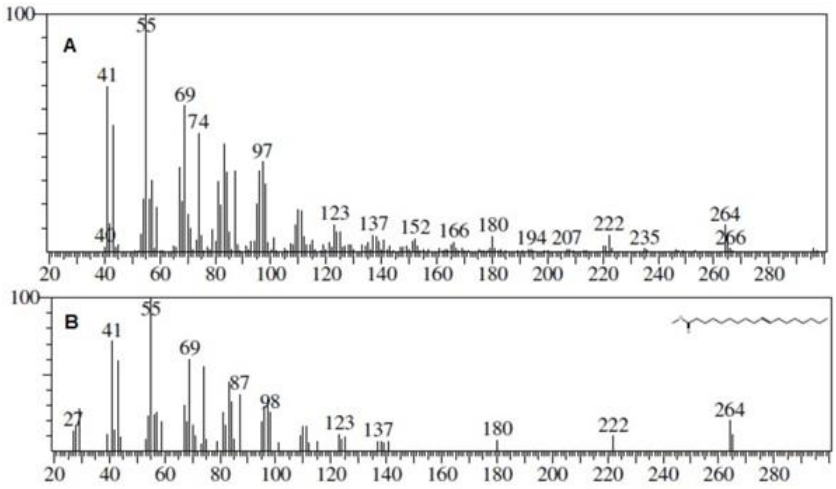

Figure 4. A) Mass spectrum of the major compound methyl octadec-10-enoate (45.53%) present in the seed oil. B) Mass spectrum obtained from the library NIST08.

Table 3. Antioxidant activity of fixed oil of the leaves and seeds of *T. gardneriana.*

Oils / Standards	DPPH (% AA)	β-carotene (%AA)
OF	-12.37±12.59	-1.39±1.38
OS	-101.19±16.78	-0.14±2.09
Ascorbic acid	93.39±0.88	8.50±0.75
BHA	67.63±8.41	19.15±8.63
BHT	73.56±1.83	25.14±15.0

Values are presented as mean ± SD (n=3). AA%= percentage of antioxidant activity; OF= fixed oil of the leaves; OS= fixed oil of the seeds; BHA (butylated hydroxyanisole); BHT (butylated hydroxytoluene).

Table 4. Antibacterial activity of fixed oil of the leaves and seeds of *T. gardneriana.*

Microorganisms	Fixed oil seeds	Fixed oil leaves
Staphylococcus aureus	1562.50	1562.50
Staphylococcus epidermidis	1562.50	1562.50
Enterococcus faecalis	781.35	781.35
Escherichia coli	-	-
Klebsiella pneumoniae	781.35	-
Salmonella enterica	-	3125.00
Staphylococcus aureus resistant methicillin (MRSA)	1562.50	1562.50

Values MBC expressed as µg/ml.

composition of oils obtained from different parts of the plant has not interfered in results of biological activities, because both oils showed moderate bacterial activity and showed no antioxidant activity.

Conclusion

This study described for the first time the identification and quantification of components obtained from fixed oil from the leaves and seeds of *T. gardneriana*. The fatty acid composition of the fixed oils showed differences, noting a greater variety of constituents in the leaf oil. In the leaves major constituents were methyl palmitate and metyl oleate, in the seeds oil was the methyl octadec-10-enoate. Neither of the oils showed antioxidant activity, but both exhibited moderate antibacterial activity. Therefore,

even though the fixed oils do not have antioxidant activity, the class of fatty acids present in the fixed oils has important physiological functions for our body and can be an alternative for pharmaceutical applications. The presence of these compounds in the studied plant is important phytochemically because contribute to the chemical and pharmacological knowledge of this specie.

Conflicts of Interests

The authors have not declared any conflicts of interests.

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