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STUDY OF THE BIOLOGICAL EFFECTS OF AN ESSENTIAL OIL PROCESSED FROM LEAVES OF EUGENIA ASTRINGENS CAMBESS SPECIMENS. (MYRTACEAE)

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ABSTRACT
The essential oil (EO) from the leaves of *Eugenia astringens* Cambess. It was obtained by hydrodistillation using Clevenger apparatus modified. Essential oil chemical composition was analyzed by GC and GC / MS, which showed a yield of 0.17%. The major component was found to be the α -pinene. The result of quantification of the EO sample, the calibration curve showed that the percentage of α -pinene is present in the essential oil of 77%. Cytotoxic potential and genotoxic of the essential oil of *E. astringens* Cambess. was assessed *in vitro*, by indirect diffusion in agarose gel in a lineage of *Staphylococcus aureus*. In the cytotoxic test, the essential oil obtained have antibacterial activity *in vitro* relevant for high dosage (25 µL), and the combination of EO associated with the amoxicilina, both in the dosage of 12.5 µL, was observed that antibiotic action was not potentiated. The rating of genotoxic test associated with H_2O_2 and stannous chloride ($SnCl_2$) showed that with 12.5 µL the essential oil potentiated the genotoxic effect of $SnCl_2$. In the dosage of 8.5 µL, OE showed genotoxity action when tested with the composts cited. There were cytotoxic and genotoxic tests with the α – pinene, under the same conditions EO, in order to compare the effectiveness of both.

KEYWORDS: *Eugenia*. GC/MS. Cytotoxic. Genotoxic. α – pinene.

INTRODUCTION
The cosmetics industry in Brazil consists of 1,659 companies, operating in personal hygiene, perfumery and cosmetics market. Brazil is the third largest consumer market for beauty products, standing behind the United States and China, with a total market higher rated than US $ 8.5 billion.

The market for cosmetics is growing and offers consumers increasingly concerned about the origin and type of assets that make up the product, it was observed an increase in the consumption of natural products, which must have quality standards, lack of toxicity, besides being chemically validated (GIMENEZ *et al*., 2013).

Cosmetic products like has no purchase restrictions, they are used too often and infinitely variably. Therefore these products should have a safety margin that ensures the use of cosmetic under the conditions directed by the manufacturer. The warranty on the product is made by the appropriate choice of ingredients and proof of security through pre- clinical or clinical trials with the analysis of the results (ANVISA, 2007).

According to ANVISA (2003) the effects observed in finished products are largely from its ingredients. So it

needs the toxicological knowledge of each raw material and its features. For this are made pre- clinical in vitro and in vivo preliminary. The main in vitro cytotoxicity tests are tests genotoxicity and phototoxicity.
The choice for the use of EO (essential oil) is given by the wide range of components with high potential for action and economic interest. The essential oil production in Brazil has highlight, beside the more world producers such as India, China and Indonesia (Bizzo *et al*., 2009).

Essential oils are complex mixtures of volatile substances, lipophilic, liquid and odoriferous. According to Internationational Standards Organization (ISO) volatile oils are products obtained according to its location in the plant. They are volatile substances embedded in a complex and photosensitive array.

The Myrtaceae family are approximately 3000 species and about 100 kind, one of the most important families because to their great distribution by the the Brazilian ecosystem (BENFATTI, *et al*., 2010). Among the genera of this family presents the *Eugenia* (MACHADO, 2005).

The *Eugenia astringens* Cambess. (Synonymous: *E. rotundifolia* Casar; *E. umbellilora* O. Berg, *E. cassinoides* O. Berg, *E. apiocarpa* O. Berg, *E. cyclophylla* O. Berg) It is popularly known as araponga (DEFAVERI *et al*., 2011; SOBRAL *et al*., 2015). It was the high yield of essential oil, and easy plant located in the state of Rio de Janeiro, which facilitates harvesting and transport, reducing process costs. Besides few published with its essential oil, no other studies of cytotoxicity and genotoxicity.

This paper will present the results of the chemical analyzes and the cytotoxicity and genotoxicity tests with statistical analysis.

MATERIAL AND METHODS

Plant material

The fresh leaves of the *E. astringens* was obtained from the collection of plants, the Dr. Marcelo de Souza Costa, an specialist on Myrtaceae, located in Barra de Guaratiba, Rio de Janeiro, Brazil. The collection was conducted in July 2012, about 7:00 in the morning. A voucher specimen was deposited in the herbarium of RBR Rural Federal University of Rio de Janeiro and identified at Drº Marcelo de Souza Costa, with Souza 233 registration code.

Essential Oil Extraction

Fresh leaves of *E. astringens* (704.46g) were subjected to hydrodistillation the 4 hour period, the modified Clevenger type apparatus, initial temperature of 100°C, keeping the boiling until the end of the extraction process.

Analysis of the essential oil by the gas chromatography technique (GC) and gas chromatography coupled to the mass spectrum (GC/MS).

The GC analysis was performed on a Shimadzu 6890 gas chromatograph using a fused silica capillary column DB -5 (30 cm x 0.25 mm ID, film thickness of 0,25µm). Helium was used as carrier gas at a flow rate of 1.0 ml/min. The oven temperature was programmed from 40°C (10 minutes) to 260°C at 3°/min.

The temperatures of the injector and detector was 270°C and 280°C respectively. Analysis by gas Chromatography and Mass Spectrometry (GC-MS) was performed on Shimadzu QP- sum system and 5000-Quadrupole MS operating at 70 eV ionization energy. It was used fused silica capillary column DB -5 (30 mm x 0.25 mm ID, film thickness of 0,25µM); Helium as the carrier gas, 1mL/min with Split. The temperatures of the injector and detector was 270°C and 280° C respectively. The oven temperature was programmed from 40°C (10 min) at 260°C at 3°/min. The temperatures of the injector and detector was 270°C and 280°C respectively.

Retention index

The components were identified based on retention index (RI) determined by using a calibration curve of a homologous series of n- alkanes (C7-C30) injected under the same chromatographic conditions of the sample and the spectra fragmentation models mass , both compared with literature data (ADAMS, 1995). The concentration of the components were calculated through the full area of the respective peaks related to the total area of all the constituents of the sample obtained by chromatographic analysis of the gas phase system (VIEGAS & BASSOL, 2007).

Calibration curve

To assess the linearity of the method was taken in quadriplicata analysis of five different concentrations of α- pinene (100, 200, 300, 400 and 500µg/ml). They prepared three calibration curves on three different days to check for possible differences in behavior with linear variation on the day of analysis (ICH, 1995; LEITE, 2002; RIBANI *et al*., 2004). The statistical data was performed by Statistica software.

The standard solutions used for the verification were prepared from a standard stock solution containing α -pinene. The α -pinene and OE standard were weighed on an analytical balance Sartorius CP225D model, standard solutions were prepared in volumetric flasks (5, 10, 25, 50 and 100 mL) and Volumetric pipettes (1, 2, 3, 4, 5 mL) calibrated. Solvent dilution of the solutions was dichloromethane.

The concentrations of standard solutions for injection were 100, 200, 300, 400 and 500µg/mL α-pinene. The preparation of the samples for measurement was conducted with dilution of EO in dichloromethane, increasing the concentration for injection 10mg/mL.

Determination of microbial activity

The microbiological evaluation adapted to the Ministry of Health Protocol (1990), using strains of *Staphylococcus aureus* in the middle of Agar Mannitol culture in order to isolate the colonies and then subject them to incubation in soybean casein (TSB) in order to achieve MC Farlland scale (0.5) with NaCl 0.9%.

The strain used was *Staphylococcus aureus* ATCC 8096. Initially set up a quantity of TSB in the test tube and then incubated strains S. aureus ATCC 8096 was for 24 hours at 35°C in an oven. After checking the growth of the colonies, they were plated with the aid of a bacteriological loop, mannitol agar plate and incubated for 24 hours at 35°C. The formation of isolated colonies was observed. Isolated colonies were resuspended in NaCl (0.9%). It was verified the turbidity of the suspension in saline according coma scale McFarland (0.5) corresponds to a concentration of approximately 10^8 colony forming units - (CFU/mL). The turbidity was considered satisfactory.

Cytotoxicity Test

The cytotoxicity test was done with the application of essential oil, fresh and Amoxicillin, on the area of bacteriological discs in contact with the cells of *Staphylococcus aureus* ATCC 8096. The culture medium used was the Müller- Hinton agar (MH). The application of OE and amoxicillin in the MH surface generated halo and its diameter corresponds cytotoxicity of the tested product.

Amoxicillin was used at a concentration of 50mg / mL. The plates with MH were seeded with bacteria *S. aureus* with a swab. Was employed at the different volumes of disks following samples: 25µL NaCl at a concentration of 0.9% (control) and 25µL 12,5µL amoxicillin 12,5µL and 25µL essential oil and the essential oil blend and amoxicillin both with the volume of 12,5µL. Then the plates were numerically identified and placed in the greenhouse for 24 hours. The cytotoxic test was performed in duplicate.

The cytotoxic activity was assessed by parameters determined in Resolution 1480/1990 of the Ministry of Health.

Genotoxicity test

The genotoxic evaluation of essential oil was made following the established protocol for cytotoxicity test, but passing some changes. This test objetive to comply with the standards determined by ANVISA (2003) and also assess the essential oil antioxidant activity *E. astringens*.

Stannous chloride ($SnCl_2$) was used at a concentration of 50mg/mL. The applications were made on bacteriological disks medium seeded with *Staphylococcus aureus* ATCC 8096 in duplicate. The essential oil was employed, fresh, and genotoxic substances stannous chloride ($SnCl_2$) and hydrogen peroxide (H_2O_2) in 8,5µL, 12,5µL and 25µL, accompanied by the control NaCl 0.9%.

The validated protocol was applied to the α -pinene component being: stannous chloride ($SnCl_2$), hydrogen peroxide (H_2O_2) and α -pinene in 8,0µL, 12,5µL and 25µL, accompanied by the control NaCl 0.9% respectively.

RESULTS AND DISCUSSION

The essential oil obtained by hydrodistillation of the leaves yielded 1,2 ml. The result of the oil yield obtained for *E. astringens* it was 0.17% based on the weight of the biomass used. The yield of essential oil observed at the end of the extraction will depend on the effectiveness of the process, the plant species and the conditions, as well as seasonality. The hydrodistillation with fresh leaves of *E. uniflora* had yield of 0.74%, while *E. racemosa* obtained 0.3% (BRUN & MOSSI, 2007; SENNA *et al*., 2011). Lopes (2008) conducted the same process with dried leaves of *E. uniflora* with yield of 2.06% and 1.67% in January and September respectively.

It was found in the chromatographic profile of the essential oil the monoterpene composition is higher than the sesquiterpene. The major constituent present in this oil was α–pinene (Figure 1). This result was similar to that found by Ramos et al (2010).

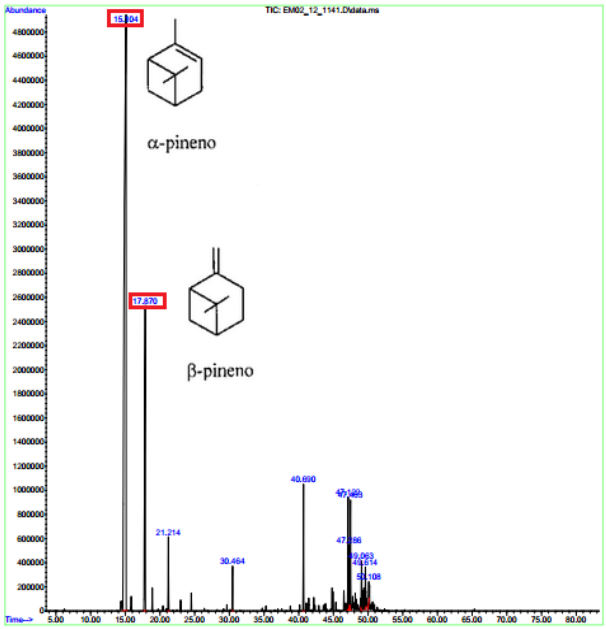


Figure 1: Chromatogram of total ion essential oil of the *E. astringens* Cambess.

The concentration of α -pinene (77.57%) was greater than that described by Defaveri *et al*. (2007) in the essential oil. 8 compounds (Table 1) were detected. The value of Kovats index of the components found was compared to those described by Adams (1995) and were

similar to those identified in the work Defaveri *et al*. (2007).

Table 1: Composition of essential oil of *Eugenia astringens*. KIC = Kovats indexvalue calculated (IK_C) and literature (IK_L) (%). Corresponds to normalized values of the areas.

Componente	IK_c	IK_L	(%)
α-pinene	930	939	77.57
α-pinene	978	980	12.49
myrcene	993	991	0.56
Terpinene	1062	1062	1.67
terpinen-7-al	1288	1287	0.83
Sesquisabinene hydrate (cis)	1544	1545	2.07
Farnesol (Z,Z)	1708	1713	1.60
Farnesol (E,E)	1716	1722	2.78
Total			99.57

The microbiological evaluation of *Staphylococcus aureus*, the colonies showed growth of rounded and yellowish colonies, with color change of the medium to yellow, being characteristic of this strain.

In testing conducted cytotoxic antibiotics (Table 2) and the oil in a greater dose (25µL) were respectively circle greater than 50mm and 28mm. It was observed that the essential oil and the antibiotic in 12,5µL dose showed circle greater than12mm and 52mm, respectively. The mixture of essential oil with antibiotics, both volume 12,5µL formed a 50mm circle. This final result demonstrates that the essential oil (EO) does not interfere in the effect of the antibiotic.

Tabela 2: Response Index (RI) to determine the percentage of degenerated cells of the citotoxy test with Amoxicilin and Essential Oil.

Sample	Zona Index (ZI)	Lise Index (LI)
Control (NaCl 0.9%)	0mm	-
Amoxicilin 12.5µL	>50mm	Greater than 80%
Amoxicilin 25µL	>50mm	Greater than 80%
EO 12.5µL	12mm	Less que 80%
EO 25µL	28mm	Greater than 80%
Amoxicilin 12.5µL + EO 12.5µL	50mm	Greater than 80%

The cytotoxicity assay with α -pinene showed that in 12,5µL and 25µL was not as cytotoxic as amoxicillin. The association of the α-pinene with amoxicillin (12,5µL each) there was a decrease of halo size, compared to the same antibiotic volume, showing that there was an interaction between the essential oil and amoxicillin (Table 3)

Table 3: Response Index (RI) to determine the percentage of degenerated cells of the citotoxy test with α-pinene and Amoxicilin.

Sample	Zona Index (ZI)	Lise Index (LI)
Control (NaCl 0.9%)	0mm	-
α-pinene 12.5µL	12mm	Less than 80%
α-pinene 25µL	20mm	Less than 80%
Amoxicilin 12.5µL	50mm	Greater than 80%
Amoxicilin 25µL	>50mm	Greater than 80%
Amoxicilin 12.5µL + α-pinene 12.5µL	50mm	Greater than 80%

The stannous chloride, in two volumes, showed a lysis index greater, than 80%, thereby genotoxic as described in the literature. The OE potentiated the genotoxic effect of $SnCl_2$ 12.5 µL. EO was 12.5 µL bit genotoxic as it had the lowest index lysis than 80%. The $SnCl_2$ 8.5 µL wasn't genotoxic. The H_2O_2 was genotoxic in the amounts used.

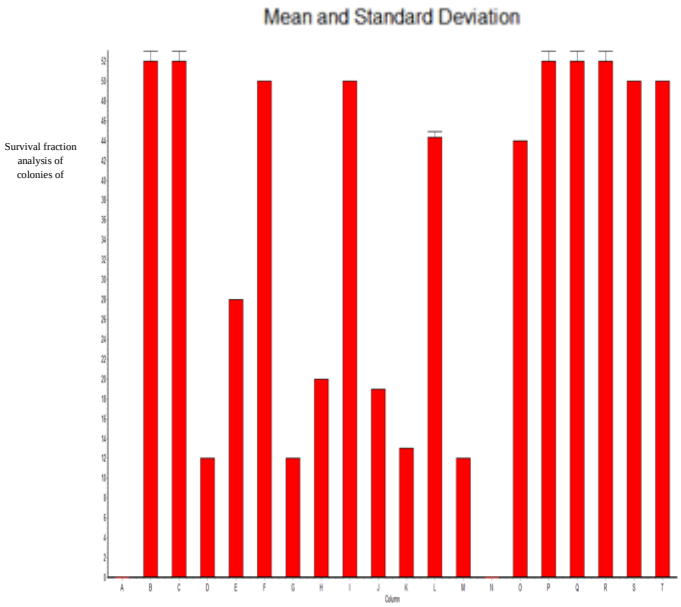
The H_2O_2 + OE 12.5 µL both, there was no increase in the halo size. Since the $SnCl_2$ + H_2O_2 there was a decrease of halo, namely an inhibition of genotoxicity. In

$SnCl_2$ OE + H_2O_2 + 8.5 µL, it is believed that the essential oil decreased the action of H_2O_2, acting as an antioxidant.

Table 4: Response Index (RI) to determine the percentage of degenerated cells of the genotoxicity test with the essential oil, $SnCl_2$ e H_2O_2.

Sample	Zona Index (ZI)	Lise Index (LI)
$SnCl_2$ 25uL	19mm	Less than 80%
$SnCl_2$ 12.5uL	13mm	Less than 80%
$SnCl_2$ 12.5uL + OE 12.5uL	44mm	Greater than 80%
OE 8.5uL	12mm	Less than 80%
$SnCl_2$ 8.5uL	0mm	Less than 20%
H_2O_2 8.5uL	44mm	Greater than 80%
H_2O_2 25uL	>50mm	Greater than 80%
H_2O_2 12.5uL	>50mm	Greater than 80%
H_2O_2 12.5uL + OE 12.5uL	>50mm	Greater than 80%
$SnCl_2$ 12.5uL + H_2O_2 12.5uL	50mm	Greater than 80%
$SnCl_2$+ H_2O_2 + OE 8.5uL	50mm	Greater than 80%
Control (NaCl 0.9%)	0mm	-

So far, have not found other work besides this, on the cytotoxicity and genotoxicity of essential oil of *E. astringens* Cambess. It needs other tests as to complement this study, as the Minimum Inhibitory Concentration (MIC) and *in vivo* assays.



Graphic 1- Comparative study regarding the biological effects of the essential oil of Eugenia astringens.

Survival fraction analysis of colonies of *Staphylococcus aureus* incubated under various experimental conditions as discrimando in the legend of the graph referring to the letters A to T.

In relation to the above chart, the columns represent the experimental conditions outlined in Tables 1, 2, 3 and 4, as detailed below.

A- Control; B- Amoxacilin (12.5 µL); C- Amoxacilin (25 µL); D- Essential Oil (12.5 µL); E- Essential Oil (25 µL); F- Amoxacilin and Essential Oil (12.5 µL); G- α-pinene (12.5 µL); H- α-pinene (25 µL); I- amoxacilin and α-pinene (12.5 µL); J- $SnCl_2$ (25 µL); K- $SnCl_2$ (12.5 µL); L-$SnCl_2$ and Essential Oil (12.5 µL); M- Essential Oil (8.5 µL); N- $SnCl_2$ (8.5 µL);O- H_2O_2 (8.5 µL); P-H_2O_2 (25 µL); Q- H_2O_2 (12.5 µL); R-H_2O_2 and Essential Oil (12.5 µL); S- $SnCl_2$ and H_2O_2 (12.5 µL); T-$SnCl_2$ and H_2O_2 (8.5 µL).

Based on statistical analysis of the results from the application of the statistical test of Tukey Kramer, one can point out that the essential oil extract at a concentration of 12.5% exhibited a similar bactericidal effect of amoxicillin to antibiotics ($p < 0.001$) not interfering with the action of said antibiotic when

incubated along with it. Similar result was obtained with the extract containing alpha pinene molecule. It was observed that the essential oil extract showed a more potent bactericidal oxidative effect that the solution of stannous chloride at a concentration of 8.5% ($p < 0.001$). It can be seen that the effect of the concentration of the essential oil extract and alpha pinene molecule is dependent extract concentration, having their proportionate effect to the increased concentration ($p < 0.001$) optimizing discretely in this way the effect of antibiotic.

CONCLUSION

Based on the Decree 1480/90 (MS, 1480/90) it is observed that the essential oil of *E. astringens* Cambes. It has antibacterial activity *in vitro* above 80% in the high dose (25µL). It was found that the use of essential oil (12.5 µL) along with the antibiotic (amoxicillin, 12,5µL) did not potentiate the drug action, this remaining unchanged. The EO has genotoxic activity dependent on dosage and it is believed that when there is an association of stannous chloride, hydrogen peroxide and the essential oil, the latter acts as an antioxidant.

The result of quantification of the EO sample from the calibration curve and Kowats index revealed that the percentage of α -pinene in the essential oil is 77%.

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