



African Journal
of Pharmacy and
Pharmacology

Volume 9 Number 6, 15 February, 2015

ISSN 1996-0816



*Academic
Journals*

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah

*Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius*

Himanshu Gupta

*University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of
Pharmacy Aurora, CO 80045,
USA*

Dr. Shreesh Kumar Ojha

*Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA*

Dr.Victor Valenti Engracia

*Department of Speech-Language and
Hearing Therapy Faculty of Philosophy
and Sciences, UNESP
Marilia-SP, Brazil.*

Prof. Sutiak Vaclav

*Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia*

Dr.B.RAVISHANKAR

*Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)*

Dr. Manal Moustafa Zaki

*Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt*

Prof. George G. Nomikos

*Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT
CENTER, INC. 675 North Field Drive Lake Forest, IL
60045
USA*

Prof. Mahmoud Mohamed El-Mas

Department of Pharmacology,

Dr. Caroline Wagner

*Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil*

Editorial Board

Prof. Fen Jicai

School of life science, Xinjiang University, China.

Dr. Ana Laura Nicoletti Carvalho

Av. Dr. Arnaldo, 455, São Paulo, SP. Brazil.

Dr. Ming-hui Zhao

*Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.*

Prof. Ji Junjun

Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences, China.

Prof. Yan Zhang

*Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.*

Dr. Naoufel Madani

*Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V
Souissi, Rabat,
Morocco.*

Dr. Dong Hui

Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University, China.

Prof. Ma Hui

School of Medicine, Lanzhou University, China.

Prof. Gu HuiJun

School of Medicine, Taizhou university, China.

Dr. Chan Kim Wei

*Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.*

Dr. Fen Cun

Professor, Department of Pharmacology, Xinjiang University, China.

Dr. Sirajunnisa Razack

Department of Chemical Engineering, Annamalai University, Annamalai Nagar, Tamilnadu, India.

Prof. Ehab S. EL Desoky

Professor of pharmacology, Faculty of Medicine Assiut University, Assiut, Egypt.

Dr. Yakisich, J. Sebastian

Assistant Professor, Department of Clinical Neuroscience R54 Karolinska University Hospital, Huddinge 141 86 Stockholm , Sweden.

Prof. Dr. Andrei N. Tchernitchin

Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA University of Chile Medical School, Chile.

Dr. Sirajunnisa Razack

Department of Chemical Engineering, Annamalai University, Annamalai Nagar, Tamilnadu, India.

Dr. Yasar Tatar

Marmara University, Turkey.

Dr Nafisa Hassan Ali

Assistant Professor, Dow institute of medical technology Dow University of Health Sciences, Chand bbi Road, Karachi, Pakistan.

Dr. Krishnan Namboori P. K.

Computational Chemistry Group, Computational Engineering and Networking, Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112 India.

Prof. Osman Ghani

University of Sargodha, Pakistan.

Dr. Liu Xiaoji

School of Medicine, Shihezi University, China.

ARTICLES

Research Articles

- In vitro* antioxidant action of the crude ethanolic extract from the leaves of *Ocotea pulchella* (Nees & Mart.) and their fractions** 145
Augusto Cesar Rebouças, Cristiane Bezerra da Silva, Luciane Dalarmi, Maislian de Oliveira, Katlin Suellen Rech, Josiane de Fátima Gaspari Dias, Sandra Maria Warumby Zanin, Vitor Alberto Kerber, Obdulio Gomes Miguel and Marilis Dallarmi Miguel
- Evaluation of the cicatrizant activity of a semisolid pharmaceutical formulation obtained from *Platonia insignis* Mart.** 154
Monica Cristiane Soares Mendes, Guilherme Antônio Lopes de Oliveira, Joselma Sousa Lacerda, Luís Mário Rezende Júnior, Mirna Luciano de Gois da Silva, Mayara Ladeira Coêlho, Adriana da Rocha Tomé, Joaquim Soares da Costa Júnior, Alexandre de Barros Falcão Ferraz, Jorge Maurício David and Rivelilson Mendes de Freitas
- Simple potentiometry and phenolphthalein-based titrimetric methods of analysis for Lisinopril tablets** 164
Ayodeji W. Olalowo¹, Olayemi M. Adegbolagun and Oluwayemisi A Bamiro
- Stability and disinfecting proprieties of the toothbrush rinse of the essential oil of *Protium heptaphyllum*** 173
Fernanda Endringer Pinto, Otávio Arruda Heringer, Mírian Almeida Silva, Tadeu Uggere Andrade, Juliano Souza Ribeiro, Dominik Lenz, Fernanda Campos Rosetti Lessa and Denise Coutinho Endringer

ARTICLES

Research Articles

- Mechanically separated fillet and meat nuggets of Nile tilapia treated with homeopathic product** **182**
Denise P. Lima¹, Mariana M. Fuzinato, Ana P. Andretto, Graciela L. Braccini, Ricardo H. Mori, Cristiane Canan, Saraspathy N. T. G. de Mendonça, Carlos A. L. de Oliveira, Ricardo R. Pereira and Lauro Vargas

Full Length Research Paper

***In vitro* antioxidant action of the crude ethanolic extract from the leaves of *Ocotea pulchella* (Nees & Mart.) and their fractions**

Augusto Cesar Rebouças*, Cristiane Bezerra da Silva, Luciane Dalarmi, Maislian de Oliveira, Katlin Suellen Rech, Josiane de Fátima Gaspari Dias, Sandra Maria Warumby Zanin, Vitor Alberto Kerber, Obdulio Gomes Miguel and Marilis Dallarmi Miguel

Department of pharmacy, Federal University of Paraná, Pós-graduation program in Pharmaceutical Sciences, Rua Prefeito Lothário Meissner 632, CEP: 80210-170, Curitiba, Paraná, Brasil.

Received 2 October, 2014; Accepted 3 February, 2015

The antioxidant potential of the crude ethanolic (CCE), hexane (HF), chloroform (CLF), ethyl acetate (EAF) and remaining extracts (RF), from the leaves of *Ocotea pulchella*, was verified using seven antioxidant assays: DPPH (2,2-diphenyl-1-picrylhydrazyl), phosphomolybdenum complex, reducing power (prussian blue), lipid peroxidation (TBARS- thiobarbituric acid reactive substances), deoxyribose oxidation and H₂O₂ scavenging. The obtained results showed that the EAF was more active in the DPPH, phospho-molybdenum, lipid peroxidation, deoxyribose oxidation and H₂O₂ scavenging methods; and the CLF showed to be more active in the reduction potential method. The hierarchical cluster analysis (HCA) and principal component analysis (PCA) analysis demonstrated that DPPH is the method which requires less extract and fractions concentrations to show antioxidant potential and that deoxyribose oxidation and H₂O₂ scavenging showed a relative antioxidant activity when compared to other methods. The reduction potential and phosphomolybdenum complex showed less antioxidant potential. The cluster analysis also shows that the EAF possesses an antioxidant activity similar to the tested standards. These results indicate that the EAF may contain promising antioxidant agents that can also be useful for the treatment of diseases caused by free radicals.

Key words: free radicals, antioxidant assays, PCA, HCA.

INTRODUCTION

The harmful effects of free radicals have drawn the attention of scientists to the importance of antioxidants in preventing and treating diseases (Niki, 2010). Hence,

there is an increased interest in searching for natural products which can be used as antioxidants, preventing the oxidative damage (Lindberg and Bertelsen, 1995;

*Corresponding author. E-mail: aug_cesar@yahoo.com.br

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](http://creativecommons.org/licenses/by/4.0/)

Moure et al., 2001). Several studies were performed with components derived from natural products, in order to unravel the components which have antioxidant properties and low cytotoxicity (Hazra et al., 2008). Natural antioxidants are important for daily consumption, while synthetic antioxidants, such as butyl hydroxyanisole (BHA) may act as carcinogenic in human beings (Ani and Naidu, 2011).

The oxidative stress leads to serious damage to macromolecules, such as proteins and DNA. However, free radical production can be eliminated by the action of endogenous enzymes, as well as by synthetic antioxidant activity (Halliwell 1990; Halliwell 1997). Antioxidants act by several mechanisms, including the prevention of transition metal ions- chelation catalysts, peroxidase decomposition and free radicals elimination (Valko et al. 2006).

Ocotea pulchella is popularly known as aloe cinnamon (canela-babosa), laurel avocado (louro-abacate), brown cinnamon (canela-parda), chilli cinnamon (canela-pimenta), guaiacá, cane-goiacá, amansa-aiui-saiiu. In Argentina, it is known as laurel-amarillo, also canelinha and inhumirim. The species occurs in Southern America, being common in Argentina, Paraguay, Uruguay and Brazil. In the latter, it is distributed in the Middle-Western (Goiás), Southeastern (Minas Gerais, Espírito Santo, São Paulo, Rio de Janeiro) and Southern (Paraná, Santa Catarina, Rio Grande do Sul) regions (Botega et al., 1993). No study concerning the phytochemical composition of its leaves has been reported so far, and benzyloquinoline alkaloids, such as 1-(*p*-methoxybenzoyl)-6,7-methylenedioxyisoquinoline and 1-(hydroxy-*p*-methoxybenzyl)-6,7-methylenedioxyisoquinoline were isolated from this species' bark (Botega et al., 1993). Thereby, investigations about the biological activity of *O. pulchella* are interesting, as the great part of species from this genus are useful in popular medicine (Ballabeni et al., 2010; Cabral et al., 2010; Coy-Barrera et al., 2009; Garcez et al., 2009; Guterres et al., 2013; Neto et al., 2011; Rolli et al., 2014).

MATERIALS AND METHODS

Plant

Leaves from *O. pulchella* Mart. were collected in April, 2010, in Pontal do Paraná, a municipality located in the coast of Parana state, Brazil, 25° 39' 54" S, 48° 27' 13" W, at an altitude of 3 m. The botanical determination was performed by biologist Osmar dos Santos Ribas, from the Municipal Botanical Museum of Curitiba, in the Parana state. The voucher was registered under the number 356741.

Preparation of the crude extract and fractions

The collected material was oven-dried at 35°C, then milled and

submitted to exhaustive extraction with ethanol in a soxhlet equipment. After extraction, the solvent was evaporated in a rotary evaporator (Rotavapor) at 50°C, and the ethanolic extract, already dry, was submitted to fractioning in soxhlet, with solvents of different levels of polarity. Then, the solvents were evaporated, and the hexane (HF), chloroform (CLF), ethyl acetate (EAF) and remaining alcoholic (FR) fractions were obtained.

Antioxidant activity by the DPPH method

The quantitative antioxidant capacity to reduce the DPPH radical was measured in visible UV spectrophotometry (Mensor et al., 2001). The DPPH solution was prepared some minutes before the assay at 0.03 mmol/ml in absolute ethanol. Five dilutions of each sample were prepared in concentrations which varied from 5.0 to 200 µg/ml. In each assay tube, 2.5 ml of each sample and 1 ml of the DPPH solution were added. The same diluted samples (2.5 ml) with 1.0 ml of solvent (CEE, EAF and RF: ethanol and HF and CLF: hexane), without reacting with DPPH, constituted the blank of the assay. The control corresponded to 2.5 ml of solvent with 1.0 ml of DPPH solution. These were left to react resting for 30 min at room temperature. As standards, vitamin C and rutin were used. After 30 min, the solution absorbances (Abs) were measured at 518 nm. All steps were performed in triplicate. The percentage of antioxidant activity (AA%) was measured with the formula:

$$AA\% = \frac{100 - (\text{Sample Abs} - \text{Blank Abs})}{\text{Positive Control Abs}} \times 100$$

The IC₅₀ value, which represented the concentration of the extracts (tested samples) that caused 50% reduction of the initial DPPH concentration was calculated from the non linear regression curve of log concentration of the tested extract (µg/ml) against the mean percentage of the radical scavenging activity (AA%) of the samples in each concentration. The line equation for this graph, where $y = ax + b$, is a basis for determining the IC₅₀ value.

Antioxidant activity by the phosphomolybdenum complex method

For the assay by the phosphomolybdenum method, the samples were dried in water bath (40°C), and using the dry material, methanolic solutions were prepared with a final concentration of 200 µg/ml. From these solutions, 0.3 ml was added to the phosphomolybdenum complex reaction solution. The tubes were closed and maintained in water bath at 95°C for 90 min. After cooling, the reading was made at 695 nm, in an UV-1601 Shimadzu® spectrophotometer to obtain the absorbances, using 0.3 ml of methanol from the reagent as blank. The sample antioxidant capacity is expressed in relation to rutin (200 µg/ml), which was used as standard, and vitamin C (200 µg/ml) which reference antioxidant activity was considered at 1.00 (Prieto et al., 1999). Results were expressed as relative antioxidant activity (AAR%) of the sample, related to vitamin C, following the formula:

$$AAR\% \text{ Related to vitamin C} = \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{blank})}}{\text{Abs}_{(\text{Vitamin C})} - \text{Abs}_{(\text{blank})}} \times 100$$

Antioxidant activity by the reduction potential method (Prussian blue)

During the antioxidant activity test by Prussian blue, extracts and fractions, in concentrations of 200 µg/ml were transferred to 25 ml-assay tubes, potassium phosphate buffer at 0.2 M (pH 7.0) and potassium ferricyanide at 1.0% were added. The mixture was incubated at 45°C for 20 min and then trichloroacetic acid at 1.0 % was added to the tubes. About 2.5 ml were transferred to 5.0 ml-assay tubes, and 1.5 ml of distilled water, 1.0 ml of ethanol and 0.5 ml of FeCl₃ at 1.0% (p/v) were added. The reading was made at 700 nm (Yen and Chen 1995). The results were expressed as relative antioxidant activity (AAR%) of the sample, related to vitamin C, following the formula:

$$\text{AAR\%} = \frac{\text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{Vitamin C})} - \text{Abs}_{(\text{blank})}} \times 100$$

Antioxidant activity by the TBARS (Thiobarbituric acid reactive substances) method

For the antioxidant activity assay by the lipid peroxidation inhibition method, the TBARS method was used. About 3.0 mg of the samples (extract and fractions) and BHT standard were diluted in 1.0 ml of ethanol. All the procedures were performed in triplicate. A volume of 0.5 ml of homogenized egg yolk solution (5% m/v), lipid rich medium, and 0.1 ml of each sample and control were added to assay tubes. Then, each tube received 0.05 ml of 2,2'-Azobis(2-methylpropionamide) dihydrochloride (ABAP) (0.035%) solution in order to induce lipid peroxidation. Then, were added 1.5 ml of acetic acid 20% (pH 3.5), 1.5 ml of thiobarbituric acid (TBA) (0.4% m/v) in a sodium dodecyl sulfate (SDS) (0.55% m/v) solution and 400 µl of distilled water were added. The prepared material was submitted to water bath (95°C) for 1 h under agitation. After cooling, each tube received 1.5 ml of n-butanol and was centrifuged during 3 min at 3,000 rpm, followed by supernatants spectrophotometric reading at 532 nm (Morais et al. 2006). The inhibition percentage in lipid peroxidation was calculated following the formula:

$$\% \text{ Inhibition} = \frac{1 - \text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{control})}} \times 100$$

Antioxidant activity by the thiocyanate oxidation method

During the antioxidant activity by ferric thiocyanate, extracts and fractions in a 200 µg/ml concentration were diluted in 1.0 ml of ethanol and then 250 µl of ethanol, 550 µl of linoleic acid at 2.51% (p/v) in ethanol (99.0% v/v), 2.0 ml of phosphate buffer 0.05 mol/L (pH 7.0) and 900 µl of distilled water were added to the tube and left in water bath at 50.0 ± 0.5°C. For 25 µl of this solution, 3.25 ml of ethanol 75% (v/v), 25 µl of ammonium thiocyanate at 30% (p/v) and 25 µl of ferrous chloride 0.02 mol/L were added. After 3 min of reaction at room temperature (25.0 ± 0.5°C), the samples were transferred to microplates and the absorbance was read at 500 nm in a microplate reader. The reading was repeated each 24 h, until the purple color of the control reached a value (Kikuzaki and Nakatani, 1993; Raymundo et al., 2004). The antioxidant butylated hydroxytoluene (BHT) was used as standard for these assays. The percentage of inhibition in lipid peroxidation was calculated

following the formula:

$$\text{AAR\% Related to vitamin C} = \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{blank})}}{\text{Abs}_{(\text{control})}} \times 100$$

Antioxidant activity by the deoxyribose oxidation method

The capacity of the extracts to avoid the decomposition of deoxyribose induced by Fe²⁺ and H₂O₂ was evaluated (Preusser et al., 2011). For the *O. pulchella* extract and fractions assays, 500 µg/ml of solution were added in 120 µl of deoxyribose solution 20 µmol/L, 0.125 ml of sodium phosphate buffer 0.1 mol/L, 40 µl of hydrogen peroxide 20 µmol/L, 40 µl of ferrous sulfate 500 µmol/ml and 500 µl of distilled water. The mixture was incubated for 30 min at 37°C. Then, 500 µl of trichloroacetic acid 2.8% and 400 µl of thiobarbituric acid 0.6% were added. The mixture was incubated once more at 30°C for 20 min. The reading was performed at 532 nm. The antioxidant vitamin C was used as standard, in a concentration of 500 µg/ml.

Antioxidant activity by the H₂O₂ scavenging method

The ability of the extracts to scavenge hydrogen peroxide was determined with a 4 mmol/L solution of hydrogen peroxide, which was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was spectrophotometrically determined at a wavelength of 230 nm (Ruch et al., 1989). For every 100 µl of the extraction solution and semipurified fractions of *O. pulchella* in a 500 µg/ml concentration, 900 µl of ethanol and 600 µl of hydrogen peroxide were added. Vitamin C was used as standard in a 500 µg/ml concentration. After 10 min of reaction at room temperature (25.0 ± 0.5°C), the reading was performed in a spectrophotometer, at a wavelength of 230 nm, against the blank solution containing the extracts and fractions in a buffer solution without hydrogen peroxide. The calculation of the scavenging activity was by the formula:

$$\% \text{ of H}_2\text{O}_2 \text{ scavenging} = \frac{100 - \text{Abs}_{(\text{control})} - \text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{control})}} \times 100$$

Statistical analysis

For the antioxidant assays, samples of extracts and fractions were evaluated in triplicate. Data were submitted to analysis of variance and when the effects of the treatments were significant, related to the control, averages were compared by Tukey test. All results were analyzed considering a significance level α = 5%. The obtained results were expressed as mean ± standard deviation.

Chemometric treatments

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) data was based on biological activity information. For each activity, two chemometric matrices were evaluated: first, the % antioxidant values, IC were interpreted to delineate the general behavior of each extract and fractions against the antioxidant

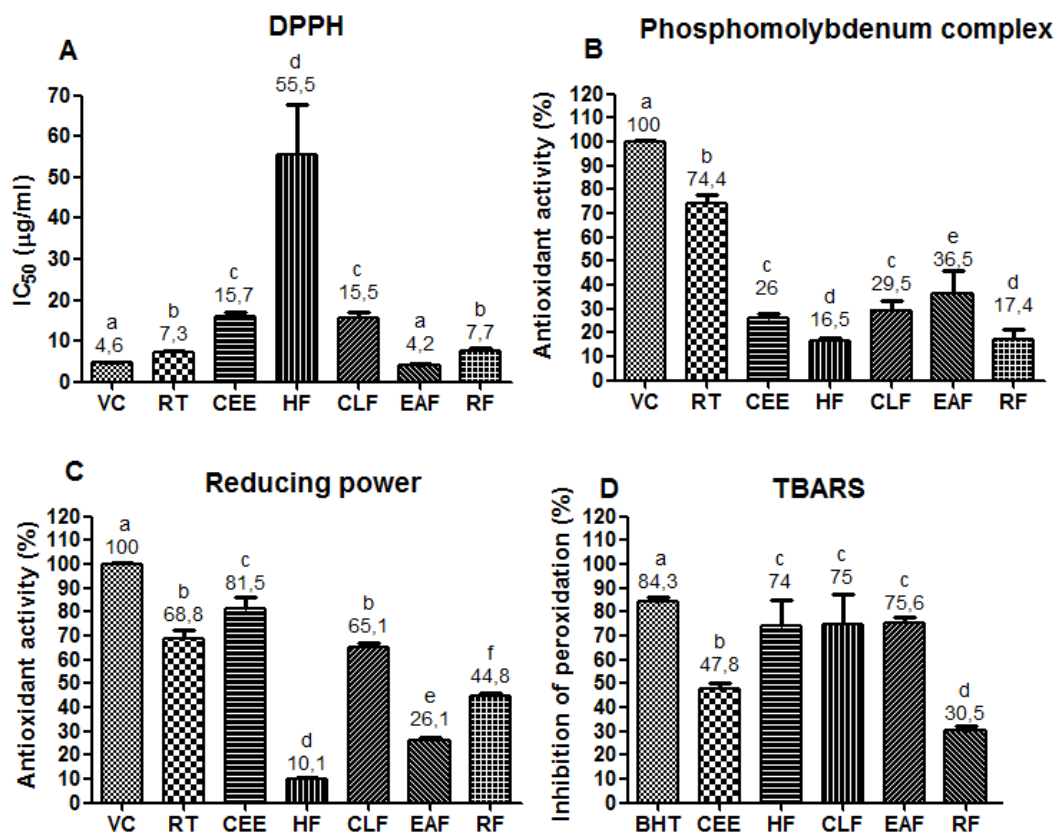


Figure 1. Antioxidant activity of the crude ethanolic extract (CEE), hexane (HF), chloroform (CLF), ethyl acetate (EAF) and remaining fractions (RF) of *O. puberulla*, by A: DPPH, B: Phosphomolybdenum complex, C: Reducing Power and D: TBARS. Means followed by the same letter do not differ each other by the Tukey test, $p < 0.005$.

antioxidant methods, bacterial and cellular lineages. Then, data of each activity were evaluated to specify the antioxidant, antimicrobial and cytotoxic potential, against the antioxidant methods, bacterial and cellular lineages. Hence, a PCA was performed through pre-processing of self-adjusted means with varied scales, which attribute to the same variant charge. For HCA, matrices were also auto-adjusted and Euclidean distances among extracts and fractions were calculated. The incremental linker method (an approach of the sum of squares to calculate intercluster distances) was applied to generate clusters (Brandão et al., 2013; Monteiro et al., 2008).

RESULTS

The extract and fractions originated from *O. pulchella* showed an antioxidant activity which was dependent of the used method. The DPPH showed that the ethyl acetate (EAF) possesses an antioxidant action which is similar to vitamin C, with an IC_{50} value of 4.2 $\mu\text{g/ml}$ and the RF to the rutin standard ($IC_{50} = 7.7 \mu\text{g/ml}$). Although the crude ethanolic extract (CEE) and chloroform (CLF) did not show a similar activity to standards, only 15.7 and

15.5 $\mu\text{g/ml}$ were necessary to achieve the IC_{50} (Figure 1A). No extract or fraction showed antioxidant activity similar to the standards vitamin C and rutin using the phosphorus-molybdenum complex method, and a small activity was observed for the EAF (36.5% of activity) (Figure 1B). The reduction potential method (Prussian blue) showed that CLF had a similar activity to the standard rutin (65.1% of activity). CEE presented an antioxidant activity of 81.5%, considered more active when compared to the fractions (Figure 1C). For the lipid peroxidation assay, HF (74%), FCL (75%) and EAF (75.6%) showed an inhibition activity of more than 50%. However, these values were not superior to the standard BHT (84.3%) (Figure 1D).

Similar to the lipid peroxidation inhibition method, EAF presented an antioxidant activity of 52% by the thiocyanate inhibition method. HF (92.2%) and CLF (98.7%) had similar activities to BHT (92.9%) (Figure 2A) in the thiocyanate inhibition method. For the deoxyribose method, only FH did not show an inhibition superior to 50%. Only EAF showed an activity of 97.3% and its

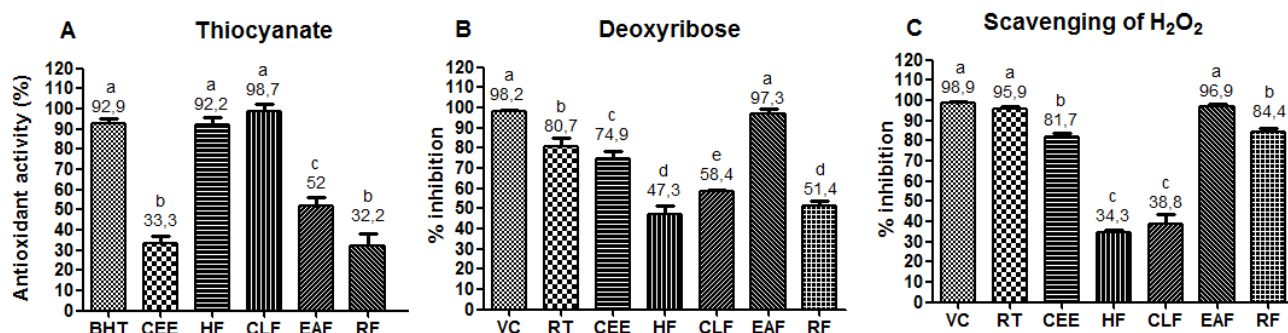


Figure 2. Antioxidant activity of the crude ethanolic extract (CEE), hexane (HF), chloroform (CLF), ethyl acetate (EAF) and remaining (RF) fractions of *O. puberulla*, by the methods A: thiocyanate inhibition, B: deoxyribose inhibition and C: hydrogen peroxide screening. Averages followed by the same letter do not differ each other by the Tukey test, $p < 0.005$.

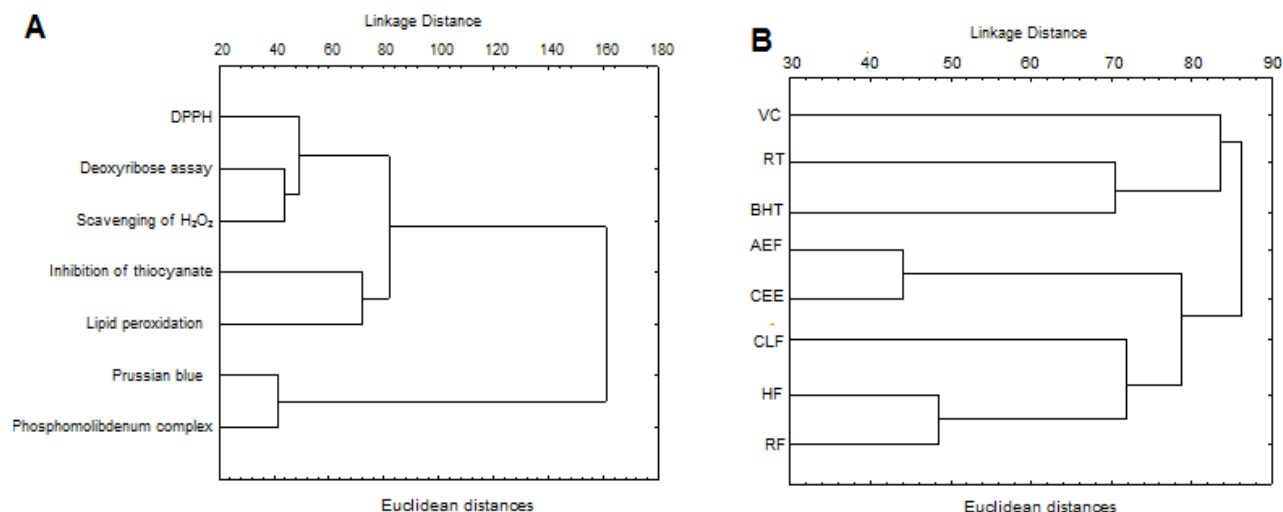


Figure 3. Built Cluster, from antioxidant activity data, for the tested antioxidant methods (A) and standards Vitamin C (VC), rutin (RT) and BHT, ethanolic crude extract (CEE), hexane (HF), chloroform (CLF), ethyl acetate (EAF) and remaining (RF) fractions (B) of *O. pulchella*.

activity was considered similar to vitamin C (98.2%). CEE (74.9%), CLF (58.4%) and remaining fractions (RF) (51.4%) showed an activity superior to 50% as well (Figure 2B). EAF showed an antioxidant activity similar to vitamin C and rutin, with an inhibition of 96.9% of the hydroperoxide radical (Figura 2C). A low activity was verified for HF and CLF, demonstrating that these fractions are not considered active by this method. CEE and RF showed activity between 81.5 and 84.4%.

A hierarchical cluster analysis (HCA) was performed for all the experimental data, taking the Euclidean distance as metric and the complete linkage method, as an amalgamation method. The obtained results were divided in three distinct groups, which correspond to three anti-

oxidant levels of extracts and fractions. As demonstrated in Figure 3, the DPPH method was characterized as the method which detects a higher antioxidant action, when compared to the others, followed by the deoxyribose reduction and H₂O₂ scan. The thiocyanate inhibition method and lipid peroxidation show as medium activity methods, and the reduction potential and phosphorus-molybdenum complex demonstrated to be less effective for the EEB and fractions of *O. pulchella* (Figure 3A).

When the antioxidant action among CEE and fractions was compared, a grouping indicating that EAF and CEE presented a higher antioxidant potential by the tested methods. HF and CLF would be considered fractions with an intermediate activity and FR the fraction which showed

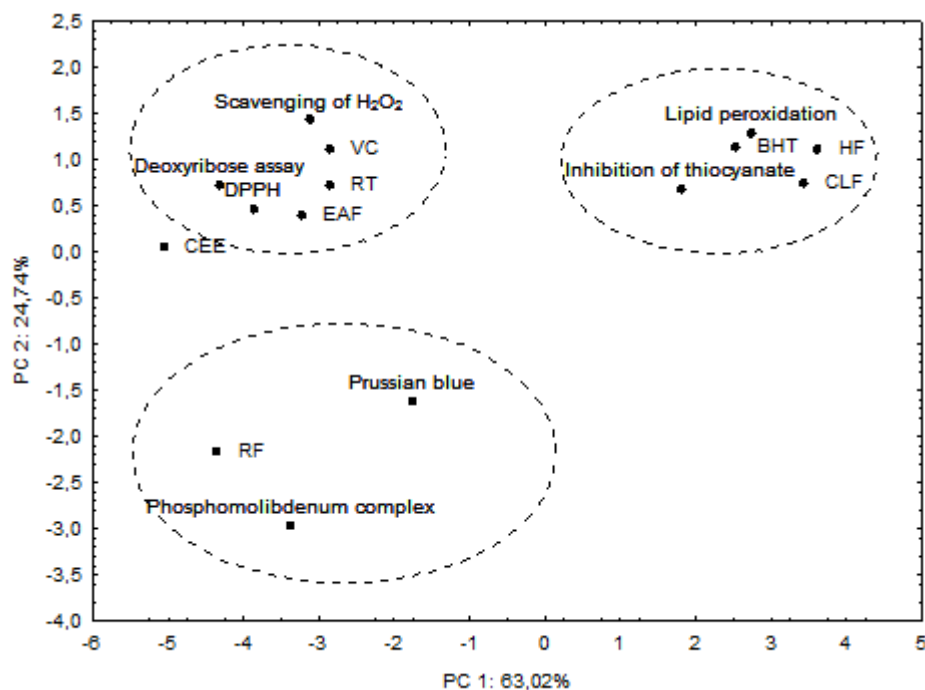


Figure 4. PCA which represents the evaluated parameters to the antioxidant activity with data parcels for the standard and extracts. The parcels show 63.02 and 24.74% of the total variance explained in PC1 and PC2, respectively.

showed smaller activity (Figure 3B). The cluster was performed with Euclidean distances, using an incremental linkage method to generate clusters. Branches represent stronger activity (upper portion, near to DPPH and standard vitamin C methods), intermediate and weak activities.

Data obtained in the antioxidant activity evaluation were used to perform a graph, where the loadings of the principal component analysis (PCA) correspond to antioxidant activity. The PC1 axis corresponds to 63.02% of variance, while the PC2 axis represents 24.74% and these axis explain the antioxidant effect under CEE and tested fractions. The extracts and fractions designated to elevated values over the PC2 axis have a pronounced antioxidant activity in EAF, CEE and standards vitamin C and rutin, indicating that these fractions and standard showed a higher activity for DPPH, deoxyribose inhibition and H₂O₂ scan methods. The methods which are closer to negative values for PC₂ and PC1 indicate a less antioxidant potential for RF and Prussian blue and phosphomolybden complex. The BHT, HF and CLF standards, localized on the positive values of PC1 and PC2, indicates high activity for the methods of lipid peroxidation (TBARS) and thiocyanate inhibition (Figure 4).

DISCUSSION

The analysis of the crude ethanolic extract and fractions obtained from leaves of *O. pulchella* indicated that the activity is concentrated on the crude extract and in the ethyl acetate fraction. The main reason for a less antioxidant activity of the hexane and chloroform fractions against the DPPH, reduction potential, phosphomolybden and H₂O₂ radical potential may be correlated to solubility and stability. These pharmacokinetic properties are closely related to the pharmacological efficacy, as the antioxidant efficacy depends on the capacity of the compounds to penetrate the cellular membrane (Takamatsu et al., 2003). Hence, this study directs the antioxidant potential of each fraction, when evaluated by the tested methods, and these results were confirmed by PCA analysis. The high activity of CEE and EAF for the DPPH, deoxyribose inhibition and hydroperoxide radical scavenging methods demonstrate their use in hydrophilic systems.

The DPPH is simple and largely employed, where DPPH is a stable free radical, not natural, whose properties differ from the highly reactive oxygen radicals such as hydroxyl (is capable of oxidizing a wide variety of organic compounds to CO₂, H₂O and inorganic ions from

heteroatoms), alcoxyl (may withdraw hydrogen atoms of other polyunsaturated fatty acids and lipid hydroperoxides, forming other lipid radical ($L\bullet$) and radical $LOO\bullet$) and superoxide, which play an important role in biological oxidative processes. Several series of chemical compounds showed a close correlation among the DPPH scavenging and antioxidant activities determined in biological and non-biological models (Malterud et al., 1993). Thus, the scavenging activity assay of the free radical DPPH is an antioxidant test which can be used for the screening of synthetic chemical compounds and natural products, being important as a preliminary test to determine the antioxidant potential of an extract and/or fractions, or even a pure substance. Other assays, such as the phosphomolybden complex determines the total antioxidant capacity of a sample against a redox reaction. It allows the evaluation of components both lipophilic and hydrophilic. This assay is based upon the reduction of molybden VI to molybden V, in the presence of some substances with antioxidant capacity, with the formation of a green complex between phosphate/molybden (V), in acid pH, spectrophotometrically determined at 695 nm (Prieto et al., 1999).

The potential reduction method (Prussian blue) is an important method as well, and is based on the reduction of Fe^{3+} , which determines the reduction power are also used for the antioxidant activity assessed. such methods evaluate the capacity of phenolic compounds to reduce Fe^{3+} , with a consequent formation of a colored complex with Fe^{2+} (Roginsky and Lissi, 2005). The reduction power is observed in the direct donation of electrons, during the potassium ferricyanide $[Fe(Cn)_6]^{3-}$ reduction to potassium ferrocyanide $[Fe(Cn)_6]^{4-}$. The product is visualized by the addition of Fe^{3+} ions, which after the reduction reaction, forms the Prussian blue complex, $Fe_4[Fe(Cn)_6]^{3-}$. The reduction potential assay becomes important in substance analysis, to be compared to DPPH activity. The advantage of this assay is that it allows for less soluble fractions to have their activity determined.

Methods which evaluate the lipid peroxidation are important, as they measure the action of a free radical under lipids of cellular membranes, leading to destruction of its structure, failure of metabolite-exchanging mechanisms and, in extreme conditions, cellular death (Benzie, 1996). In the ferricyanide methodology, peroxide oxidize Fe^{2+} to Fe^{3+} , which is dosed by colorimetry (500 nm) under chloride or ferricyanide (Silva et al., 1999). In this assay, hydroperoxides generated during the oxidation of linoleic acid react with ferrous sulfate, originating ferric sulfate and then to ferric thiocyanate, of a blood-red color. The absorbance decrease reveals the interruption in the oxidation due to linoleic acid unavailability in the middle of the reaction and to the onset of secondary products coming from hydroperoxide degradation (Chen et al.,

1996).

During TBARS assay, MDA-derived products are formed, which usually requires a low pH and elevated temperatures (80 to 100°C), some fluorescent substances derived from MDA may be generated in neutral pH and lower temperature (37°C). In neutral pH, at 37°C, a Hantzsch-like condensation reaction between MDA and primary amines produces fluorescent compounds. The non-specific reaction of MDA with amino-primary groups occurs in several biomolecules (proteins, nucleic acids, amines and phospholipids) producing complexes which are Schiff-conjugated bases (Janero, 1990). The MDA evaluation by the TBA test is not specific, because many other substances which occur in biological materials also react with TBA. This nonspecific is particularly important in lipids containing auto oxidation agents, such as secondary decomposition products, including a complex mixture of saturated short-chain compounds (alkanes), unsaturated (alkenes and dienes) and monofunctional aldehydes, in addition to MDA. Some monofunctional aldehydes are also reactive to TBA in acid medium and at elevated temperature used in the SRATB test, forming TBA derived products with a spectrum in the visible similar to that of MDA:TBA (1:2) complex (Janero, 1990). Therefore, the spectrophotometric test with TBA may measure a variety of species that absorb at 532 nm. Another possible interference in this method is due to the presence of transition metals which enable MDA generation during analysis. It is essential to include enough amount of antioxidant to prevent the auto oxidation of lipids and subsequent MDA formation during the heating phase of the assay. Additionally, it is also advisable to treat the reagents with transition metals chelates, to hinder their transference. Another disadvantage of the thiobarbituric acid reactive species test (TBARS) is the instability of the chromogen, which absorbs at 532 nm (Fukunaga et al., 1995).

The deoxyribose assay is a method where this sugar is degraded when exposed to the hydroxyl radical, generated by a mixture which contains Fe^{3+} , ascorbate and H_2O_2 in the presence of a small amount of EDTA. If the resulted mixture is heated in acid conditions, the malondialdehyde is formed, which can be detected by its ability to react with thiobarbituric acid (TBA), forming a rosy chromogen. Any compound added to the reactional mixture and able to react with OH will compete with deoxyribose for this radical, thus diminishing the degradation of this sugar and MDA formation. This method measures the combination of two factors: the ability in removing iron ions from deoxyribose and the ability in turning such ions inactive or poorly active for OH generation (Halliwell et al., 1987).

The obtained results of antioxidant action for different methods (independent of action mechanisms) is similar to EAF regarding the reduction of free radicals. The presented

antioxidant mechanisms may occur in parallel in all systems (although a mechanism can be predominant for some assay), or this may be determined by the chemical structure or solubility of the substances. These discussions recommend that only one antioxidant assay is enough to quantitatively detect or precisely determine the potential of an extract or fraction. Based on this information, it is plausible to infer that EAF is an efficient antioxidant, as it had more activity compared to the other fractions, and that its use should be investigated for the isolation and identification of substances present in it. This work provides a general vision of the several assay methods for antioxidant activity / capacity which are used to measure the non-enzymatic potential in food complex / biological matrices, including the general characteristics, comparisons and correlations of these methods.

ACKNOWLEDGMENTS

We are thankful to Paraná Federal University – Paraná – Brazil, and Capes/ Reuni for the research grant.

Conflict of interest

The authors declared no conflict of interest.

REFERENCES

- Ani V, Naidu KA (2011) Antioxidant potential of bitter cummin (*Centratherum anthelminticum* (L.) Kuntze) seeds in *in vitro* models. BMC Complement. Altern. Med. 11:40.
- Ballabeni V, Tognolini M, Giorgio C, Bertoni S, Bruni R, Barocelli E (2010). Ocotea quixos Lam. essential oil: In vitro and in vivo investigation on its anti-inflammatory properties. Fitoterapia 81(4):289-295.
- Benzie IF (1996). Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. Int. J. Food sci. Nutr. 47(3):233-61.
- Botega C, Pagliosa FM, Bolzani VdS, Yoshida M, Gottlieb OR (1993). Benzylisoquinoline alkaloids and eudesmane sesquiterpenes from *Ocotea pulchella*. Phytochemistry 32(5):1331-1333.
- Brandão LF, Alcantara GB, Matos Mde F, Bogo D, Freitas Ddos S, Oyama NM, Honda NK (2013). Cytotoxic evaluation of phenolic compounds from lichens against melanoma cells. Chem. Pharm. Bull. 61(2):176-83.
- Cabral MM, Barbosa-Filho JM, Maia GL, Chaves MC, Braga MV, De Souza W, Soares RO (2010). Neolignans from plants in northeastern Brazil (Lauraceae) with activity against *Trypanosoma cruzi*. Exp. Parasitol. 124(3):319-324.
- Chen H-M, Muramoto K, Yamauchi F, Nokihara K (1996). Antioxidant Activity of Designed Peptides Based on the Antioxidative Peptide Isolated from Digests of a Soybean Protein. J. Agric. Food Chem. 44(9):2619-2623.
- Coy-Barrera ED, Cuca-Suárez LE, Sefkow M (2009). PAF-antagonistic bicyclo[3.2.1]octanoid neolignans from leaves of *Ocotea macrophylla* Kunth. (Lauraceae). Phytochemistry 70(10):1309-1314.
- Fukunaga K, Takama K, Suzuki T (1995). High-performance liquid chromatographic determination of plasma malondialdehyde level without a solvent extraction procedure. Anal. Biochem. 230(1):20-3.
- Garcez WS, Garcez FR, da Silva LMGE, Hamerski L (2009). Larvicidal activity against *Aedes aegypti* of some plants native to the West-Central region of Brazil. Bioresour. Technol. 100(24):6647-6650.
- Guterres ZDR, da Silva AFG, Garcez WS, Garcez FR, Fernandes CA, Garcez FR (2013). Mutagenicity and recombinogenicity of *Ocotea acutifolia* (Lauraceae) aporphinoid alkaloids. Mutation Research/Genetic Toxicol. Environ. Mutagen. 757(1):91-96.
- Halliwell B (1990). How to characterize a biological antioxidant. Free Rad. Res. Commun. 9(1):1-32.
- Halliwell B (1997). Antioxidants: the basics--what they are and how to evaluate them. Adv. Pharmacol. 38:3-20.
- Halliwell B, Gutteridge JMC, Aruoma OI (1987). The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Analyt. Biochem. 165(1):215-219.
- Hazra B, Biswas S, Mandal N (2008). Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Complement. Altern. Med. 8(1):1-10.
- Janero DR (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol. Med. 9(6):515-540.
- Kikuzaki H, Nakatani N (1993). Antioxidant Effects of Some Ginger Constituents. J. Food Sci. 58(6):1407-1410.
- Lindberg MH, Bertelsen G (1995). Spices as antioxidants. Trends Food Sci. Technol. 6(8):271-277.
- Malterud KE, Farbrøt TL, Huse AE, Sund RB (1993). Antioxidant and radical scavenging effects of anthraquinones and anthrones. Pharmacology 47 Suppl 1:77-85.
- Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, Leitão SG (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15(2):127-30
- Monteiro MR, Ambrozini ARP, Lião LM, Boffo EF, Tavares LA, Ferreira MMC, Ferreira AG (2008). Study of Brazilian Gasoline Quality Using Hydrogen Nuclear Magnetic Resonance (1H NMR) Spectroscopy and Chemometrics. Energy Fuels 23(1):272-279.
- Morais SMD, Catunda Júnior FEA, Silva ARAd, Martins Neto JS, Rondina D, Cardoso JHL (2006). Atividade antioxidante de óleos essenciais de espécies de Croton do nordeste do Brasil. Quím. Nova 29:907-910.
- Moure A, Cruz JM, Franco D, Domínguez JM, Sineiro J, Domínguez H, Núñez MJ, Parajó JC (2001). Natural antioxidants from residual sources. Food Chem. 72(2):145-171.
- Neto RLM, Sousa LMA, Dias CS, Filho JMB, Oliveira MR, Figueiredo RCBQ (2011). Morphological and physiological changes in *Leishmania promastigotes* induced by yangambin, a lignan obtained from *Ocotea duckei*. Exp. Parasitol. 127(1):215-221.
- Niki E (2010). Assessment of Antioxidant Capacity in vitro and in vivo. Free Radic. Biol. Med. 49(4):503-515.
- Preusser M, de Ribaupierre S, Wohrer A, Erridge SC, Hegi M, Weller M, Stupp R (2011). Current concepts and management of glioblastoma. Ann. Neurol. 70(1):9-21.
- Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem. 269(2):337-41.
- Raymundo MdS, Horta P, Fett R (2004). Atividade antioxidante in vitro de extratos de algumas algas verdes (Chlorophyta) do litoral catarinense (Brasil). Rev. Brasil. Ciên. Farm. 40:495-503
- Roginsky V, Lissi EA (2005). Review of methods to determine chain-breaking antioxidant activity in food. Food Chem. 92(2):235-254.
- Rolli E, Marieschi M, Maietti S, Sacchetti G, Bruni R (2014). Comparative phytotoxicity of 25 essential oils on pre- and post-emergence development of *Solanum lycopersicum* L.: A multivariate approach. Ind. Crops Prod. 60(0):280-290.
- Ruch RJ, Cheng SJ, Klauing JE (1989). Prevention of cytotoxicity and

- inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 10(6):1003-8.
- Silva FAM, Borges MFM, Ferreira MA (1999). Métodos para avaliação do grau de oxidação lipídica e da capacidade antioxidante. *Quím. Nova* 22:94-103.
- Takamatsu S, Galal AM, Ross SA, Ferreira D, ElSohly MA, Ibrahim AR, El-Ferally FS (2003). Antioxidant effect of flavonoids on DCF production in HL-60 cells. *Phytother. Res.* 17(8):963-6.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Interact.* 160(1):1-40.
- Yen G-C, Chen H-Y (1995) Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. *J. Agric. Food Chem.* 43(1):27-32.

Full Length Research Paper

Evaluation of the cicatrizant activity of a semisolid pharmaceutical formulation obtained from *Platonia insignis* Mart.

Monica Cristiane Soares Mendes¹, Guilherme Antônio Lopes de Oliveira²,
Joselma Sousa Lacerda³, Luís Mário Rezende Júnior³, Mirna Luciano de Gois da Silva²,
Mayara Ladeira Coêlho², Adriana da Rocha Tomé⁴, Joaquim Soares da Costa Júnior⁵,
Alexandre de Barros Falcão Ferraz⁶, Jorge Maurício David⁷ and Rivelilson Mendes de Freitas^{1,8*}

¹Pharmaceutical Sciences Department, Federal University of Piauí, Teresina, Piauí, Brazil.

²Biotechnology Department - RENORBIO, Federal University of Piauí, Teresina, Piauí, Brazil.

³Scientific Initiation Scholarship - PIBIC-UFPI, Teresina, Piauí, Brazil.

⁴State University of Ceará, Fortaleza, Ceará, Brazil.

⁵Federal Institute of Piauí, Teresina, Piauí, Brazil.

⁶Lutheran University of Brazil, Laboratory of Genetic Toxicology, Rio Grande do Sul, Brazil.

⁷Federal University of Bahia, Salvador, Bahia, Brazil.

⁸Federal University of Piauí, Teresina, Piauí, Brazil.

Received 3 September, 2014; Accepted 3 February, 2015

The use of natural products for skin treatments has been increased. Mainly, those of topical action incorporated to pharmaceutical forms, since they allow the restoring of skin integrity after possible attacks. In this context, the plant *Platonia insignis* Mart has been widely used for the treatment of various skin diseases and as wound healing medicine in folk medicine. The aim of this study is to evaluate the wound healing activity of a cream formulated containing triglyceride isolated from *P. insignis* Mart called 1,3-distearoyl-2-oleoylglycerol (TG1) in skin lesions induced in Wistar rats by macroscopic and histological analysis of the wounds. The animals were randomly divided into 5 groups according to the treatment to be performed. The negative control received saline solution, the positive control received collagenase and the other groups received cream containing the triglyceride at concentrations of 5, 10 and 15%. The results showed effectiveness in the healing of wounds through reduction of their diameter compared to negative control. Histological analysis demonstrated the inflammatory and proliferative phenomena of healing in wounds of rats. The cream with TG1 in three concentrations demonstrated efficacy in wound healing, as evidenced by macroscopic and microscopic analyses of lesions in Wistar rats. Based on this, a further development of phytomedicines for wound care is suggested.

Key words: Cutaneous wounds, healing, *Platonia insignis*.

INTRODUCTION

Natural products from plants, animals and microorganisms are a huge source of different chemicals

that can be used by pharmaceutical industries for production of medicines (Ogbourne and Parsons, 2014; Fonseca et al., 2014). Bioactive substances, such as alkaloids, steroids, glycosides, saponins, tannins and flavonoids present mainly in plants, are potential therapeutic agents that can help in wound healing (Adele et al., 2014; Kuntal, 2013). The healing process involves migration of inflammatory cells, synthesis of granulation tissue, deposition of collagen and proteoglycans and scar maturation, being associated with intense refurbishment. The search for treatments using medicinal plants as a healthier lifestyle alternative has led to complementary medicine to be used as alternative to treat wounds (Mukherjee et al., 2013; Furumoto et al., 2014; Santos et al., 2006). In this context, the plant *Platonia insignis* Mart has been widely used in treatments of several dermatoses and as a preparation for healing wounds in folk medicine. This species, popularly known as "bacurizeiro", is a monotype vegetal belonging to the Clusiaceae family. It is a fruit tree species and timber found in the Northeast region of Brazil, mainly in the states of Piauí and Maranhão. Its seeds are used in the processing of oil or "bacuri lard". Recent studies have shown the effectiveness of the oil from seeds in increasing the healing process of skin wounds in rats (Souza et al., 2013; Moura et al., 2007; Santos et al., 2013; Clerici et al., 2011).

Given the growing use of natural products for dermatological treatments, the topical action incorporated in dosage forms should be highlighted because they allow the restoration of skin integrity after possible attacks (Karodi et al., 2009). Thus, the semi-solid pharmaceutical formulations using a natural and typical product as active principle may be an alternative for caring of local population. In addition, it may contribute to the regional flora valorization showing its economic and biological potential. In this scenery, the aim of this study was to evaluate the wound healing activity of a formulated cream containing TG1 (1,3-distearoyl-2-oleoylglycerol) (Figure 1) in skin lesions of Wistar rats by macroscopic and histological analysis.

MATERIALS AND METHODS

Plant

The seeds were obtained from fruits in the city of Barras, Piauí, Brazil, in March 2013. A voucher specimen was deposited in the Herbarium Graziella Barroso of Federal University Piauí (UFPI), N° ICN TEPB 27,164.

Extraction and isolation of TG1

The *P. insignis* seeds were dried at 55°C and powdered, yielding 848 g, and then extracted with hexane in Soxhlet for 8 h. The extract was concentrated in vacuum rota evaporator (534.24 g, yield 63%) and stored at 8°C. A formation of a white precipitate was observed, consisting of TG1. The white precipitate was fractionated by chromatography over Si gel, using n-hexane-EtOAc (19:1) yielded TG1. Its spectra of NMR ^1H NMR (Figure 2), ^{13}C NMR (Figure 3), DEPT 135 (Figure 4), COSY (Figure 5) and HMBC (Figure 6) were obtained for identification of TG1: $\text{C}_{57}\text{H}_{108}\text{O}_6$. NMR spectra description of ^1H is ^{13}C NMR (CDCl_3 , ppm, 125 MHz): $\delta_{\text{H}} = 5.45\text{-}5.35$ (4H, m, $-\text{CH}-\text{CH}-$), $5.30\text{-}5.28$ (1H, m, $-\text{CH}_2-\text{CH}-\text{CH}_2-$), $4.34\text{-}4.30$ (2H, dd, $-\text{CH}_2-\text{CH}(\text{O})-\text{CH}_2-$), $4.19\text{-}4.15$ (2H, dd, $-\text{CH}_2-\text{CH}(\text{O})-\text{CH}_2-$), $2.35\text{-}2.31$ (6H, t, $-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-$), $2.04\text{-}1.96$ (4H, m, $=\text{CH}_2-\text{CH}_2-$), $1.65\text{-}1.62$ (6H, m, $-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-$), $1.32\text{-}1.29$ (nH, m, $-\text{CH}_2-$), $0.92\text{-}0.89$ (9H, t, $-\text{CH}_3$). NMR spectra description of ^{13}C is ^{13}C NMR (CDCl_3 , ppm, 500 MHz), $\delta_{\text{C}} = 173.31$ (C-1, sn 1,3); 172.87 (C-1, sn 2); 34.06 (C-2, sn 1,3); 34.21 (C-2, sn 2); 24.88 (C-3, sn 1,3); 24.88 (C-3, sn 2); 29.22 (C-4, sn 1,3); 29.14 (C-4, sn 2); 29.50 (C-5, sn 1,3); 29.50 (C-5, sn 2); 29.35 (C-6, sn 1,3); 29.35 (C-6, sn 2); 29.70 (C-7, sn 1,3); 29.70 (C-7, sn 2); 29.72 (C-8, sn 1,3); 29.72 (C-8, sn 2); 29.72 (C-9, sn 1,3); 129.69 (C-9, sn 2); 29.72 (C-10, sn 1,3); 130.03 (C-10, sn 2); 29.72 (C-11, sn 1,3); 29.72 (C-11, sn 2); 29.38 (C-13, sn 1,3); 29.38 (C-13, sn 2); 31.94 (C-14, sn 1,3); 31.94 (C-14, sn 2); 22.71 (C-15, sn 1,3); 22.71 (C-15, sn 2); 31.94 (C-16); 22.71 (C-17); 14.13 (C-18); 68.88 (C-2, CHO); 62.10 (C-1 and 3, CH_2O).

Cream preparation formulation

The emulsions developed were O/A type in the pharmaceutical form of creams. It was chosen a nonionic-base cream that allows the vehicle general cosmetic principles (Brazil, 2011). To prepare the nonionic basic cream, the emulsion components were separately weighed on an analytical scale and classified into two distinct phases: aqueous phase and oil phase. The oil and aqueous phases were heated at 80 and 85°C in two beakers, respectively. Subsequently, the internal phase (oil) was poured under the external phase (aqueous) under stirring, and the product was taken from heating. After cooling, with moderate stirring, the preservatives were added at approximately 40°C to reach the room temperature. The base was then homogenized. The cream-based active TG1 were initially weighed 2.5, 5 and 7.5 g of TG1 in order to acquire the formulations at concentrations of 5, 10 and 15%. The active was slowly incorporated to the base under constant homogenization, using an adjuvant (propylene glycol).

Animals

A total of 50 adult male and female Wistar rats (250 ± 50 g) were used in this study. Animals were kept in well-ventilated cages (Alesco, São Paulo) under standard conditions of light (12 h with alternative day and night cycles) and temperature ($24 \pm 1^\circ\text{C}$), and they were housed with access to commercial rodent stock diet (Nutrilabor, São Paulo, Brazil) and water *ad libitum*. The

*Corresponding author. E-mail: rivmendes@hotmail.com. Tel. +55 86 3215 5870.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

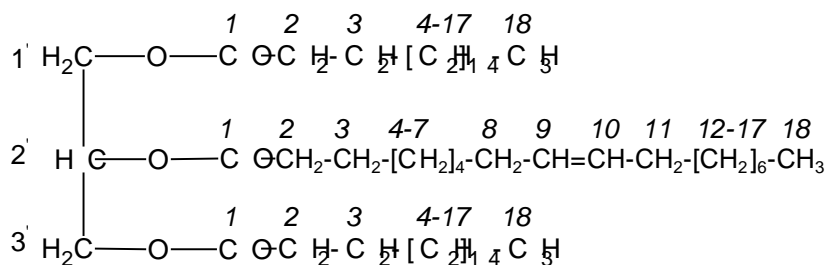


Figure 1. Chemical structure of TG1.

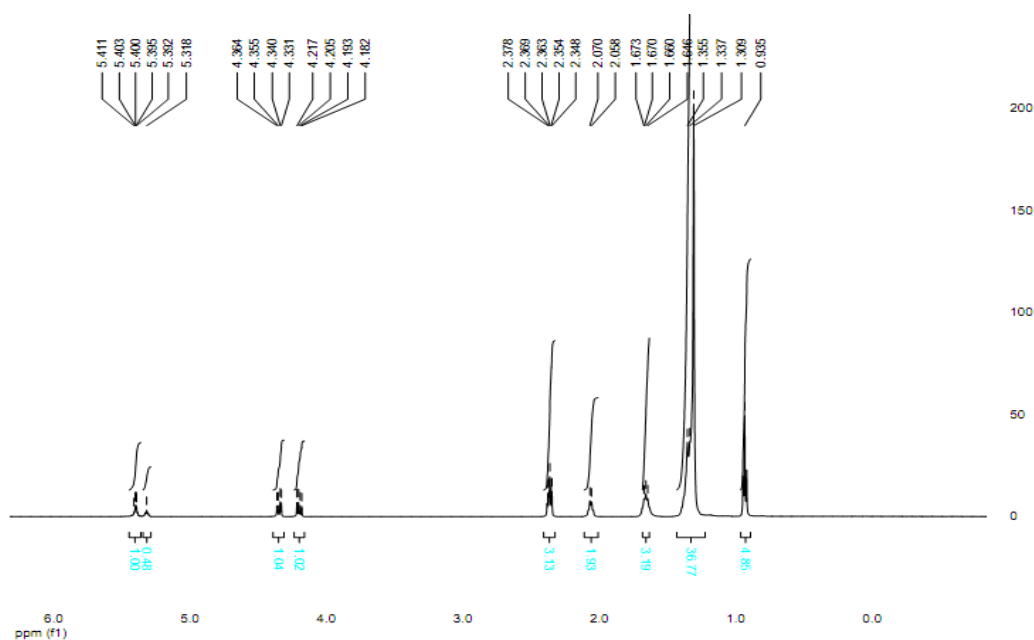


Figure 2. ^1H NMR Spectrum of TG1 in CDCl_3 , 500 MHz

investigational protocols were approved by the local Ethical Committee on Animal Research at Federal University of Piauí (078/2012). Animals were randomly divided into 5 groups according to the treatment ($n = 10$ rats/group) (Figure 7).

Procedure for injury obtaining

After intraperitoneal anesthesia of the rats with sodium pentobarbital (40 mg/100 g), it was performed trichotomy and epilation in the dorsal region, removing skin circular diameter of 4 mm with the aid of a punch (tool provided with a circular cutting surface) to expose the dorsal muscle fascia. Afterwards, animals were put back in their cages and treated with the samples (Figure 7) observed daily for the 7th, 14th and 21st days.

Wound care

The wounds were treated with a bandage, which were composed of gauze (first layer) and crepe bandage (second layer), and evaluated. The treatment was topically administered in the injured

area once a day during 14 days. The wounds were cleaned with 0.9% saline every new application (Santos et al., 2002; Rahal et al., 2011). Furthermore, in order to avoid interference with wounds regarding a possible cross contamination, the autoclaving of wood shavings was carried out daily. This procedure improves asepsis and reduces friction of animals inside cages.

Macroscopic evaluation of skin lesions in rats

Treated animals were monitored daily and observed for the repair of the lesion. The observed parameters were changes in the presence or absence of edema, exudate and crust, and wound color. Digital photographic of the wound were recorded during treatments. In addition, the lesions were measured with analogical caliper in 7th, 14th and 21st day of treatment.

Histological evaluation of skin lesions in rats

A rectangular skin fragment (0.5 cm \times 2.0 cm) from the back of each animal was dried. Each segment contained an injured central

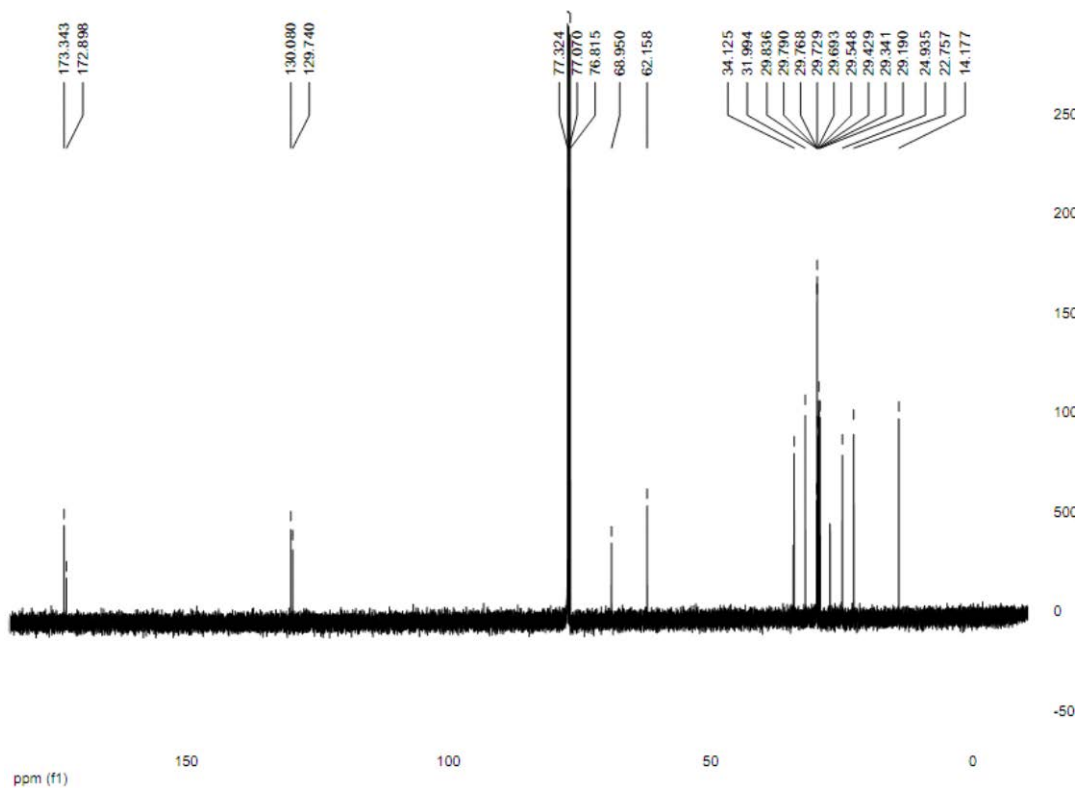


Figure 3. ^{13}C NMR Spectrum of TG1 in CDCl_3 , 125 MHz

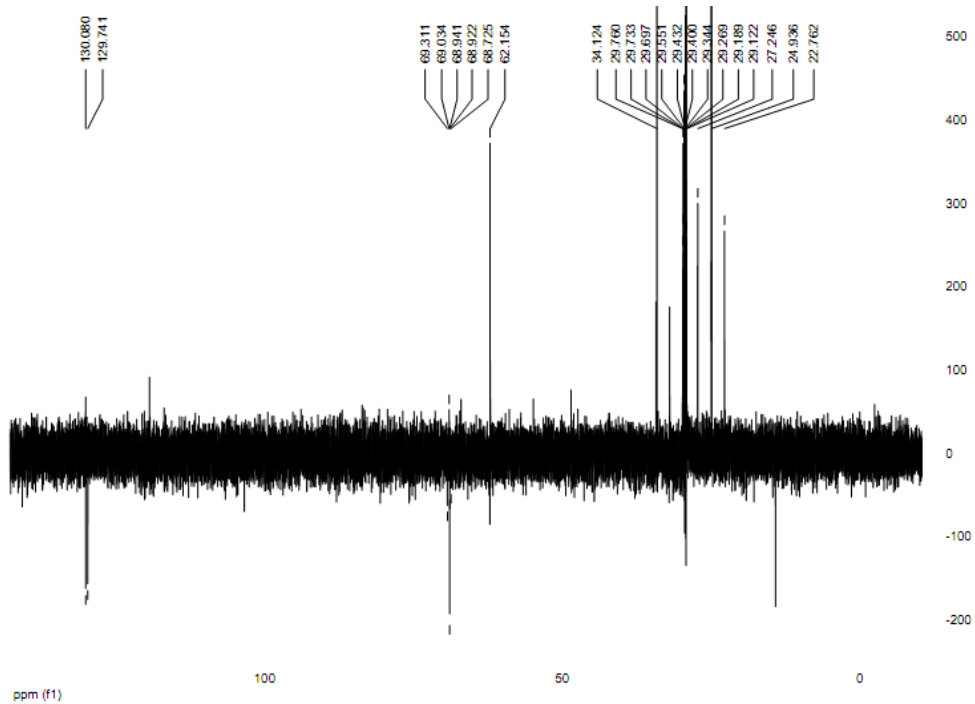


Figure 4. DEPT 135° Spectrum of TG1 in CDCl_3 , ^{13}C NMR (CDCl_3 , ppm, 125 MHz) MHz.

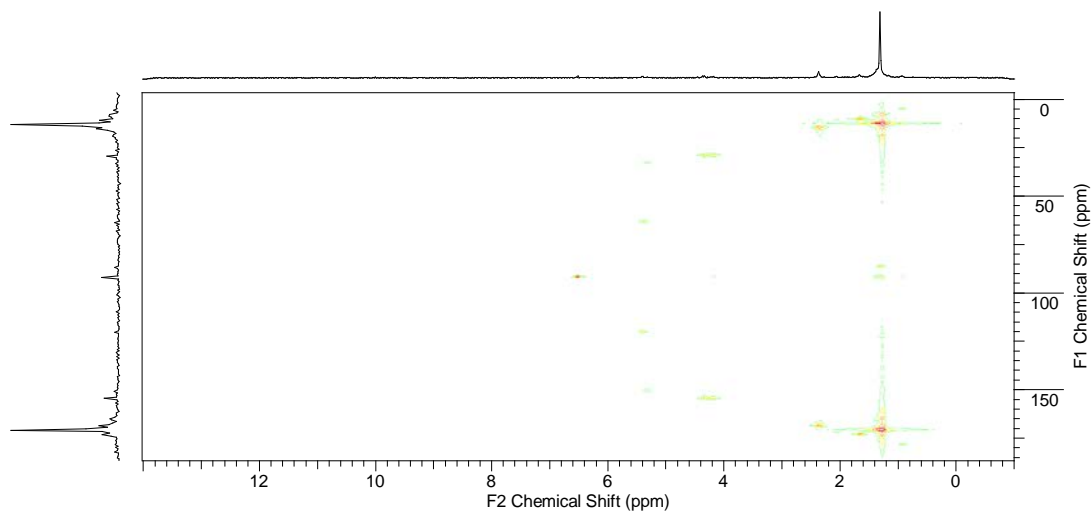


Figure 5. HMBC Spectrum of TG1 in CDCl_3 , 500 MHz.

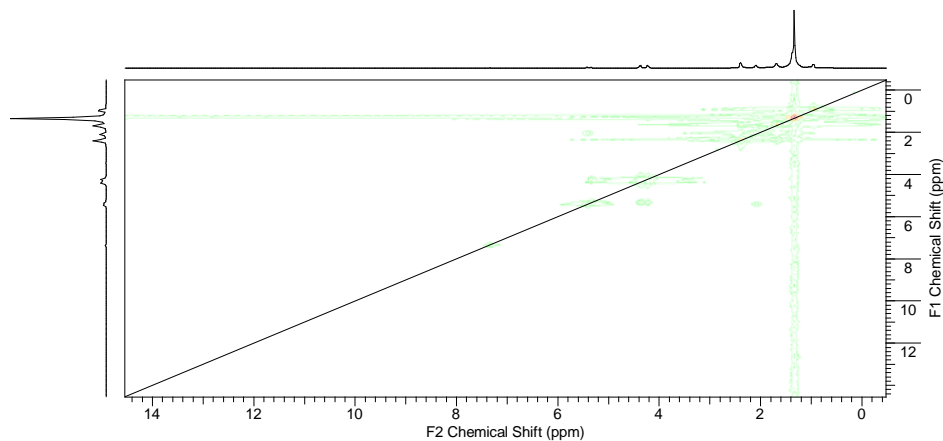


Figure 6. ^1H - ^1H COSY Spectrum of TG1 in CDCl_3 , 500 MHz.

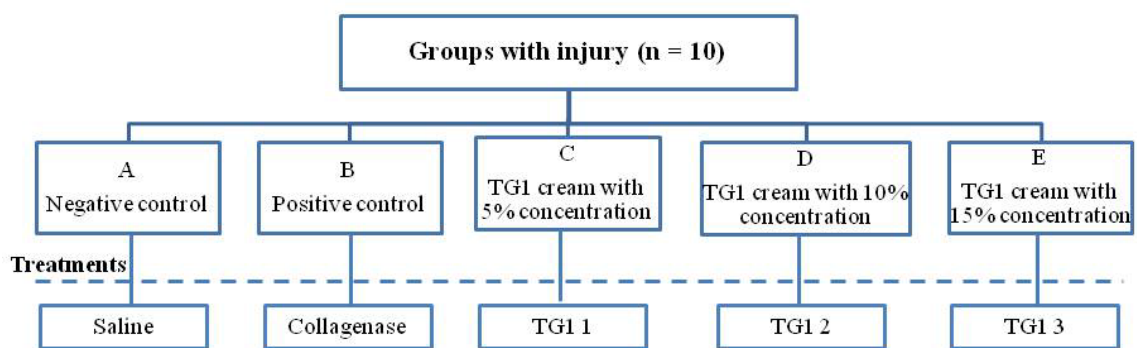


Figure 7. Distribution of the experimental groups for evaluation of cutaneous wound healing in rats.

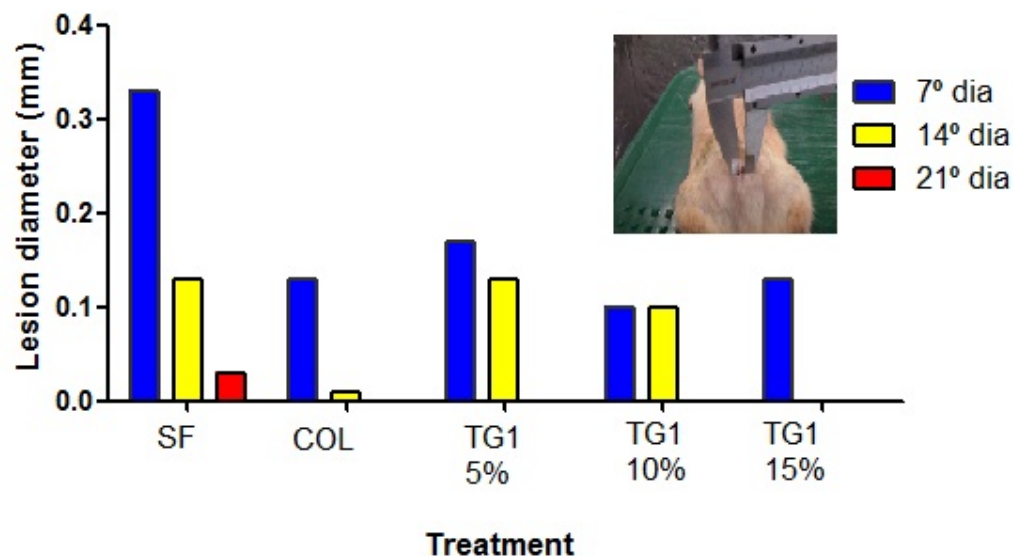


Figure 8. Diameter of skin lesions in animals on the 7th, 14th and 21st days of treatment with cream containing TG1 (1,3-distearoyl-2-oleoylglycerol).

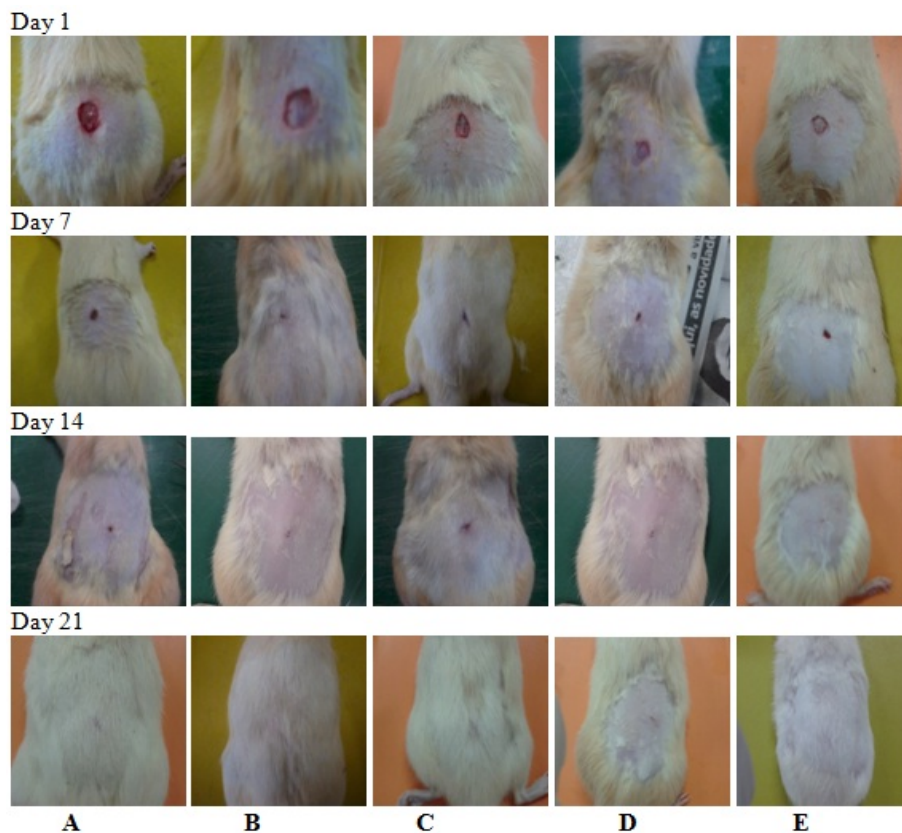


Figure 9. Macroscopic analysis of the cutaneous lesions of animals after 1, 7, 14 and 21 days of daily treatment. Legend - A: Treatment with collagenase; B: Treatment with saline; C: Treatment with cream containing 5% TG1; D: Treatment with cream containing 10% TG1, E: Treatment with cream containing 15% TG1.

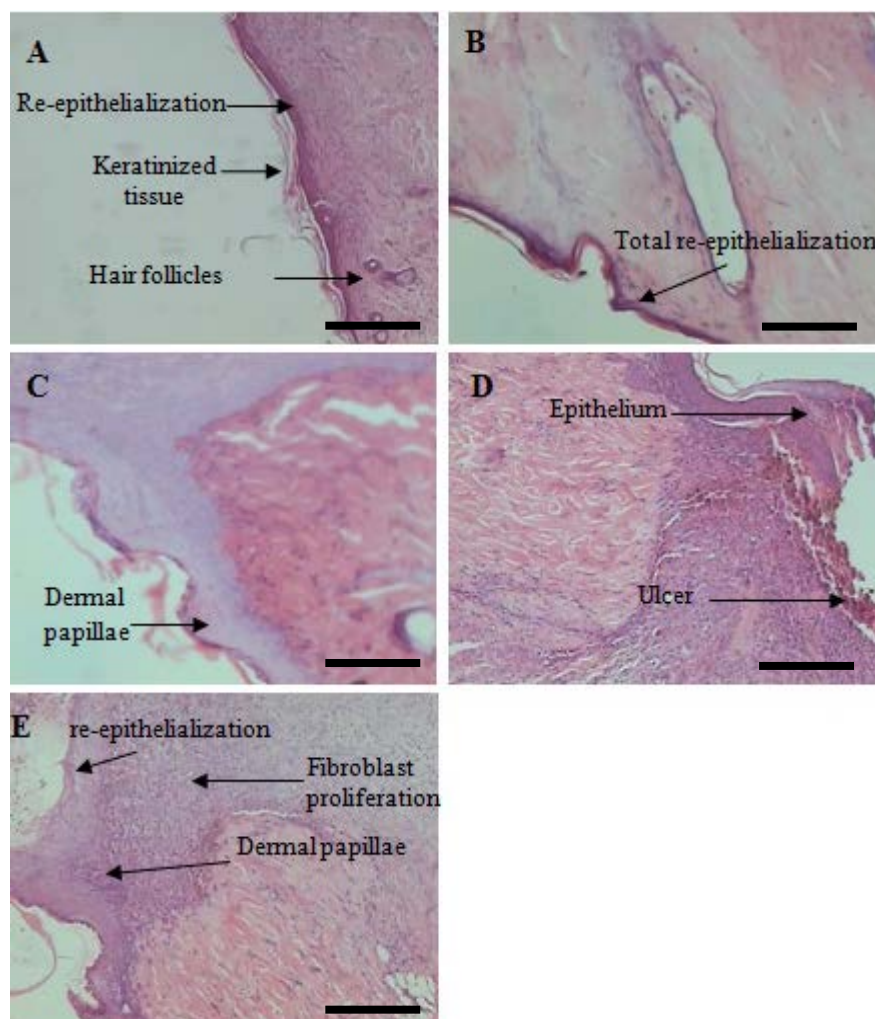


Figure 10. Histological analysis on the 7th day of treatment of skin wounds in rats. Legend - A: Saline; B: Collagenase; C: Cream with 5% TG1, D: Cream with 10% TG1 and E: Cream with TG115%. Hematoxylin & Eosin staining (H&E, Scale bar, 10 μm). Magnification, 100 x. One representative experiment with n = 5 is shown.

area and a non-injured peripheral area of skin to serve as controls for 7 days, 14 and 21 days of treatment (Coelho et al., 2010). All skin lesion samples obtained were fixed in formalin and sent for histological preparation at the State University of Ceará (UECE). The analysis was performed under optical microscope at (100x magnification) in order to observe inflammatory and healing processes such as: presence of granulation tissue, vascular proliferation, acute and chronic inflammation, presence of collagen and re-epithelialization.

Statistical analysis

Statistical analyses were performed with the Graph Pad Prism software, version 5.0 (San Diego, CA, USA). Results are expressed as mean ± standard error of mean (SEM). Data were analyzed by analysis of variance (ANOVA) followed by Student-Newman-Keuls.

Differences were considered statistically significant when $p < 0.05$.

RESULTS

Macroscopic evaluation of skin lesions, the groups that received collagenase (positive control) and cream in three concentrations showed wounds with about 0.1 to 0.2 mm in diameter, while the group that received saline had lesions with about 0.3 mm in diameter (Figure 8). Thus, it was noted that on the 7th day of treatment with collagenase and cream preparations (5, 10 and 15%), the wounds presented smaller diameters when compared with the group treated with saline only (negative control). The macroscopic lesions of rats treated with saline

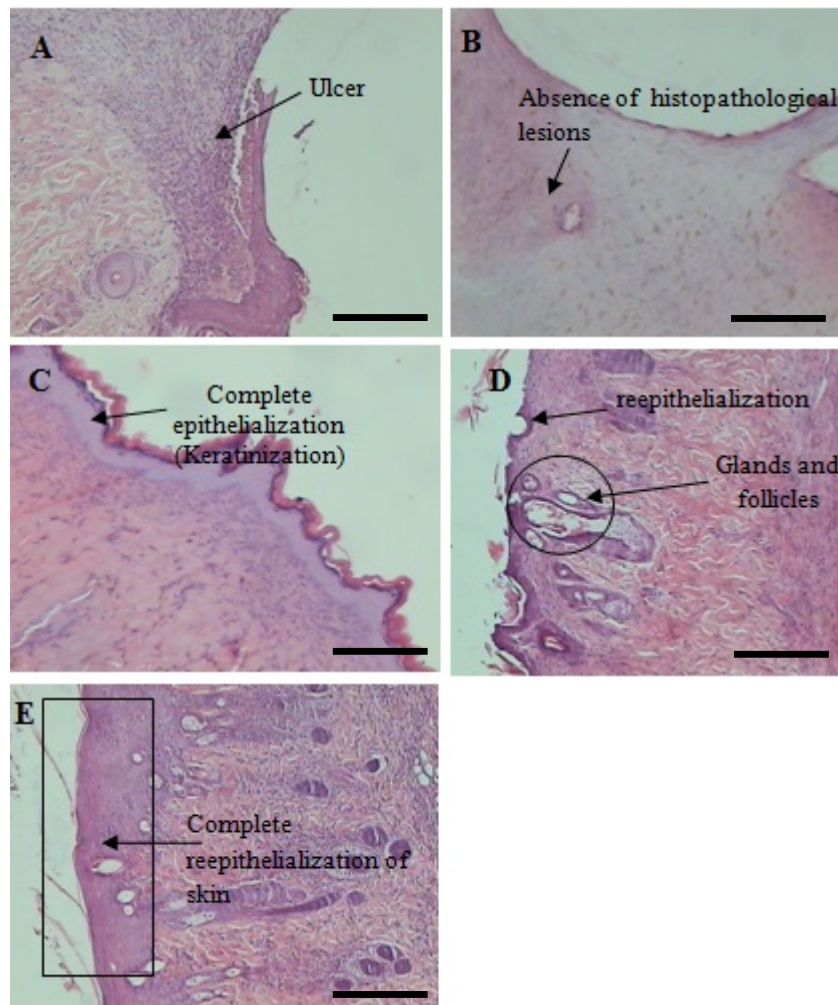


Figure 11. Histological analysis of the 14th day of treatment of skin wounds in rats. Legend - A: Saline; B: Collagenase; C: Cream with 5% TG1, D: Cream with 10% TG1 and E: Cream with 15% TG1. Hematoxylin & Eosin staining (H&E, Scale bar, 10 μm). Magnification, 100x. One representative experiment with n = 5 is shown.

(negative control), collagenase and cream in three concentrations allowed realizing evolution of the tissue repair. The groups treated with collagenase and cream in a concentration of 5% did not show edema or hemorrhage in the lesion. The groups treated with saline and cream in concentrations of 10 and 15% showed edema and purulent secretion on the 7th day of treatment. After a week of administration, there was formation of brownish crust in the majority of groups. There was no evidence of granulation during the evaluations (Figure 9). Figures 8 and 9 indicate that increasing concentration of cream accelerates the healing process, since decreased wounds compared to the three concentrations were noted. This supports the

cream effectiveness on the wound healing. In the concentration of 15%, it was observed the absence of injury on the 14th day of treatment; a similar outcome was obtained with the positive control. The analysis of scar diameter revealed early lesion reduction on the 7th day (Figure 10).

Histological analysis on the 14th day of treatment with TG1 showed better results compared with the 7th day. It was possible to observe healing with formation of dermal papillae and hair follicles and total reorganization of collagen with a keratinized and recovered epithelium in the area where previous injury had been done (Figure 11). On day 21 of treatment with cream TG1 (Figure 12) total histofunctional restructuring with angiogenesis and

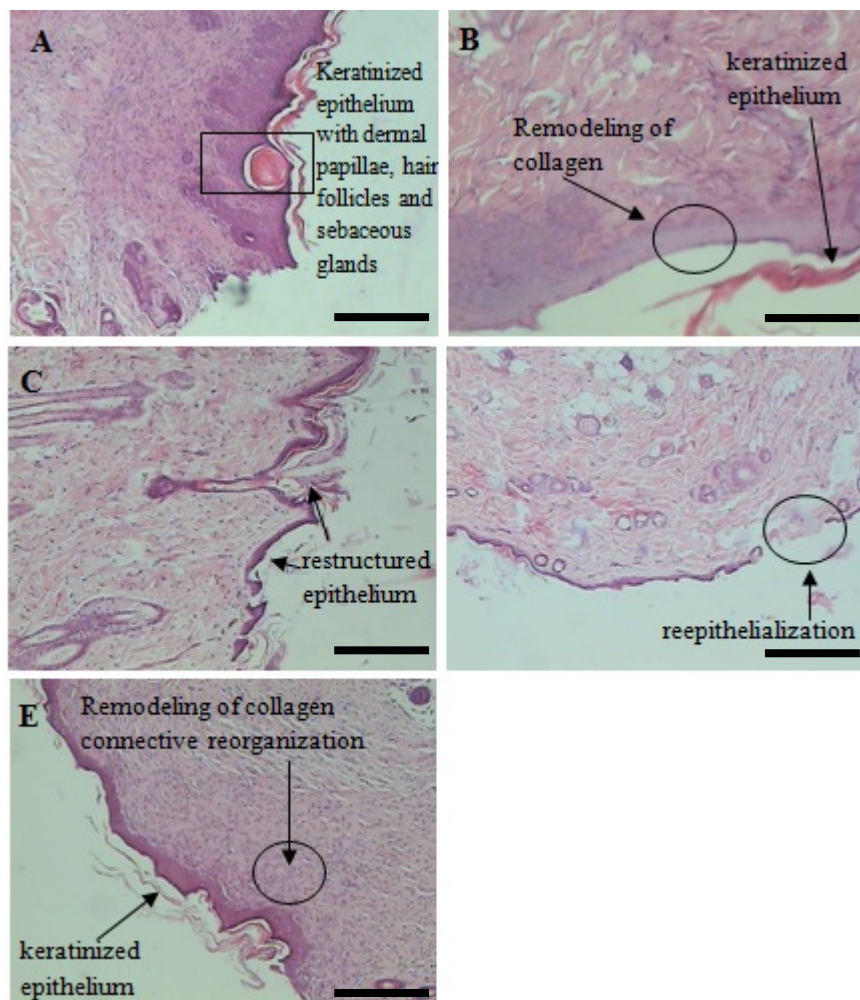


Figure 12. Histological analysis of the 21st day of treatment of skin wounds in rats. Legend - A: Saline; B: Collagenase; C: Cream with 5% TG1; D: Cream with 10% TG1 and E: Cream with 15% TG1. Hematoxylin & Eosin staining (H&E, Scale bar, 10 μm). Magnification, 100x. One representative experiment with n = 5 is shown.

absence of inflammatory foci and edema was observed.

DISCUSSION

The assessment of the characteristics that the active ingredient incorporated into the base in semisolid pharmaceutical has fundamental importance in the process of wound healing in rats. It allows the verification of the feasibility of the planned formulation for use in other pharmacological models and preclinical trials. Mice and rats are routinely used as an experimental tool to study novel bioactive medicines and healing remedies (Surey et al., 2014; Fernandes et al., 2014; Sá et al., 2012; Magalhães et al., 2010). The Wistar rats were

chosen for this study for being small, easy to purchase and due to the standardization concerning age, weight, sex, food, accommodation, cleaning care and experimental manipulation. They have also good resistance to handling, surgical injury and infections (Santos et al., 2006).

Monitoring of histological attributes, the identification of cellular elements and the content of collagen production are parameters usually used for wound healing studies (Rawat et al., 2012; Akela et al., 2012). Thus, histological analysis of groups treated with the cream of TG1 allowed realizing effective healing of the skin lesions. It is noteworthy that the response to tissue injury, whether traumatic or surgical, is composed of three phases: the inflammatory phase, the proliferative phase and remodeling

phase, all aiming at the definitive tissue repair. The inflammatory phase may take 24 to 48 h and consists of hemostatic mechanisms. The following steps are related to collagen production and tissue remodeling (Guo and Dipietro, 2010). Thus, it is possible to realize a principle of re-epithelialization on the 7th day of treatment of wounds with cream containing TG1. A study with different extracts of *Martynia annua* Linn leaves demonstrated wound healing in rats. It showed fibroblast proliferation, collagen maturation and epithelialization in the histological analyses, which corroborate to the results of this study (Santram and Singhai 2011).

Other studies also showed intense healing on day 14 of treatment. The treatment of induced diabetic wounds in rats with the flavonoid fraction of leaves of *M. annua* Linn demonstrated organization of collagen fibers, fibroblasts, and increased angiogenesis. The use of crude extract of *Jatropha gossypifolia* L. also showed intense fibroblastic proliferation and epithelialization on the 14th day (Santos et al., 2006; Santram and Singhai 2013). It is noteworthy that the last phase of tissue repair process (maturation phase) is characterized by endothelial regression. On the 21 day of treatment, there was continuous process of re-epithelialization of the injured area with keratinization in all groups, promoting the corneum stratum, primarily composed of keratin, a protein responsible for the skin impermeability (Garros et al., 2006; Moura et al., 2014).

The angiogenesis is a process of proliferative phase in which new blood vessels are formed and involves the migration and proliferation of endothelial cells. Topical treatment with substance P (a neuropeptide produced by both neuronal and immune cells) in skin wounds of rats allowed realizing effective wound healing with increased fibroblast proliferation, angiogenesis and collagen deposition (Kant et al., 2013). Therefore the treatment with cream containing TG1 in the three concentrations used demonstrated efficacy in wound healing. It was evidenced by macroscopic and microscopic analyses of lesions and wound retraction. Histological analysis showed healing morphological phenomena (intense fibroblast proliferation, angiogenesis and reepithelialization) with all doses tested. The present study showed a possible healing activity of the cream containing TG1, enabling the advanced development of phytomedicines.

ACKNOWLEDGMENT

We acknowledge the financial support of the Coordination of Improvement of Higher Education (CAPES), the Foundation for Research Support of Piauí (FAPEPI) and Federal University of Piauí (UFPI).

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

- Adiele LC, Adiele RC, Enye JC (2014). Wound healing effect of methanolic leaf extract of *Napoleona vogelii* (Family: Lecythidaceae) in rats. *Asian Pac. J. Trop. Med.* 7:620-624.
- Akela A, Nandi SK, Banerjee D, Das P, Roy S, Joardar SN, Mandal M, Das PK, Pradhan NR (2012). Evaluation of autologous bone marrow in wound healing in animal model: a possible application of autologous stem cells. *Int. Wound J.* 9:505-516.
- National Health Surveillance Agency (2011). Herbal form of Brazilian Pharmacopoeia. 1:117-119.
- Clerici MTPS, Carvalho-Silva LB (2011). Nutritional bioactive compounds and technological aspects of minor fruits grown in Brazil. *Food Res. Int.* 44:1658-1670.
- Coelho JM, Antonioli AB, Silva DN, Carvalho TM, Pontes ERJCP, Odashiro AN (2010). The effect of silver sulfadiazine, extract ipe purple and arbatimão extract on cutaneous wound healing in rats. *J. Braz. Coll. Surg.* 37:45-51.
- Fernandes TRMO, Santos IKJP, Silva BSL, Carvalho LO, Plapler H (2014). Cutaneous changes in rats induced by chronic skin exposure to ultraviolet radiation and organophosphate pesticide. *Acta Cir. Bras.* 29:07-15.
- Fonseca F, Freitas C, Dutra L, Guimarães R, Carvalho O (2014) Spatial modeling of the *Schistosomiasis mansoni* in Minas Gerais State, Brazil using spatial regression. *Acta Trop.* 133:56-63.
- Furumoto T, Ozawa N, Inami Y, Toyoshima M, Fujita K, Zaiki K, Sahara S, Akita M, Kitamura K, Nakaoji K, Hamada K, Tamai K, Kaneda Y, Maeda A (2014). *Mallotus philippinensis* bark extracts promote preferential migration of mesenchymal stem cells and improve wound healing in mice. *Phytomedicine* 21:247-253.
- Garros IC, Campos ACL, Tambara EM, Tenório SB, Torres OJM, Agulham MA, Araújo ACS, Santis-Isolan PMB, Oliveira RM, Arruda ECM (2006). *Passiflora edulis* extract on the healing of open wounds in rats: Morphological and histological study. *Surg. Braz. Minutes* 21: 55-65.
- Guo S, Dipietro LA (2010) Factors Affecting Wound Healing. *J. Dental Res.* 89:219-229.
- Kant V, Gopal A, Kumar D, Bag S, Kurade N, Kumar A, Tandan S, Kumar D (2013) Topically applied substance P enhanced healing of open excision wound in rats. *Eur. J. Pharmacol.* 715:345-353.
- Karodi R, Jadhav M, Rub R, Bafna A (2009). Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. (Indian madder) in mice. *Int. J. Appl. Res. Nat. Prod.* 2:12-18.
- Kuntal D (2013). Wound healing potential of aqueous crude extract of *Stevia rebaudiana* in mice. *Rev. Bras. Farmcognosia* 23:351-357.
- Santram L, Singhai AK (2011). Preliminary pharmacological evaluation of *Martynia annua* Linn. leaves for wound healing. *Asian Pac. J. Trop. Biomed.* 1:421-427.
- Santram S, Singhai AK (2013) Wound healing effect of flavonoid rich fraction and luteolin isolated from *Martynia annua* Linn. on streptozotocin induced diabetic rats. *Asian Pac. J. Trop. Biomed.* 1:253-259.
- Magalhães HIF, Ferreira PMP, Moura ES, Torres MR, Alves APNN, Pessoa ODL, Costa-Lotufo LV, Moraes MO, Pessoa C (2010). *In vitro* and *in vivo* antiproliferative activity of *Calotropis procera* stem extracts. *Ann. Braz. Acad. Sci.* 82:407-416.
- Moura DJ, Richter MF, Boeira JM, Pêgas HJA, Saffi J (2007) Antioxidant properties of β -carboline alkaloids are related to their antimutagenic and antigenotoxic activities. *Mutagenesis* 22:293-302.
- Moura LIF, Dias AMA, Suesca E, Casadiegos S, Leal EC, Fontanilla MR, Carvalho L, Sousa HC, Carvalho E (2014). Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice. *Biochim. Biophys. Acta* 1842: 32-43.
- Mukherjee H, Ojha D, Bharitkar YP, Ghosh S, Mondal S, Kaityc S, Dutta S, Samantac A, Chatterjee TK, Chakrabarti S, Mondal NB, Chattopadhyay D (2013). Evaluation of the wound healing activity of *Shorea robusta*, an Indian ethnomedicine, and its isolated constituent(s) in topical formulation. *J. Ethnopharmacol.* 149:335-343.

- Ogbourne SM, Parsons PG (2014). The value of nature's natural product library for the discovery of New Chemical Entities: The discovery of ingenol mebutate. *Fitoterapia* 98:36-44.
- Rahal SC, Rocha NS, Blessa EP, Iwabe S, Crocci AJ (2011). Natural organic ointment or isotonic saline in the treatment of clean wounds induced in rats. *Rural Sci.* 31:1007-1011.
- Rawat S, Singh R, Takhur P, Kaur S, Senwal A (2012). Wound healing Agents from Medicinal Plants: A Review. *Asian Pac. J. Trop. Biomed.* 1:910-917.
- Sá CG, Cardoso KMF, Freitas RM, Feitosa CM (2012). Effect of oral treatment with essential oil of *Citrus sinensis* (L) Osbeck in the retention of spatial memory in rats evaluated in the Morris water maze. *Rev. Ciênc. Farm. Básica Apl.* 33:211-215.
- Santos LOM, Simões MLPB, Machado APB, Máticoski Filho GR, Endo PC, Gruen GR, Cipriani VR, Mesquita LD (2002). Effect of somatotropin on wound healing in rats. *Surg. Braz. Minutes* 17:220-224.
- Santos MFS, Czecko NG, Nassif PAN, Filho JMR, Alencar BLF, Malafaia O, Ribas CAPM, Trautwein VM, Henriques GS, Maia JMA, Bittencourt RCA (2006). Evaluation of the use of the crude extract of *Jatropha L. gossypifolia* in cutaneous wound healing in rats. *Minutes Suppurative Braz.* 21:2-7.
- Santos PRP, Carvalho RBF, Costa Júnior JS, Freitas RM, Feitosa CM (2013). Lifting of the physicochemical and pharmacological properties of extracts and compounds isolated from *Platonia insignis* Mart. A perspective for the development of phytomedicines. *Braz. J. Pharm.* 94:161-168.
- Souza IG, Souza, VA, Lima PS (2013). Molecular characterization of *Platonia insignis* Mart. ("bacurizeiro") using inter simple sequence repeat (ISSR) markers. *Mol. Biol. Rep.* 40:3835-3845.
- Surey S, Berry M, Logan A, Bicknell R, Ahmed Z (2014). Differential cavitation, angiogenesis and wound-healing responses in injured mouse and rat spinal cords. *Neuroscience* 275:62-80.

Full Length Research Paper

Simple potentiometry and phenolphthalein-based titrimetric methods of analysis for Lisinopril tablets

Ayodeji W. Olalowo¹, Olayemi M. Adegbolagun^{1*} and Oluwayemisi A Bamiro²

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Ibadan, Oyo State, Nigeria.

²Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria.

Received 31 January, 2014; Accepted 21 January, 2015

A colour indicator-based assay was developed and validated for the quantitative analysis of Lisinopril in tablet in aqueous medium. The proposed procedure involved a reaction between the acidic functional group (COOH) of Lisinopril drug and standardised aqueous sodium hydroxide. The method involved dissolution of powdered Lisinopril tablet in water followed by filtering; the obtained filtrate was titrated with aqueous sodium hydroxide, and the end point was determined using phenolphthalein as indicator. The method which was applicable over a concentration range of 0.2 to 1.12 mg/ml gave an inter-day percentage of relative standard deviation (%RSD) of 0.11 to 1.67, while that of intra-day was 0.27 to 1.72 across the different concentrations used for the determinations. Similarly, the percentage of relative error (%RE) were 0.38 to 2.58 and 0.38 to 2.60 for the inter-day and intra-day assays, respectively. This indicates good accuracy and precision for the method. Furthermore, water soluble excipients did not interfere with the end point determination. Slight modification of the method involving potentiometrically determining the end point using glass calomel electrode system gave similar results. The application of both methods; potentiometry and phenolphthalein indicator-based to the chemical content assay of nine different brands of Lisinopril tablets showed no statistically significant difference between the two methods.

Key words: Lisinopril, phenolphthalein-based assay, titrimetry, potentiometry.

INTRODUCTION

The prevalence of hypertension in Nigeria and Africa is put at 12.4 to 34.8% in the south western part of Nigeria as at 2009 (Ekwunife and Aguwa, 2011) with the prevalence increasing with age (Hall, 2006; Ulasi et al., 2011). It is also the most common cause of hospital admissions in Nigeria (Kolo et al., 2012; Ukoh, 2007; Ike,

2009). The chronic nature of hypertension requires that the therapeutic objective should be dependent on dosage regime and duration of therapy. Effective management of most chronic diseases like hypertension is strongly influenced by the assurance of the quality drugs used in such disease conditions.

*Corresponding author. E-mail: duplag03@yahoo.com.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](http://creativecommons.org/licenses/by/4.0/)

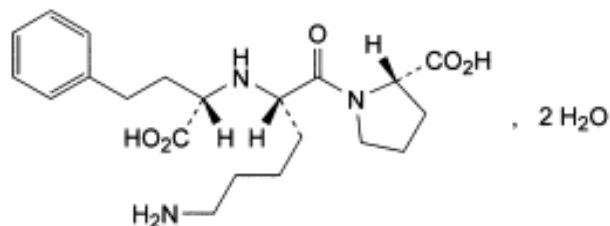


Figure 1. Lisinopril dihydrate.

Quality assurance of drugs depends on maintaining established quality standards based on standardized laboratory tests; physical, chemical, biopharmaceutical and biological procedures. This could be achieved for pharmaceutical products by concise determination of its chemical contents through classical (titrimetry, gravimetry, colourimetry, electrochemistry and polarography) and instrumental methods (spectrofluorometry, spectrophotometry, mass spectrometry and chromatography) (Olaniyi, 1993). An important step in the assessment of quality of drug product is the determination of chemical content of such products. A highly sensitive procedure for detecting variations between different batches of pharmaceutical products to ensure uniformity and consistency among drug batches is an essential component of quality control of drug products.

A major setback in the quality control of pharmaceutical products in developing countries is the unavailability of analytical equipments, unskilled personnel and inadequate infrastructures (Jegade, 1998). This has led to distribution of fake, substandard and poor quality drug products.

Lisinopril (2S)-1-[(2S)-6-Amino-2-[[[(1S)-1-carboxy-3-phenylpropyl] amino] hexanoyl] pyrrolidine-2-carboxylic acid dihydrate (Figure 1) is a lysine analogue of enalaprilat, the active metabolite of enalapril, which exist as a dihydrate salt. It is a long-acting, dicarboxyl-containing angiotensin-converting enzyme (ACE) inhibitor (Widimsky, 2009).

Lisinopril dihydrate is an important member of the angiotensin converting enzyme inhibitors (ACEIs) class of drugs used as first line drug in the management of hypertension and congestive heart failure; they act by reducing peripheral vascular resistance and blood volume (Hall, 2006; El Gindy et al., 2001).

The official method for the chemical content determination of pure Lisinopril dihydrate involves the use of potentiometry and high performance liquid chromatography (HPLC) (British Pharmacopoeia, 2009; United States Pharmacopoeia, 2000). Furthermore, various analytical techniques have been reported for the tablet dosage form, these includes spectrophotometry (Asad, et al., 2005; El- Yazbi et al., 1999; El-Gindy et al., 2001; Stanis, 2004; Dinc et al., 2013; Shinde et al., 2007; Ahmed Ali and Elbashir, 2012; Devi et al., 2003;

Fawzy et al., 1999; Čakar and Popović, 2012), liquid chromatography (El-Gindy et al., 2001; Fawzy et al., 1999; United States Pharmacopoeia, 2000; Ali et al., 2004; Sagirli and Ersoy, 2004; Ivanovic et al., 2007; Japanese Pharmacopoeia, 1993), gas chromatography (Avadhanutu and Pantulu, 1993), spectrofluorometry (El Gindy et al., 2001; El- Yazbi et al., 1999; Jamakhandi et al., 2010; Esra et al., 2003; Constantinos et al., 2004), derivative spectrophotometry (Abdel-Razak et al., 2003), and polarography (Abdel-Razak et al., 2003; El-Enany et al., 2003), capillary electrophoresis and fluoroimmunoassay (Gotti et al., 2000; Yuan and Gilbert, 1996).

Most of these methods are sophisticated, costly, tedious, time consuming, and or require certain reagents, equipments as well as skilled personnel which may not be easily available in many developing countries where prevalence of hypertension is on the increase. This may result in inadequate control of the quality of the drug compound with the accompanying therapeutic failure, which may lead to patients developing complications that may be life threatening and even fatal.

Guidelines for global standardization and requirements for the registration, assessment, marketing, authorization and quality control of drug products have been issued by WHO Report (1996). However, many developing countries do not have the technical, financial, or human resources required to monitor the quality of drug products being distributed within their regions. Hence, the need for a simple, rapid, economical and selective method, that can easily be used for routine field assessment of the quality of Lisinopril.

This study was aimed at developing a simple, fast, sensitive and cost effective method for the determination of Lisinopril in raw and pharmaceutical formulations, which can compare favourably with official methods. The method was applied to the analysis of nine brands of Lisinopril tablets.

MATERIALS AND METHODS

Average weight determination

The average weight and percentage deviation of the Lisinopril dihydrate tablets (Zestril[®]) brand was determined according to the official method (British Pharmacopoeia, 2009).

Isolation of pure Lisinopril dihydrate (2^o Standard) from tablets

Fourteen Lisinopril tablets were powdered, transferred into an extraction tube and extracted with methanol. The solution was decanted, filtered and dried under nitrogen gas; the dried residue was recrystallised using chloroform-methanol (1:1, v/v).

Pure Lisinopril powder obtained was dried under nitrogen gas. The identification of the recrystallised Lisinopril was determined using melting point (Stuart apparatus, England), thin layer chromatography (TLC) (Silica gel GF254 using butanol: ethylacetate: glacial acetic acid: water [5:5:5:5] as mobile phase), infrared (Buck, England), ultraviolet-visible spectrophotometry (Pye Unicam, Stoke, England) (British Pharmacopoeia, 2009) and high

performance liquid chromatography (Agilent series 1100, Waldbronn, England) (Japanese Pharmacopoeia, 1993). Assay of the pure Lisinopril (2° Standard) was determined using potentiometry (British Pharmacopoeia, 2009) and HPLC (Japanese Pharmacopoeia, 1993).

Development of new titrimetric assay techniques

Colour indicator based technique using phenolphthalein indicator

Pure Lisinopril dihydrate (2° Standard): pure Lisinopril powder (25 mg) dissolved in distilled water (25 ml) was titrated with NaOH (0.01 M); the end point was determined using phenolphthalein solution (1%) as indicator with colour change from colourless to pink.

Lisinopril tablet in the presence of excipients: powdered Lisinopril tablet (Zestril®) (equivalent to 25 mg pure Lisinopril) was dissolved in 25 ml of distilled water with shaking. Few drops of phenolphthalein indicator were added and the mixture was titrated with NaOH (0.01 M), and the end point was determined by the change in colour from colourless to pink. Triplicate assay was carried out.

Lisinopril tablet in the absence of excipients: powdered Lisinopril tablet (Zestril®) (equivalent to 25 mg pure Lisinopril) was dissolved in 25 ml of distilled water with shaking. The mixture was filtered before titration with NaOH (0.01 M); the end point was determined using phenolphthalein indicator with colour change from colourless to pink. Triplicate assay was carried out.

Using methyl orange indicator

The above procedure for pure Lisinopril and powdered tablet was repeated using methyl orange indicator.

Potentiometry technique

Lisinopril dihydrate pure (2° standard): the pure procedure for pure Lisinopril was repeated but the end point was determined potentiometrically (British Pharmacopoeia, 2009). Triplicate assay was carried out.

Lisinopril tablet: Lisinopril tablets (in the presence and absence of excipients) was repeated, but the end point was determined potentiometrically (British Pharmacopoeia, 2009). Triplicate assay was carried out.

Validation of the selected method

Calibration curve: Lisinopril pure powder; 5, 12 and 28 mg were weighed and dissolved in 25 ml of distilled water, giving 0.2, 0.48 and 1.12 mg/ml solution, respectively. Few drops of phenolphthalein solution (1% w/v) used as indicator was added to each solution and titrated with 0.01 M NaOH. The end point was determined by the colour change from colourless to pink. Triplicate assay was carried out at the different concentration.

A calibration curve was generated using a graph of mean end point volume (MEPV) of the titrant (ml) against the corresponding concentration (mg/ml) of Lisinopril. The regression line equation and correlation coefficient was obtained from the curve. A three-day recovery study was done and the accuracy and precision were determined.

Application of the method to nine brands of Lisinopril tablets

Average weights of nine brands of Lisinopril tablets procured from retail pharmacies were determined. The presence of Lisinopril dihydrate was determined using TLC. The amount of Lisinopril in each brand was determined using the calibration curve as following.

Colour based titrimetric technique: powdered Lisinopril tablet equivalent to 25 mg pure Lisinopril was dissolved in 25 ml of distilled water with shaking. The mixture was filtered before titration with NaOH (0.01 M); the end point was determined using phenolphthalein as indicator. Triplicate assay was carried out.

Potentiometry technique: powdered Lisinopril tablet equivalent to 25 mg pure Lisinopril was dissolved in 25 ml of distilled water with shaking. The mixture was filtered before titration with NaOH (0.01 M), and the end point was determined potentiometrically. Triplicate assay was carried out.

HPLC assay method: test solutions (equivalent pure Lisinopril, 0.2 mg/ml) and internal standard (caffeine, 0.05 mg/ml) were prepared in distilled water. Chromatographic analysis was performed as earlier described. The peak areas, peak heights and retention times were measured and the percentage content of the Lisinopril was calculated with reference to the internal standard (Japanese Pharmacopoeia, 1993).

Statistical analysis

Students' t-test and one-way analysis of variance (ANOVA) was used for the statistical analysis, $p < 0.05$ was taken as the significant level.

RESULTS

The extracted Lisinopril dihydrate (2° standard) gave a melting point of 160 to 162°C with λ_{\max} 209 nm (Figure 2), prominent infra red bands at 1376.50, 1461.84, and 2724.15 cm^{-1} using KBr disc (Figure 3) and a single peak with retention time of 5.05 min with HPLC analysis, while caffeine internal standard was 17.39 min (Figure 4). Lisinopril dihydrate content was 99.40±9.06 and 102.88% w/w using potentiometry and HPLC procedures, respectively. The obtained value with the potentiometry complied with official specification of 98.5 to 101.5% w/w (British Pharmacopoeia, 2009), while that of the HPLC was slightly higher.

The colour based reaction using methyl orange as indicator could not determine the end point as there was no change in colour in all the determinations of the pure Lisinopril and tablet dosage forms. However, defined change in colour from colourless to pink was observed with phenolphthalein indicator at the end point which corresponds with the end point volume obtained with the potentiometric technique. The colour based titrimetric techniques using phenolphthalein indicator gave Lisinopril content of 97.14 ± 1.83% w/w for the pure Lisinopril (2° standard).

Application of the procedures to tablet dosage form involving the use of one brand of Lisinopril tablet gave 110.10±1.74 and 95.27±1.85% w/w in the presence and

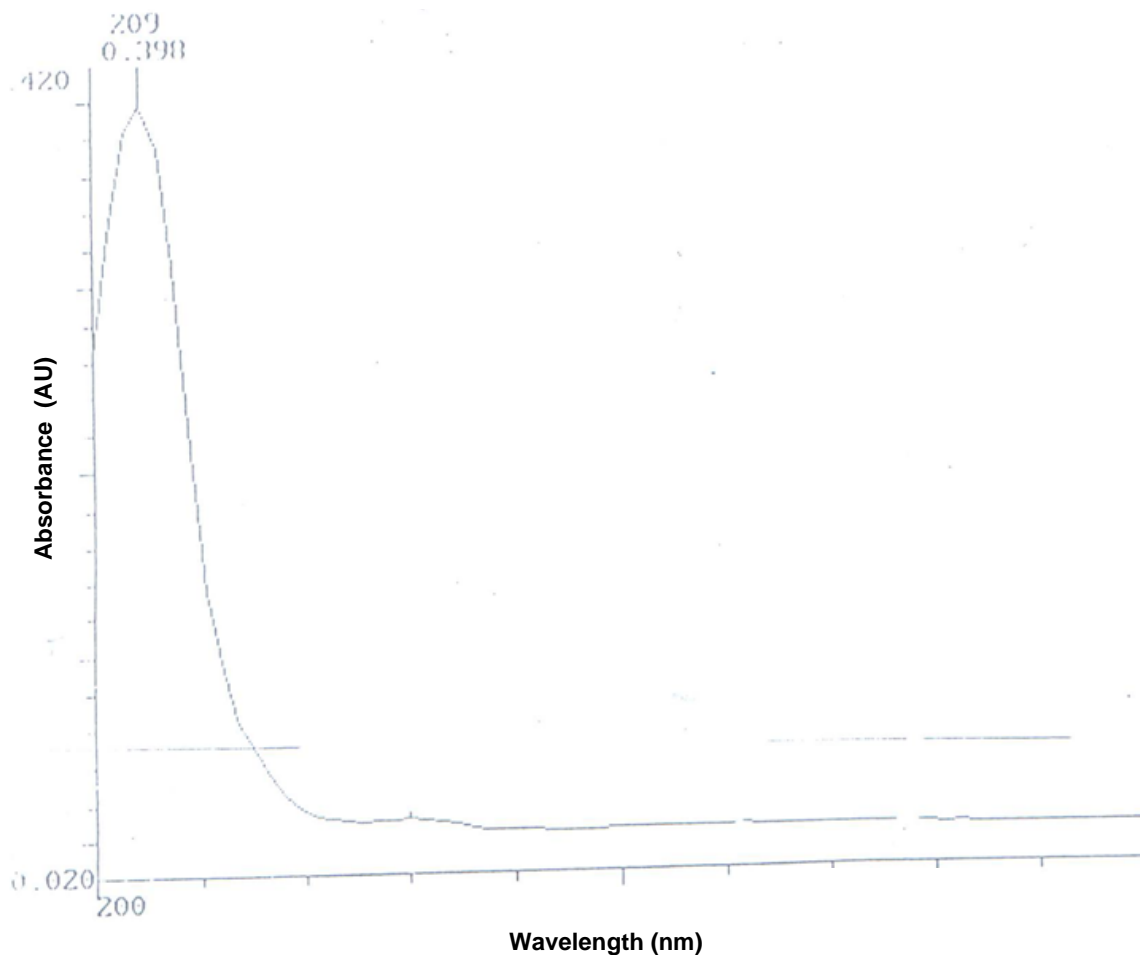


Figure 2. Ultraviolet spectrum of the pure Lisinopril dihydrate (2° standard).

absence of excipients, respectively using potentiometry, while the colour based titrimetry technique gave 115.22 ± 9.06 and $99.35 \pm 9.02\%$ w/w, respectively.

Validation of the proposed phenolphthalein based titrimetric procedure gave a calibration curve with linear response; $y = 6.286x + 0.064$ ($r^2 = 0.998$) (Figure 5). The inter-day relative standard deviation (%RSD) was 0.11 to 1.67, while that of intra-day was 0.27 to 1.72 across 0.2 to 1.12 mg/ml used for the determinations. Similarly, the percentage of relative error (%RE) were 0.38 to 2.58 and 0.38 to 2.60 for the inter-day and intra-day assays, respectively (Table 1).

The result of the application of the proposed procedures: potentiometry and phenolphthalein indicator based titrimetry and HPLC to nine brands of Lisinopril tablets is presented as shown in Table 2. The obtained results showed that there was no significant difference between potentiometry and phenolphthalein indicator based procedures ($p = 0.6028$), while the obtained values HPLC method were significantly higher than the potentiometry and phenolphthalein indicator based procedures ($p = 0.0051$ and 0.0075 , respectively).

DISCUSSION

The absence of reliable drug quality control systems in many developing countries is a major contributor to the prevalence of fake and sub-standard drug compounds, which has accounted for treatment failures especially with chronic diseases such as hypertension, diabetes, etc. Multi-sourcing of drug compounds have long been implicated in the rising cases of distribution of fake and substandard drugs, especially in poor resourced economies where access to appropriate quality control technologies are not available. Thus the need for simple, cost effective and reliable methods of assay for the quality control of drug compounds in developing countries cannot be over emphasised.

Lisinopril (an ACEI), is a first line drug in the management of hypertension and coronary heart diseases which is available in various brands as multi-sourced drug. The official methods and earlier reported methods involve the use of high technology equipments and procedures. Thus this study was carried out to proffer an alternative and equally reliable method assay

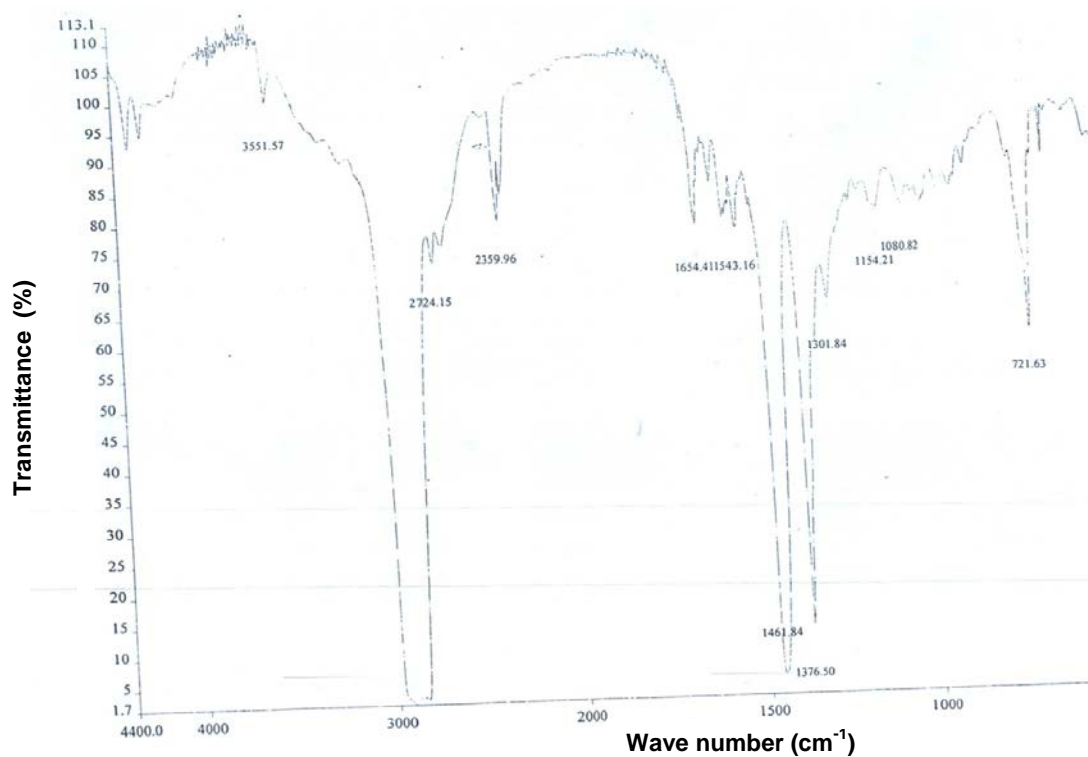


Figure 3. Infrared spectrum of the pure Lisinopril dihydrate (2° standard).

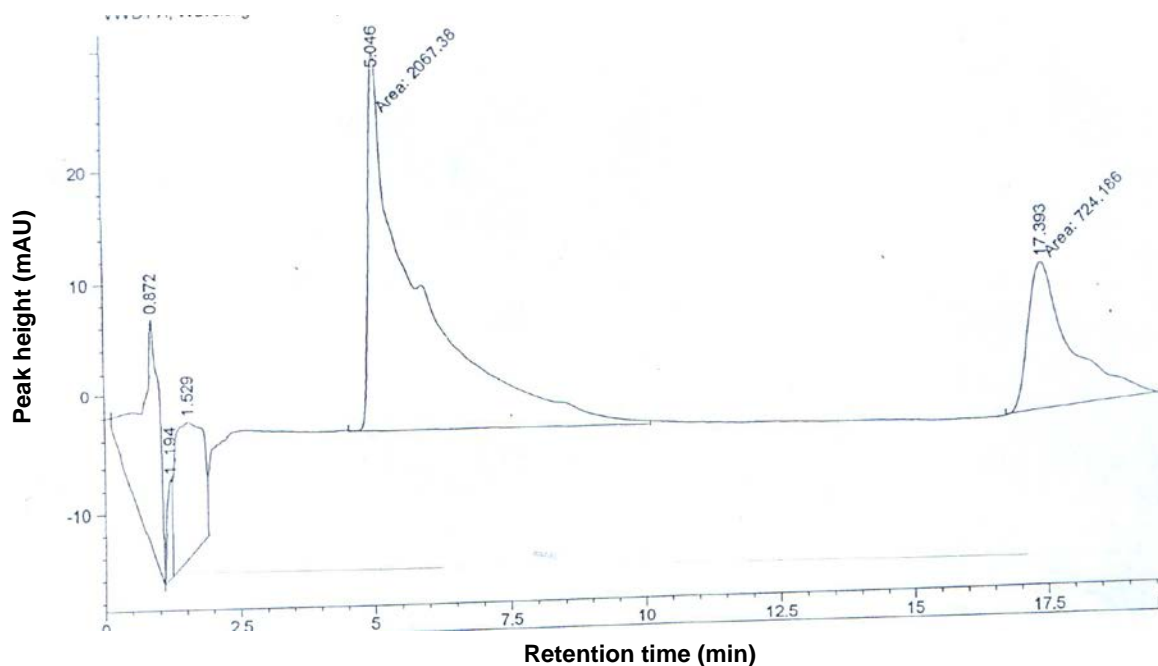


Figure 4. HPLC chromatogram of the pure Lisinopril dihydrate (2° standard) using caffeine as internal standard.

for Lisinopril tablets.

Physicochemical analysis of the Lisinopril pure powder (2° standard) extracted from the tablet gave melting point

of 160 to 162°C, λ_{\max} of 209 nm and infrared bands which are characteristic of Lisinopril (Japanese Pharmacopoeia, 1993). The chemical content determination gave 99.40±9.06

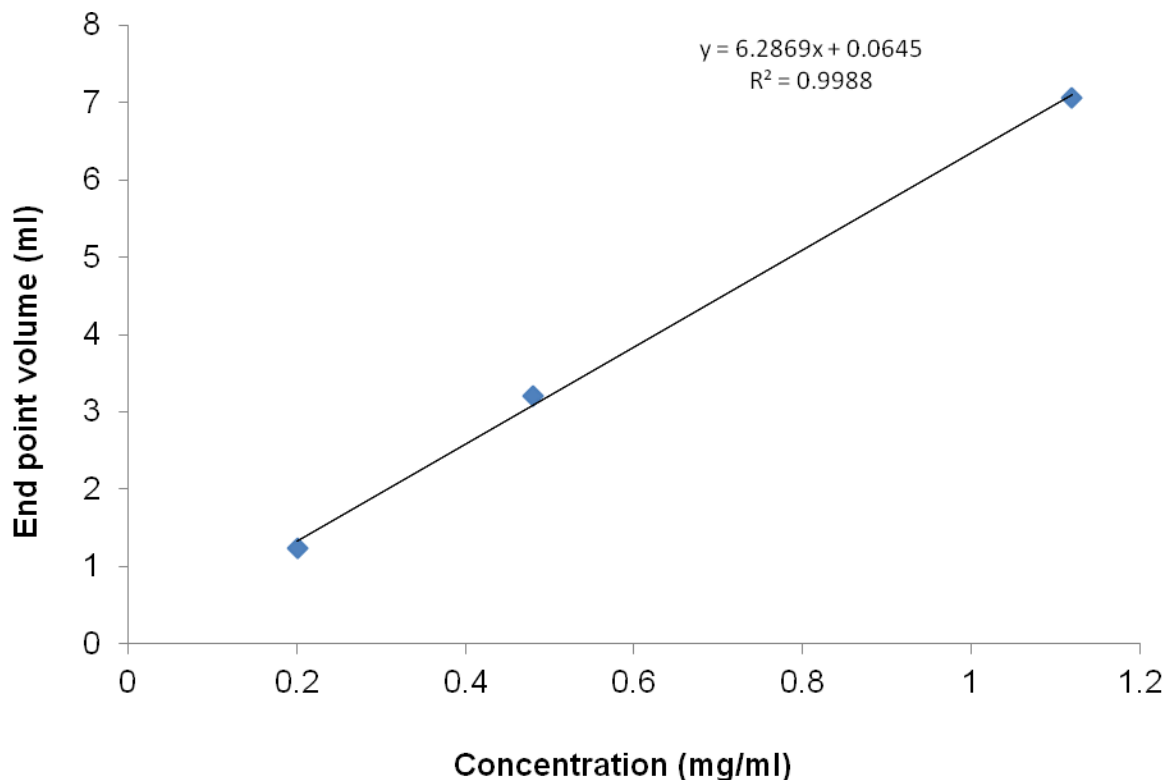


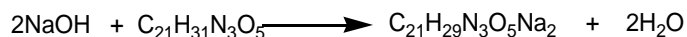
Figure 5. Calibration curve of Lisinopril dihydrate using phenolphthalein-based titration technique in aqueous medium.

Table 1. Inter-day and intra-day validation of the phenolphthalein indicator titrimetric assay method.

Concentration (mg/ml)	Inter-day assay			Intra-day assay		
	% Recovery (Mean±SD)	%RSD	%RE	% Recovery (Mean±SD)	RSD	%RE
0.2	97.42±1.53	1.57	2.58	97.42±0.0030	1.54	2.60
0.48	99.03±1.65	1.67	1.37	99.14±0.0082	1.72	0.85
1.12	99.52±0.104	0.11	0.38	99.62±0.0030	0.27	0.38

and 102.88% w/w using the official potentiometric and HPLC methods, respectively. These results confirm the purity and suitability of the extracted Lisinopril as a secondary reference standard for this study.

Lisinopril, a common antihypertensive drug compound is an amphoteric compound, possessing both acidic and basic properties; it has two carboxylic acids in its structure which can ionise in basic medium. The reaction of these carboxyl groups with sodium hydroxide is the basis for the proposed assay technique, which is a slight modification of the official potentiometric method for pure Lisinopril dihydrate (British Pharmacopoeia, 2009):



The modification involved filtering the tablet solution and the use of colour indicator to determine the end point.

Titrimetric assay using colour indicator for the pure Lisinopril (2° standard) gave 97.14±1.83% w/w using phenolphthalein indicator with a colour change from colourless to pink, while the methyl orange did not show any colour change. This shows that methyl orange is not suitable for determination of the end point using this procedure.

Furthermore, the excipients were observed to interfere with the end point determination using the proposed potentiometry and colour indicator based methods, hence, the need to filter the solution before the assay. Thus, the procedure was repeated after filtration to remove the insoluble excipients.

Validation of the proposed methods showed consistency on a three-day assessment at the three different concentrations: 0.2, 0.48 and 1.12 mg/ml. A positive correlation of end point volume (ml) against concentration

Table 2. Chemical content determination of Lisinopril in nine brands of Lisinopril tablets using potentiometry, phenolphthalein indicator and HPLC methods.

Brand	Chemical content (% w/w of labelled claim \pm SD)		
	Proposed method		Official method
	Potentiometry	Colour indicator based titrimetry	(HPLC)
A	98.30 \pm 8.48	98.80 \pm 4.25	111.30
B	93.10 \pm 4.60	90.50 \pm 4.61	110.50
C	101.70 \pm 7.80	101.70 \pm 1.74	112.60
D	100.7 \pm 4.20	100.7 \pm 4.25	114.40
E	99.5 \pm 3.64	100.7 \pm 4.25	95.40
F	100.7 \pm 4.20	98.8 \pm 4.25	105.00
G	98.3 \pm 4.20	98.3 \pm 4.25	105.20
H	98.4 \pm 4.55	98.5 \pm 4.59	109.50
I	99.7 \pm 3.44	100.4 \pm 2.76	102.40

(mg/ml) obtained and coefficient of determination of 0.998 showed that the method is accurate and precise. The %RSD of 0.11 to 1.67 and 0.27 to 1.72 for inter-day and intra-day, respectively at 0.2 to 1.12 mg/ml range was used for the determinations. Similarly, %RE were 0.38 to 2.58 and 0.38 to 2.60 for the inter-day and intra-day assays, respectively. This showed that the methods gave good accuracy and precision.

Application of the proposed methods to nine other brands of Lisinopril tablets, whose content of Lisinopril had earlier been confirmed through TLC showed slight variations in the end point colour; five brands showed a colour change from colourless/white to pink, while one brand gave a colour change from peach to pink, and the remaining three showed variations in the colour changes. The change in colour though different was well defined in all the samples, could be attributed to differences in the formulations.

The chemical content ranged from 93.1 \pm 4.60 to 101.7 \pm 7.80% w/w for all the brands using the proposed potentiometric method, while the phenolphthalein indicator method gave 90.5 \pm 4.61 to 101.7 \pm 1.74% w/w. On the other hand, 95.4 to 114.4% w/w was obtained for the nine brands using an HPLC method (Japanese Pharmacopoeia, 1993) (Table 2). Statistical comparison of the proposed methods: phenolphthalein indicator based titrimetry and potentiometry, and HPLC, showed that there was no significant difference in the obtained results for potentiometry and colour indicator based titrimetry ($p=0.6028$), however, a significant difference was observed when compared with HPLC method ($p<0.05$).

The obtained results from this study are in agreement with another report on the use of titrimetric technique in the analysis of Lisinopril tablets using benzene: methanol (3:1) mixture as solvent (Basavaiah et al., 2010). However, the proposed method from this study is in aqueous medium which has a great advantage over the earlier titrimetric report because of the issue of solvent

cost and safety with regards to benzene.

Titrimetric techniques involving the use potentiometry and colour indicator for the chemical content determination of some drug compounds; salbutamol (Pungal, 2013), hydroxyzine hydrochloride (Rajendraprasad et al., 2013), pheniramine maleate (United States Pharmacopoeia, 2000, Raghu et al., 2012), in pure and dosage forms have been reported. These methods were reported to exhibit very good correlation with instrumental methods in terms of accuracy, robustness and precision. In all the methods as observed in the current proposed methods, soluble excipients did not interfere with the determinations.

Although, the Lisinopril content of all the brands to which the proposed procedures were applied were within the official specification for tablets: 92.5 to 105.5% w/w (British Pharmacopoeia, 2009), the values obtained with the HPLC method was quite higher than the two methods. Furthermore, a similar trend was obtained with the nine brands in the proposed methods; Brand B gave the lowest chemical content, while Brand C gave the highest value (Table 2). Comparing the chemical content values obtained with HPLC method for the tablets with that of the pure Lisinopril showed a similar trend; the value obtained for the pure Lisinopril (2° standard) was higher than the official specification (British Pharmacopoeia, 2009).

This is a definite indication that the two proposed methods can be used to determine the chemical content of Lisinopril tablets.

Conclusion

The two proposed methods: potentiometry and phenolphthalein indicator based titrimetry, are simple, fast, cost-effective requiring minimal instrumental/technological input and thus can be adopted for use in a poor resourced economy where appropriate sophisticated

equipments and other infrastructures are inadequate.

Conflict of interest

The authors declared no conflict of interest.

REFERENCES

- Abdel Razak O, Belal F, Bedair M, Barakat S, Haggag S (2003). Spectrophotometric and polarographic determination of enalapril and Lisinopril using 2, 4-dinitrofluorobenzene. *J. Pharm. Biomed. Anal.* 31:701-711.
- Asad R, Tariq A, Atta-ur-R (2005). Spectrophotometric determination of Lisinopril in Pure and Pharmaceutical Formulations. *J. Chin. Chem. Soc.* 52(5):1055-1059.
- Avadhanu B, Pantulu R (1993). Gas liquid chromatographic estimation of lisinopril in its pharmaceutical dosage forms. *Indian Drugs* 30:646-649.
- Basavaiah K, Tharpa K, Vinay KB (2010). Titrimetric assay of lisinopril in aqueous and non-aqueous media. *Eclética Quím.* 35(2):7-14.
- British Pharmacopoeia (2009). *British Pharmacopoeia*, HM Stationary Office: London. pp. 1135-1138.
- Čakar M, Popović G (2012). Determination of lisinopril in pharmaceuticals by a kinetic spectrophotometric method. *J. Serb. Chem. Soc.* 77(10):1437-1442.
- Constantinos Z, Paraskevas T, Demtrius T, Georgios T, Anastasios E, Pantelis R (2004). Rapid spectrofluorimetric determination of lisinopril in pharmaceutical tablets using sequential injection analysis. *Anal. Bioanal. Chem.* 379:759-763.
- Devi PA, Mallikarjuna Rao GPV, Krishna Prasad KMM, Sastry CSP (2003). Four simple spectrophotometric determinations of lisinopril in pure state and in tablets. *Indian J. Pharm. Sci.* 65(3):296-99.
- Dinc S, Donmez OA, Asci Burge, Bozdogan A (2013). Spectrophotometric multicomponent resolution of a tablet formulation containing lisinopril and hydrochlorothiazide by multivariate calibration methods. *Asian J. Chem.* 25(2):999-1002.
- El-Enany N, Belal F, Al-Ghannam S. (2003). Polarographic determination of lisinopril in pharmaceuticals and biological fluids through treatment with nitrous acid. *Microchim. Acta* 141:55-61.
- El-Gindy A, Ashour A, Abdel-Fattah L, Shabana MM (2001). Spectrophotometric, septrofluorimetric and LC determination of lisinopril. *J. Pharm. Biomed. Anal.* 25:913-922.
- El-Yazbi FA, Abdine HH, Shaalan RA (1999). Spectrophotometric and spectrofluorometric methods for the assay of lisinopril in single and multicomponent pharmaceutical dosage forms. *J. Pharm. Biomed. Anal.* 19:819-827.
- Esra S, Aktas L, Lale E, Olcay S (2003). A new spectrofluorimetric method for the determination of lisinopril in tablets. *IL Farmacol.* 58:165-168.
- Gotti R, Andrisano V, Cavrini V, Bertucci C, Furlanetto S (2000). Analysis of ACE-inhibitors by CE using alkylsulfonic additives. *J. Pharm. Biomed. Anal.* 22:423-31.
- Hall J, Guyton E, Arthur C (2006). *The textbook of medical physiology*. St. Louis, Mo., Elsevier Saunders. P 228.
- Ike SO (2009). "Prevalence of hypertension and its complications among medical admissions at the University of Nigeria teaching hospital, Enugu (Study 2)." *Niger. J. Med.* 18(1):68-72.
- Ivanovic D, Medenica M, Jancic B, Knezevic N, Malenovic A, Milic J (2007). Validation of an analytical procedure for simultaneous determination of hydrochlorothiazide, lisinopril, and their impurities. *Acta Chromatogr.* 18:143-156.
- Jamakhandi CM, Javali C, Kumar S, Kumar DS. (2010). New fluorimetry method of determination for lisinopril dosage forms. *Int. J. Pharm. Sci. Drug Res.* 2(3):182-187.
- Japanese Pharmacopoeia (1993). 15th Edition. Pharmaceuticals and Medical Devices Agency Chiyoda-ku, Tokyo 100-0013 Japan.
- Jegade EC (1998). Drug regulation and control in developing countries. Buffalo Groove IL, Interpharm Press Inc. pp. 35-37.
- Kolo PM, Jibrin YB, Sanya EO, Alkali M, Peter Kio IB, Moronkola RK (2012). Hypertension-related admissions and outcome in a tertiary hospital in Northeast Nigeria. *Int. J. Hypertens.* pp. 1-6.
- Olaniyi AA (1993). Towards better quality assurance of drugs and foods. Proceedings of a National Workshop, 1993. Department of Pharmaceutical Chemistry, University of Ibadan. pp. 67-72.
- Pungal A (2013). Simple titrimetric method for the estimation of salbutamol sulphate (SBS) in pharmaceutical formulations. *Res. J. Pharm. Sci.* 2(1):11-14.
- Raghu MS, Basavaiah K, Prashanth KN, Vinay KB (2012). Acid-base titrimetric assay of pheniramine maleate in pharmaceuticals in hydro-alcoholic medium. *Der. Pharmacia. Lett.* 4(5):1523-1529.
- Rajendraprasad N, Basavaiah K, Vinay KB (2013). Acid-base titrimetric assay of hydroxyzine dihydrochloride in pharmaceutical samples. *Chem. Ind. Chem. Eng. Q.* 16(2):127-132.
- Sagirli O, Ersoy L (2004). An HPLC method for the determination of lisinopril in human plasma and urine with fluorescence detection. *J. Chromatogr.* 809(1):159-165.
- Shinde V, Trivedi A, Upadhayay PR, Gupta NL, Kanase DG, Chikate R (2007). Identification of a new impurity in lisinopril. *J. Pharm. Biomed. Anal.* 43(1):381-86.
- Stanisz B (2004). Estimation of the applicability of differential spectroscopic method for the determination of lisinopril in tablets and for the evaluation of its stability. *Acta Pol. Pharm. Drug Res.* 61(5):327-334.
- Ukoh VA (2007). Admission of hypertensive patients at the University of Benin teaching hospital, Nigeria. *East Afr. Med. J.* 84(7):329-335.
- United States Pharmacopoeia-USP-24, NF-19 (2000). Asian Edition, United States Pharmacopoeial Convention, Inc. Twin Brook Parkway, Rockville, MD, U.S.A.
- Widimsky J (2009). The combination of an ace inhibitor and a calcium channel blocker is an optimal combination for the treatment of hypertension. *Soc. Hypertens. J.* 55(2):123-130.
- WHO (1996). World Health Organization Expert Committee on specifications for Pharmaceutical Preparations, 34th Report. WHO Technical Report Series, No. 863. Geneva, Switzerland.
- Yuan AS, Gilbert JD (1996). Time-resolved fluoroimmunoassay for the determination of lisinopril and enalaprilat in human serum. *J. Pharm. Biomed. Anal.* 14:773-81.

Full Length Research Paper

Stability and disinfecting proprieties of the toothbrush rinse of the essential oil of *Protium heptaphyllum*

Fernanda Endringer Pinto¹, Otávio Arruda Heringer², Mírian Almeida Silva¹, Tadeu Uggere Andrade¹, Juliano Souza Ribeiro³, Dominik Lenz¹, Fernanda Campos Rosetti Lessa¹ and Denise Coutinho Endringer^{1,3*}

¹Pharmaceutical Science, University Vila Velha (UVV), , Rua Comissário José Dantas de Melo, 21, Boa Vista, Vila Velha-ES, Brasil, 29102-770, Brazil.

²Tommasi Analítica, Av. Luciano das Neves, 2016, Divino Espírito Santo, Vila Velha- ES, Brasil, (27) 3340-8200, Brazil.

³Federal Institute of Espírito Santo Campus Vila Velha, IFES, Av Salgado Filho, Santa Ines, 1000, Vila Velha-ES, Brazil.

Received 7 November, 2014; Accepted 6 February, 2015

***Protium heptaphyllum* (PH) is rich in essential oil, has anti-inflammatory properties and has no toxic potential. However, data is unavailable about its antiseptic effect against bacteria that cause caries. This study aimed to evaluate the antiseptic effect of the toothbrush rinse of essential oil of PH as well as its chemical stability. The toothbrush rinse was prepared with 1% essential oil of PH. The minimum inhibitory concentration (MIC) of the essential oil and toothbrush rinse were evaluated against *Streptococcus mutans* (ATCC 25175TM). The *ex vivo* study was double-blinded and randomised; the children were divided into three groups, each participating in a crossover design where all solutions (water, toothbrush rinse (1%) and chlorhexidine (0.12%) were used in all stages by different groups of children. The chemical composition of the essential oil and toothbrush rinse were analysed by gas chromatography coupled to mass spectrometry. The stability was evaluated at three time points. The essential oil and toothbrush rinse exhibited antimicrobial activity against *S. mutans*, MIC = 0.125 and 2.4 µg/ml. The toothbrush rinse showed the same effect as chlorhexidine on disinfecting the toothbrushes contaminated with mutans streptococci (pH = 57.3 ± 5.3%; chlorhexidine 55.5 ± 13.3%; water 39.4 ± 5.8%; p > 0.05). Chromatographic analysis showed that the essential oil contained monoterpenes as a major component, and the toothbrush rinse possessed the same constituents as the pure essential oil, except for α-terpineol. Storage did not cause chemical degradation of the toothbrush rinse, but decreased the concentration of the chemical constituents. The toothbrush rinse of essential oil of *P. heptaphyllum* showed antiseptic properties and exhibited antimicrobial activity against mutans streptococci.**

Key words: Mutans streptococci, *Protium heptaphyllum*, antimicrobial, monoterpenes, chemical composition, contamination of toothbrushes.

INTRODUCTION

Oral diseases such as caries and periodontitis in children and adolescents have an impact on the quality of life of

these individuals (Paula et al., 2012; Gherunpong et al., 2004). Dental caries is a multifactorial disease following Keyes's trilogy, which is based on substrate (teeth), microorganisms and diet (Keyes, 1960; Kumarihamy et al., 2011). The frequency and times when these factors of Keyes's trilogy interact determine the severity of the injury due to the effect of the acid on the mineral forming the tooth, resulting in the destruction of the tooth (Carounanidy and Sathyanaraynan, 2009). When left untreated, tooth decay can cause abscesses, pain and malocclusion as well as the loss of deciduous teeth (Kumarihamy et al., 2011).

There are more than 500 microbial species in the oral cavity (Paster et al., 2001). The tooth has an ideal environment for those species, and they live in synergism and antagonism in the biofilm in a form of homeostasis (Marsh and Devine, 2011). The primary microorganisms on the surface of the tooth belong to the mutans streptococci group, *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus mitis* and *Streptococcus mutans* (Carounanidy and Sathyanaraynan, 2009). The most common activities used to reduce the oral microbial load are brushing one's teeth with toothpaste, rinsing with mouthwash containing antiseptic solutions and dipping the brush in antiseptic solutions, such as chlorhexidine, xylitol and fluorine (Nelson-Filho et al., 2011; Subramaniam and Nandan, 2011; Efstratiou et al., 2007; Mehta et al., 2007). The latter is needed to avoid re-contamination, mainly because the brush rinse reduces the degree of contamination but also because the residual pathogens remain active. To minimise contamination on a toothbrush, the disinfection process should be initiated immediately after it is unpacked, and a daily routine of applying antiseptics should be maintained to prevent the formation of bacterial biofilms (Neal and Rippin, 2003; Nascimento et al., 2012).

Antiseptic solutions are capable of preventing the adhesion of bacteria on the surface of the teeth and their subsequent colonisation; hence, they inhibit bacterial growth (Subramaniam and Nandan, 2011). However, only a few studies of natural products in dentistry have been conducted, though these natural products have recently received more attention. The primary objective of those published studies was to find, identify and evaluate substances exhibiting antibacterial and antifungal activities (Santos et al., 2009).

A study by Santos et al. (2009) reports several plants used by a population for dental use, such as the pomegranate (*Punica granatum* L.), purple cashew (*Anacardium occidentale* L.), *juá* (*Zizyphus joazeiro* Mart),

mint leaf (*Plectranthus amboinicus* (Lour) Sprengel) and mastic (*Schinus terebinthifolius* Raddi). These plants are used because they are known for their analgesic, anti-inflammatory and wound healing activities (Santos et al., 2009). However, despite being used as a medicinal plant by many people, the majority of these plants have not yet been evaluated sufficiently in terms of their bactericidal activity. A study by Ramos et al. (2009) reported the use of essential oils and plant extracts, such as *S. mutans*, for inhibiting the growth of fungi and bacteria. Recently, our group reported the effectiveness of using a mouthwash containing guaco (*Mikania glomerata* Sprengel and *Mikania laevigata* Sch. Bip. ex Baker) to disinfect toothbrushes (Lessa et al., 2012).

The species *Protium heptaphyllum* (Aubl) March (Burseraceae family) is popularly known as putty or pitch black and is distributed in various regions of Brazil, such as the North, Northeast, Southeast and Midwest areas (Pinto et al., 2008; Vieira-Junior et al., 2005). In those regions, this species is popularly used for healing as well for an expectorant, anti-ulcerogenic and anti-inflammatory (Vieira-Junior et al., 2005). The anti-nociceptive and anti-inflammatory activities of individual components of resin *P. heptaphyllum*, such as triterpenes α - and β -amyrin, were evaluated in animal models, which had been induced with periodontitis and gingivitis (Pinto et al., 2008).

In this study, we hypothesised that the essential oil extracted from the resin of *P. heptaphyllum* and the toothbrush rinse produced from the oil exhibited antimicrobial activity and that the formulation would be stable. However, the use of toothbrush rinse prepared with essential oil extracted from the resin of *P. heptaphyllum* for the disinfection of toothbrushes has not yet been evaluated. To improve the potential for clinical application of the *P. heptaphyllum* essential oil toothbrush rinse, a formulation was prepared, and its chemical stability was evaluated for 12 months. Thus, the aim of this study was to evaluate the effect of the *P. heptaphyllum* essential oil toothbrush rinse in the disinfection of toothbrushes and to evaluate the chemical stability of the formulation over 12 months.

MATERIALS AND METHODS

Plant

The stem exudate of the *P. heptaphyllum* (Aubl.) species was collected in Guriri, São Mateus, Espírito Santo, Brazil, in May, 2010. Voucher specimens of the samples were prepared for subsequent botanical identification in the herbarium of the University Vila Velha

*Corresponding author. E-mail: endringe@gmail.com, denise.endringer@uvv.br Tel: +55-27-99246- 3366.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

Table 1. Experimental design of the crossover study (n=7).

Solutions	Week 1	Week 2	Week 3	Week 4	Week 5
Water	Group I		Group III		Group II
Rinse <i>P. heptaphyllum</i> 1%	Group II	Wash-out period	Group I	Wash-out period	Group III
Chlorhexidine 0.12%	Group III		Group II		Group I

- UVV (UVV / ES 1802).

Extraction of the essential oil

The extraction of the essential oil was performed using hydrodistillation, maintaining a minimum temperature required for boiling; the method used was based on that published by Skrubis (1982) and Ming et al. (1996), with modifications. The extraction process took two hours to complete. The hydrolate was kept in a dark environment at 4°C. The oily fraction was filtered using anhydrous magnesium sulphate. The oil was stored at 4°C in amber vials under critical cooling.

Preparation of essential oil toothbrush rinse *P. heptaphyllum*

The toothbrush rinse containing the essential oil of the species *P. heptaphyllum* was prepared by following the formulation methods of commercial mouthwashes, with modifications. Briefly, 10 g of essential oil were weighed and added to 10 ml polysorbate 20 (Vetec-Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 2 g sucrose (Vetec-Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 100 ml glycerine (dynamic, Diadema, São Paulo, Brazil) and ultrapure water (18 W, Elga Purifier) qsp 1,000 ml. The toothbrush rinse was separated into aliquots of 100 ml. One aliquot was submitted to microbiological assays and *in vivo* testing of disinfection, and the remaining aliquots underwent evaluation of stability and were stored at different temperatures and locations. The samples used to evaluate the biological activity were stored at 4°C until analysed.

Evaluation of *in vitro* antimicrobial activity of the essential oil toothbrush rinse and *P. heptaphyllum* against *S. mutans*

The antimicrobial activity analysis was performed as described by Zarai et al. (2011), with modification. The standard strain used was ATCC 25175™ *S. mutans*. We performed a serial dilution of the toothbrush rinse with a concentration range of 100 to 0.01 µg/ml to calculate the minimum inhibitory concentration (MIC) (Lessa et al., 2012). In total, 100 µl bacitracin sucrose broth (SB20), a selective enrichment broth (Davey and Rogers, 1984), 100 µl of the inoculum and 50 µl of the test solution were added to each well. As a positive control, we used 0.12% chlorhexidine solution (Periogard, Colgate, São Paulo, São Paulo, Brazil) and 10% dimethyl sulphoxide (Sigma, St. Louis, Mo., USA) as a negative control. We also evaluated the entire toothbrush rinse solution matrixes (without the addition of essential oil) in the presence of inoculum. The microbiological control solutions were also prepared by incubating the test solution in only SB20. The plates were sealed and incubated at 37°C for 48 h. After incubation, a 10 µl solution of methyl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT, 5 mg/ml) (Sigma, St. Louis, Mo, USA) was added to each well and incubated for an additional 3 h. Subsequently, 100 µl of an isopropanol solution acidified with 40 µM HCl (Merck, Darmstadt, Germany) was added to solutions of the experimental and control treatments. The analysis was performed using a microplate reader (Reader-TP, Thermoplate) at 595 nm. The assay was performed in triplicate. The MIC was defined as the lowest concentration of the tested samples to inhibit visible growth of the microorganism tested.

Clinical trial for the use of *P. heptaphyllum* toothbrush rinse to disinfect toothbrushes

This research project was approved by the local Research Ethics Committee at the University of Vila Velha (CAAE N° 03487312.2.0000.5064), and informed consent was obtained from parents or legal guardians. Children of a private school located in the city of Vitória (ES, Brazil) were preselected to participate in this study. Fifty subjects were included in this study, which consisted of both sexes, 2 to 5 years of age, who had complete primary dentition and were in good health. Children who were using antimicrobial mouthwash, who had used antibiotics in the last three months or who were not present in all phases of the study were excluded from the study. The inclusion criteria were that children should have mutans streptococci in their saliva. The presence of mutans streptococci in children was identified using the kit Dentocult® SM Strip mutans (Orion Diagnostica Oy, Espoo, Finland), which is specific for confirming the identity of this group of microorganisms in saliva (Lessa et al., 2012).

Children were randomly assigned to one of three groups, using a table of random numbers. The study consisted of a crossover design with a washout period of one week. The test was conducted in three stages, where the three solutions (sterile tap water, chlorhexidine 0.12% and toothbrush rinse of *P. heptaphyllum* 1.0%) were used in all stages, but each of them was rotated to a different group of children in each stage to minimise the occurrence of variables that could interfere with the results (Table 1). Shortly before the evaluation, the solutions were placed in individual plastic trigger spray bottles (Elyplast, São José dos Campos, SP, Brazil) under aseptic conditions, wrapped with aluminium foil and identified with numbers. The identity of each solution was only known by the person who prepared the solutions, who did not participate in any stage of the clinical experiment.

At each stage, the children received new toothbrushes with soft bristles and small heads (Infantil Leader Macia, Sanifil) that were properly coded. The children were subjected to tooth brushing, which was performed by a single professional without dentifrice in a position suggested by Starkey (1961), always following the same sequence. The brushing time was one minute, and time was kept with a standardised digital stopwatch (Lessa et al., 2012). After brushing, the toothbrushes were carefully rinsed in tap water, and the excess water was removed from the bristles by slightly beating the handle of the toothbrush against the edge of the sink. Then the

practitioner suspended the toothbrushes in a vertical position with the head facing up and at a distance of, at most, 5 cm between the brush head and the bottle. Each solution was sprayed on the bristles five times, totalling approximately 0.5 ml per toothbrush (Lessa et al., 2012).

The toothbrushes were kept in a closed vessel for 4 h while drying, simulating the average interval between brushings (Warren et al., 2001). After drying, the toothbrushes were kept in 50 ml SB20 culture medium in an upright position with the bristles totally submerged in the culture medium; the toothbrushes were incubated for four days at 37°C. After the incubation time, aliquots from each tube were submitted for an analysis of bacterial viability. Three aliquots (100 µl) of each tube were transferred to a 96-well plate, and 10 µl solution of MTT (5 mg/ml) (Sigma Chemical, St. Louis, MO, USA) was added; the solution was subsequently incubated for an additional 3 h. The final procedure was identical to the one previously described in the *in vitro* study.

As an additional control, five toothbrushes were removed from their original containers and subjected to microbiological processing without being used. This procedure was performed to verify that the toothbrushes were not contaminated from the manufacturing process and industrial packaging (Nascimento et al., 2012).

Analysis of the chemical constituents

Analysis of the chemical constituents of the oil and the toothbrush rinse of *P. heptaphyllum* was performed using a gas chromatograph (Trace Ultra, Thermo Scientific®) coupled to a mass spectrometer (MS) (DSQII, Thermo Scientific®). The samples were separated using a capillary column DB-5 ms (30 m × 0.25 mm id × 0.25 µm, J & W Scientific, Folsom California, USA). The temperature program was initiated at 60°C and increased to 240°C by a temperature ramp of 3°C/min. The final temperature was maintained for 5 min. Helium gas was used with a constant flow rate of 1 ml/min. The injector temperature was maintained at 220°C, and the temperature of the interface (GC/MS) was maintained at 250°C. The mass detector operated by ionisation with electron impact (+70 eV) using the scan mode and was held at 35 to 450 m/z. The detector voltage was set to 1.6 kV. The samples were injected into the GC/MS in duplicate and diluted in hexane (2 mg/ml), and the injected volume was 2.0 µl. The identification of the substances contained in the oil was performed by comparing similarities between data obtained in this study with mass spectra obtained from the literature (Adams, 1995). The relative percentages of these compounds were calculated from the mean areas of the chromatograms obtained.

Evaluation of the stability of essential oil toothbrush rinse of *P. heptaphyllum* with respect to time and temperature

The aliquots of toothbrush rinse *P. heptaphyllum* were submitted to different treatments: (i) extract control (CE), where the toothbrush rinse was immediately extracted and analysed at the rate it was produced; (ii) storage under controlled temperature (A2), where the temperature remained between 18 and 23°C and relative humidity between 35 and 50% (the analysis was performed after two months of storage); (iii) negative control (NC), consisting of aliquots prepared and stored in a refrigerator for one hour at a controlled temperature (2 to 8°C); (iv) positive control (PC), where aliquots were subjected to 50°C in a double boiler for two hours; and (v) storage control at room temperature (A12), where the samples were stored for 12 months at room temperature (18 to 23°C and relative

humidity between 35 and 50%). After these treatments, the samples were subjected to the extraction process consisting of adding 2 ml of hexane (high performance liquid chromatography (HPLC) grade), vigorously shaking and separating by centrifugation at 1588 × g. The supernatant was removed from an aliquot of 100 ml and added to a vial containing 890 ml of HPLC grade hexane, 10 L of internal standard and a blend of C7 to C32 alkanes (Sigma). The samples were injected using the method used to identify the constituents present in the essential oil. The stability of the substances was assessed by examining the ratio of the area of each substance and its internal standard area (Aa/Ai). Among the substances identified in the essential oil, five were selected according to their relative percentage. The substances selected, the internal standards and the ions used to obtain the area are described in Table 2.

Statistical analysis

Data from the study on the antimicrobial effect of the essential oil toothbrush rinse and *P. heptaphyllum in vitro* and the data from the toothbrush rinse treatment of *P. heptaphyllum* and chlorhexidine in disinfecting brushes were expressed as the mean ± standard error of the mean (SEM). We applied the Shapiro-Wilk normality test in the data sets. The differences were considered significant at $p < 0.05$. Comparisons between treatments for disinfecting brushes were applied using the Kruskal-Wallis one-way analysis of variance (ANOVA) followed by the Dunn's test. The differences were considered significant at $p < 0.05$. The statistical non-parametric Friedman test was applied to investigate possible differences between the solutions with respect to the inhibition or absence of cariogenic biofilm formation on the bristles of the brushes. To compare the ratio of the Aa/Ai's stability, a one-way ANOVA test was applied followed by Tukey's test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using free software and Tanagra GraphPrism® software (Prism 5 for Windows, version 5.00, 2007).

RESULTS

The MIC values against *S. mutans* for the essential oil and the toothbrush rinse of *P. heptaphyllum* were 0.13 and 2.4 µg/ml, respectively. The base toothbrush rinse showed no antimicrobial activity when in the presence of the inoculum; nor did the base toothbrush rinse show microbial growth when evaluated only in the presence of the growth medium. Of the 50 children initially enrolled in the study, 39 (78% children) tested positive for mutans streptococci (MS). However, the study was completed with 21 children, because 18 children did not attend all stages of the study (school absence during study days); thus, these students were excluded from evaluation, which was the main limitation of the study. In total, sixty-eight brushes were evaluated.

Contamination of the brushes was observed in all toothbrushes after a single brushing. The additional control toothbrushes ($n = 5$) showed no microbial contamination. The toothbrush rinse containing the essential oil of *P. heptaphyllum* was able to reduce the formation of MS. The results of the microbiological ana-

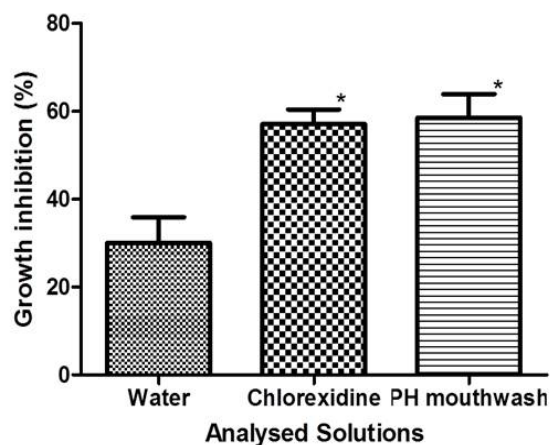


Figure 1. Microbiological evaluation of toothbrushes contaminated with mutans streptococci after use of toothbrush rinse containing the essential oil of *P. heptaphyllum* 1%. PH (*Protium heptaphyllum* 1%)* difference compared to water ($p < 0.05$).

Table 2. List of substances used for monitoring the stability of the toothbrush rinse, the internal standard and the ions (m/z).

Compound	Ions	Internal standard	Ions
p-Cymene	119	Nonane (C9)	57
Terpinolene	93	Nonane (C9)	57
p-Cymen-8-ol	135	Decane (C10)	57
α -Terpineol	121	Decane (C10)	57
Carvenone	95	Undecane (C11)	57

lysis of the toothbrushes after disinfection are shown in Figure 1. The essential oil composition of *P. heptaphyllum* showed predominant monoterpenes, with terpinolene as the primary constituent (35.1%), followed by p-cymene (26.66%), tricyclene (11.05%) and p-cymen-8-ol (10.12%) (Table 2). The toothbrush rinse containing the essential oil of *P. heptaphyllum* 1% consisted of the same constituents, except for the α -terpineol. The toothbrush rinse of *P. heptaphyllum* was a clear, colourless liquid, with the characteristic odour of pure oil. To test the stability of the toothbrush rinse, some substances were monitored, such as terpinolene, p-cymene, p-cymen-8-ol, α -terpineol and carvenone, because of their larger relative area (Table 3). Because the substance tricyclene was the first peak on the chromatogram, it was not monitored. In addition, the extraction process interfered with the relative area of α -terpineol as it was eliminated in this process (Figure 2A).

The relative amount of p-cymene was not altered by

the extraction process or the change in temperature. However, storage for 2 to 12 months resulted in a decrease of its relative area (Figure 2B). For substances p-cymen-8-ol, terpinolene and carvenone, the extraction process led to losses of these products (Figure 2C, D and E), and storage for 2 and 12 months influenced their volatility. Therefore, a period of 12 months in storage had the greatest influence on the loss of p-cymen-8-ol (Figure 2D). The overlap of all of the chromatograms obtained showed the same chromatographic profile for all samples with reduced peak areas (Figure 3).

DISCUSSION

The present study demonstrated the antimicrobial activity of essential oil extracted from the resin of *P. heptaphyllum* and a toothbrush rinse containing 1% of the essential oil. The data indicated that the chemical constituents of the essential oil of *P. heptaphyllum* exhibit antimicrobial activity against gram-positive *S. mutans*, the primary cariogenic bacteria tested. The use of this toothbrush rinse was also evaluated as an alternative treatment to disinfect toothbrushes. Several studies have evaluated substances that could be used as disinfectants for toothbrushes, seeking an easy, economical and safe use for disinfecting them and thereby maintaining oral health (Quintas et al., 2015; Nelson-Filho et al., 2011; Zarai et al., 2011; Juiz et al., 2010; Efstratiou et al., 2007; Mehta et al., 2007).

The present study showed the *in vitro* and *ex vivo* effects of *P. heptaphyllum* mouthrinse against the gram positive bacteria group MS. The experiment was designed in order to establish the MIC of pure essential oil, which was 0.013%, and thereafter, to estimate the mouthwash concentration, which was about 80-fold concentrated (1.0%), based on the predictable loss of activity and bioviability during the formulation. The MIC of the pH mouthwash was 0.24%, that is about 19-fold bigger than the pure essential oil. It is also important to highlight that with 1% essential oil of *P. heptaphyllum*, the components of the oil will be diluted in a concentration similar to a commercial mouthwash such as listerine (menthol 0.042%, thymol 0.064%, methyl salicylate 0.06% and eucalyptol 0.092%) (Pan et al., 2010; Oyanagi et al., 2012). There are some studies about essential oils mouthwash, several using a 0.2 to 1.4% concentration of essential oil (Kothiwale et al., 2014; Lobo et al., 2014; Quintas et al., 2014) and others in which the final concentration is omitted (Batista et al., 2014). An antiseptic agent to be used in children should not be harmful to the mucosa; its toxicity should be low if ingested accidentally, and it should be free from sugar and alcohol (Subramanian and Nandan, 2011). The toxicity of *P. heptaphyllum* has been evaluated by Siane

Table 3. Composition (shown as a relative percentage) of the essential oil resin of *P. heptaphyllum* and toothbrush rinse prepared with the essential oil of *P. heptaphyllum* and the IK data.

Substance	Relative percentage	IK	RT (min)
Tricyclene	11.05	926	5.6
α -Pinene	4.35	939	7.67
2- Δ -Carene	1.09	1001	7.97
<i>p</i> -Cymene	26.66	1026	8.4
Terpinolene	35.81	1088	10.50
<i>p</i> -Cymen-8-ol	10.12	1183	14.59
α -Terpineol	2.29	1189	15.19
2-Methoxy- α -methylbenzyl alcohol	1.08	NR	16.61
Carvenone	1.27	1252	18.4
Total	96.61	-	-

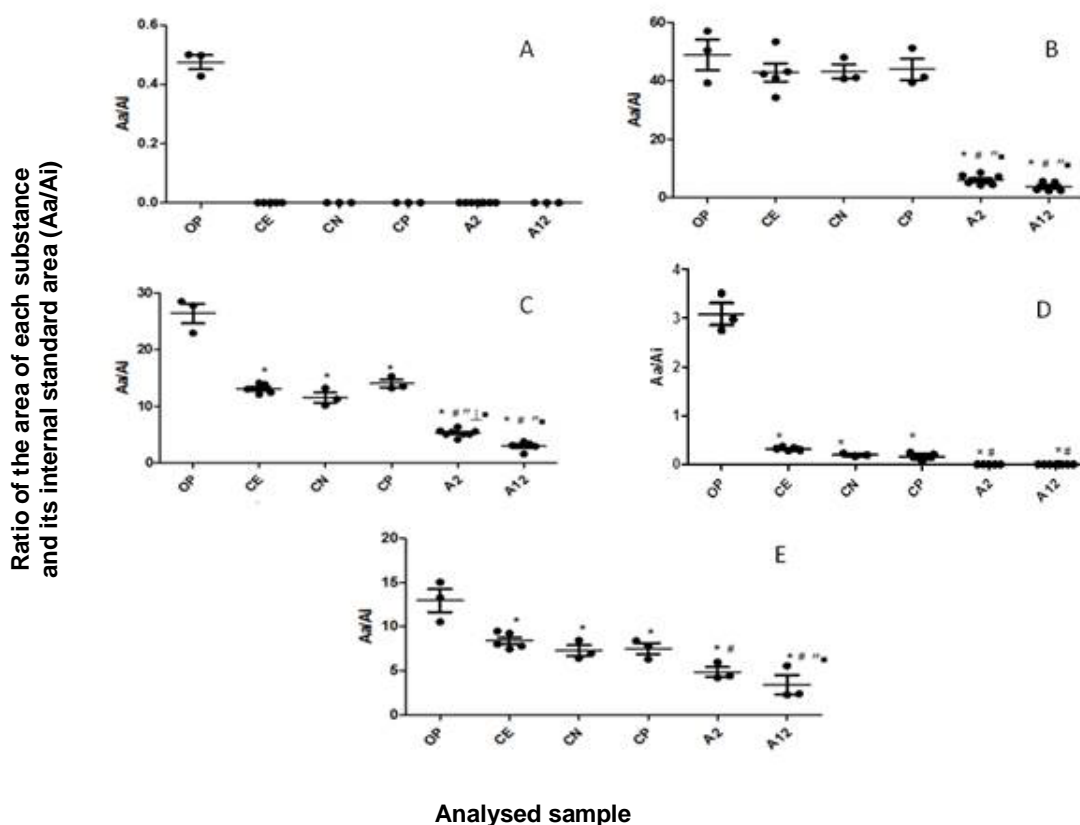


Figure 2. Evaluation of the stability of the oil *Protium heptaphyllum* (OP), the essential oil toothbrush rinse *P. heptaphyllum* (1%) (CE), kept under refrigeration (CN), subjected to heating (CP), stored at room temperature for two months (A2) and 12 months (A12). x-Axis: ratio of the area of each substance and its internal standard area (Aa/Ai) and y-axis: analysed sample. A: α -terpineol. B: *p*-cymene. C: *p*-cymen-8-ol. D: carvenone. E: terpinolene. It differs from pure oil *, # EC, ^CN, CP ^ □ A12. $p < 0.05$.

et al. (1999), who described the resin of *P. heptaphyllum* as showing no toxic potential. Polysorbate 20, a

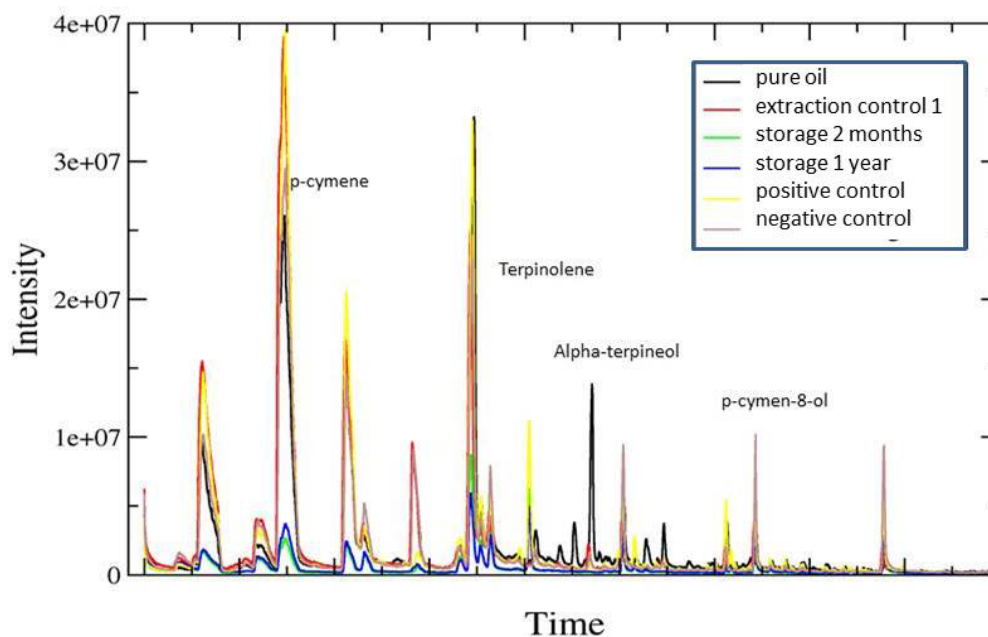


Figure 3. Overlay of chromatograms of aliquots of *Protium heptaphyllum* oil (PO), toothbrush rinse essential oil of *P. heptaphyllum* (1%) (EC), kept under refrigeration (NC), and subjected to heating (PC), stored at room temperature for two months (A2) and 12 months (A12)

solubilising agent, was used in the formulation of the toothbrush rinse because it had no toxic or irritant properties (Cordeiro et al., 2006). Previous trials evaluating mouthwash to disinfect toothbrushes specified that supervised participants used clean and new brushes, which only after brushing were placed in contact with the analyte (do Nascimento et al., 2015; Nelson-Filho et al., 2011; Mehta et al., 2007). This procedure was also adopted in this study.

Contamination of the brushes was observed in all toothbrushes after a single brushing. The toothbrush rinse essential oil of *P. heptaphyllum* showed the same efficacy in disinfecting toothbrushes contaminated with MS as did the gold standard, chlorhexidine. Both treatments were more effective than water (Figure 1). Chlorhexidine is known as the most effective commercial solution and is used as the gold standard in controlling plaque, because it is effective on gram-negative and gram-positive yeast (Nelson-Filho et al., 2011; Subramanian and Nandan, 2011; Juiz et al., 2010; Nelson-Filho et al., 2000). There are several studies which compare either the effectiveness or effect of chlorhexidine-containing mouthrinses and different products on dental plaque, gingivitis and caries prophylaxis, using a one-week period of washout (Charangundla et al., 2014; Lobo et al., 2014; Venu et al., 2013; Barnes et al., 2011; Franco Neto et al., 2008). The

studies showed that chlorhexidine significantly reduced the mutans streptococci group or other microorganisms' levels, but these levels returned to baseline (Lobo et al., 2014). The effectiveness of chlorhexidine after a 12 h application was also evaluated (Tomás et al., 2013), showing that after 12 h, the effect decreases. Since in the studies with one week washout, a difference between groups (for example, water and chlorhexidine) was observed, it is reasonable to use this period of washout in the present study. At low concentrations, chlorhexidine is bacteriostatic, while in high concentrations, it is bactericidal (Subramanian and Nandan, 2011). However, the use of chlorhexidine is associated with detrimental effects, such as a darkening of dental enamel, hyperplasia of the tongue papillae and loss of sense of taste (Juiz et al., 2010). Due to these effects, a search for other agents that have beneficial effects similar to those of chlorhexidine has been increasing.

Cordeiro et al. (2006) described the importance of using medicinal plants to support other therapies and routine prophylaxis. Batista et al. (2014) reported the use of mouthwashes of chamomile and pomegranate extracts and suggested that both extracts have anti-inflammatory and antimicrobial properties. In a recent study conducted by our group (Lessa et al., 2012), a mouthwash known as guaco was prepared with ethanolic extracts of *Mikania laevigata* and *Mikania glomerata*. It showed antimicrobial

activity against *S. mutans* and was evaluated as a toothbrush sanitiser; mouthwash prepared with *M. glomerata* had the same efficacy as the mouthwash with chlorhexidine 0.12% (gold standard).

A search for chemical markers is essential for assessing and maintaining quality. The results found for the essential oil of *P. heptaphyllum* (Table 3) are consistent with work previously described, where the components α -pinene, terpinolene, p-cymen-8-ol and α -cymene were also reported as major components of the *P. heptaphyllum* oil (Marques et al., 2010; Siane et al., 1999). However, some differences were observed in relative percentages and may be related to genetic factors, the nutritional status of the plant and the soil and climatic conditions (Figueiredo et al., 2012); medicinal plants that are derived from the same species may exhibit significant differences in quality when collected at different sites (Asghari et al., 2012). The temperature did not seem to be a factor that influenced the degradation of the substances; however, volatilisation of the chemicals might have contributed to losses, since the overlap of all chromatograms obtained observes the same chromatographic profile for all samples, with a reduction in peak area (Figures 2 and 3).

A surfactant was added to the formulations in this study (polysorbate 20) because essential oils are notoriously volatile and quickly evaporate off of surfaces (Rojo et al., 2012). However, the presence of this fixative at a concentration of 1% did not appear to be adequate in maintaining the composition for more than two months. A change in the amount of this surfactant would not be indicated, because higher concentrations of this product exhibit potential antimicrobial activity (Cordeiro et al., 2006).

Based on the results of this study, the essential oil of *P. heptaphyllum* and the toothbrush rinse made from this oil exhibited antimicrobial activity against the causative agent of caries, *S. mutans*. The effectiveness of the toothbrush rinse *P. heptaphyllum* is equal to that of chlorhexidine (0.12%). The stability of the toothbrush rinse *P. heptaphyllum* was not influenced by temperature, but was influenced by the storage time. However, the substances were volatilised instead of degraded.

ACKNOWLEDGEMENTS

The work was supported with funds from University Vila Velha. FAPES and University Vila Velha are acknowledged for the scientific scholarship. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROSUP) is acknowledged for a Masters fellowship. The authors thank Claudia Grillo Bermudez for assistance during the brushing of children.

Conflict of interest

There is no conflict of interest as regard this study.

REFERENCES

- Adams RP (1995). Identification of essential oils components by gas chromatography/quadrupole mass spectroscopy. Illinois (USA), Carol Stream: Allured Publishing Corporation p 804.
- Asghari G, Jalali M, Sadoughi E (2012). Antimicrobial activity and chemical composition of essential oil from the seeds of *Artemisia aucheri* Boiss. Jundishapur J. Nat. Pharm. Prod. 7:11-15.
- Barnes VM, Arvanitidou E, Szewczyk G, Richter R, DeVizio W, Cronin M, Michelle S (2011). Evaluation of the antiplaque efficacy of two cetylpyridinium chloride-containing mouthwashes. J. Clin. Dent. 22:200-203.
- Batista ALA, Lins RDAU, Coelho RS, Barbosa DN, Belém NM, Celestino FJA (2014). Clinical efficacy analysis of the mouth rising with pomegranate and chamomile plant extracts in the gingival bleeding reduction. Complement Ther. Clin. Pract. 20:93-98.
- Carounanidy U, Sathyanarayan R (2009). Dental caries- A complete changeover (Part I) J. Conserv. Dent. 12:46-54.
- Charangundla BR, Anjum S, Mocherla M (2014). Comparative effect of fluoride, essential oil and chlorhexidine mouthrinses on dental plaque and gingivitis in patients with and without dental caries: a randomized controlled trial. Int. J. Dent. Hyg. DOI: 10.1111/idh.12094
- Cordeiro CHG, Sacramento LVS, Corrêa MA, Pizzolitto AC, Bauab TM (2006). Análise farmacognóstica e atividade antibacteriana de extratos vegetais empregados em formulação para a higiene bucal. Rev. Bras. Cienc. Farm. 42:395-404.
- Davey AL, Rogers AH (1984). Multiple types of the bacterium *Streptococcus mutans* in the human mouth and their intra-family transmission. Arch. Oral Biol. 29:453-460.
- do Nascimento C, Paulo DF, Pita MS, Pedrazzi V, de Albuquerque Junior RF (2015). Microbial diversity of the supra- and subgingival biofilm of healthy individuals after brushing with chlorhexidine- or silver-coated toothbrush bristles. Can. J. Microbiol. 61:112-23.
- Efstratiou M, Papaioannou W, Nakou M, Ktenas E, Vrotsos IA, Panis V (2007). Contamination of a toothbrush with antibacterial properties by oral microorganisms. J. Dent. 35:331-337.
- Franco Neto CA, Parolo CC, Rösing CK, Maltz M (2008). Comparative analysis of the effect of two chlorhexidine mouthrinses on plaque accumulation and gingival bleeding. Braz. Oral Res. 22:139-144.
- Gherunpong S, Tsakos G, Sheiham A (2004). The prevalence and severity of oral impacts on daily performances in Thai primary school children Health Qual. Life Outcomes 2:57.
- Juiz PJJ, Alvez RJC, Barros TF (2010). Uso de produtos naturais no tratamento da doença periodontal Rev. Bras. Farmacogn. 20:134-139.
- Keyes PH (1960). The infectious and transmissible nature of experimental dental caries. Arch. Oral Biol. 1:304-320.
- Kothiwale SV, Patwardhan V, Gandhi M, Sohoni R, Kumar A (2014). A comparative study of antiplaque and antigingivitis effects of herbal mouthrinse containing tea tree oil, clove, and basil with commercially available essential oil mouthrinse. J. Indian Soc. Periodontol. 18:316-320.
- Kumarihamy SLM, Subasinghe LD, Jayasekara P, Kularatna SM, Palipana PD (2011). The prevalence of early childhood caries in 1-2 yrs olds in a semi-urban area of Sri Lanka. BMC Res. Notes 4:336.
- Lessa FCR, Grillo CHB, Pinto FE, Lorençon BB, Martins JDL, Bertolucci SKV, Pinto JEBP, Endringer DC (2012). Efficacy of guaco mouthwashes (*Mikania glomerata* and *Mikania laevigata*) on the disinfection of toothbrushes. Rev. Bras. Farmacogn. 22:1330-1337.
- Lobo PL, Fonteles CS, Marques LA, Jamaracu FV, Fonseca SG, de Carvalho CB, de Moraes ME (2014). The efficacy of three formulations of *Lippia sidoides* Cham. essential oil in the reduction of

- salivary *Streptococcus mutans* in children with caries: a randomized, double-blind, controlled study. *Phytomedicine* 21:1043-1047.
- Marques DD, Sartori RA, Lemos TLG, Machado LL, Souza JSN, Monte FJQ (2010). Chemical composition of the essential oils from two subspecies of *Protium heptaphyllum* Acta Amaz. 40:227-230.
- Marsh PD, Devine DA (2011). How is the development of dental biofilms influenced by the host? *J. Clin. Periodontol.* 38: 28-35.
- Mehta A, Sequeira PS, Bhat G (2007). Bacterial contamination and decontamination of toothbrushes after use. *NY State Dent. J.* 73:20-22.
- Ming L, Figueiredo R, Machado S, Andrade R (1996). Yield of essential oil of and citral content in different parts of lemongrass leaves (*Cymbopogon citratus* (D.C.) Stapf.) Poaceae. *Acta Hort.* 01:555-559.
- Nascimento C, Scarabel TT, Miani PK, Watanabe E, Pedrazzi V (2012) *In vitro* evaluation of the microbial contamination on new toothbrushes: a preliminary study. *Microsc. Res. Tech.* 75:42-45.
- Neal PR, Rippin JW (2003). The efficacy of a toothbrush disinfectant spray – an *in vitro* study. *J Dent.* 31:153-157.
- Nelson-Filho P, Macari S, Faria G, Assed S, Ito IY (2000). Microbial contamination of toothbrushes and their decontamination *Pediatr. Dent.* 22:381-384.
- Nelson-Filho P, Silva LA, Silva RA, Silva LL, Ferreira PD, Ito IY (2011). Efficacy of microwaves and chlorhexidine on the disinfection of pacifiers and toothbrushes: an *in vitro* study. *Pediatr. Dent.* 33:10-13.
- Oyanagi T, Tagami J, Matin K (2012). Potentials of mouthwashes in disinfecting cariogenic bacteria and biofilms leading to inhibition of caries. *Open Dent. J.* 6:23-30.
- Pan PC, Harper S, Ricci-Nittel D, Lux R, Shi W (2010). *In-vitro* evidence for efficacy of antimicrobial mouthrinses. *J. Dent.* 38 Suppl 1:S16-20.
- Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001). Bacterial Diversity in Human Subgingival Plaque. *J. Bacteriol.* 183:3770-3783.
- Paula JS, Leite ICG, Almeida AB, Ambrosano GMB, Pereira AC, Mialhe FL (2012). The influence of oral health conditions, socioeconomic status and home environment factors on schoolchildren's self-perception of quality of life. *Health Qual. Life Outcomes* 10:6-10.
- Pinto SAH, Pinto LM, Cunha GMA, Chaves MH, Santos FA, Rao VS (2008). Anti-inflammatory effect of α , β - amyryn, a pentacyclic triterpene from *Protium heptaphyllum* in rat model of acute periodontitis. *Inflammopharmacology* 16:48-52.
- Quintas V, Prada-López I, Prados-Frutos JC, Tomás I (2015). *In situ* antimicrobial activity on oral biofilm: essential oils vs. 0.2 % chlorhexidine. *Clin. Oral Investig.* 19:97-107.
- Ramos SCS, Oliveira JCS, Câmara CAG, Castelar I, Carvalho AFFU, Lima-Filho JV (2009). Antibacterial and cytotoxic properties of some plant crude extracts used in Northeastern folk medicine. *Rev. Bras. Farmacogn.* 19:376-381.
- Rojo SR, Varona S, Nunez M, M.J. Cocero MJ (2012). Characterization of rosemary essential oil for biodegradable emulsions *Ind. Crops Prod.* 37:137-140.
- Santos EB, Dantas GS, Santos HB, Diniz MFFM, Sampaio FC (2009). Estudo etnobotânico de plantas medicinais para problemas bucais no município de João Pessoa, Brasil. *Rev. Bras. Farmacog.* 19:321-324.
- Siane AC, Ramos MFS, Lima JR OM, Santos RR, Ferreira EF, Soares ROA, Rosas EC, Susunaga GS, Guimarães AC, Zoghbi MGB, Henriques MGMO (1999). Evaluation of anti-inflammatory related activity of essential oils from the leaves and resin of species of *Protium* *J. Ethnopharmacol.* 66:57-69.
- Skrubis BG (1982). The drying of laurel leaves. *Perfumer and Flavorist* 7:37-40.
- Starkey P (1961). Instructions to parents for brushing the child's teeth. *J. Dent. Child* 28:42-47.
- Subramaniam P, Nandan N (2011). Effect of xylitol, sodium fluoride and triclosan containing mouth rinse on *Streptococcus mutans* *Contemp Clin. Dent.* 2:287-290.
- Tomás I, García-Caballero L, López-Alvar E, Suárez-Cunqueiro M, Diz P, Seoane J (2013). *In situ* chlorhexidine substantivity on saliva and plaque-like biofilm: influence of circadian rhythm. *J. Periodontol.* 84:1662-1672.
- Venu V, Prabhakar AR, Basappa N (2013). Comparative evaluation of antibacterial property and substantivity of chlorhexidine containing dentifrices with sodium lauryl sulfate and Tween as surfactants: an *in vivo* study. *Indian J. Dent. Res.* 24:521-522.
- Vieira-Júnior GM, Souza CML, Chaves MH (2005). Resina de *Protium heptaphyllum*: isolamento, caracterização estrutural e avaliação das propriedades térmicas. *Quim Nova* 28:183-187.
- Warren DP, Goldschmidt MC, Thompson MB, Adler-Storhiz K, Keene HJ (2001). The effects of toothpastes on the residual microbial contamination of toothbrushes. *J. Am. Dent. Assoc.* 132:1241-1245.
- Zarai Z, Kadri A, Chobba IB, Mansour RB, Bekir A, Mejdoub H, Gharsallah N (2011). The *in-vitro* evaluation of antibacterial, antifungal and cytotoxic properties of *Marrubium vulgare* L. essential oil grown in Tunisia. *Lipids Health Dis.* 10:161.

Full Length Research Paper

Mechanically separated fillet and meat nuggets of Nile tilapia treated with homeopathic product

Denise P. Lima^{1,3}, Mariana M. Fuzinato³, Ana P. Andretto³, Graciela L. Braccini⁴, Ricardo H. Mori², Cristiane Canan¹, Saraspathy N. T. G. de Mendonça¹, Carlos A. L. de Oliveira⁴, Ricardo R. Pereira⁴ and Lauro Vargas^{4*}

¹Department in Food, Federal Technology University of Paraná, Medianeira - PR - Brazil.

²Aquaculture Center, Paulista State University, Jaboticabal – SP - Brazil.

³Department in Food, Maringá State University, Maringá – PR – Brazil

⁴Animal Science Department, Maringá State University, Av. Colombo, 5790, Bloco J 45 – Sala 115, CEP 87.020-900 - Maringá – PR – Brazil

Received 12 September, 2014; Accepted 26 January, 2015

The homeopathic product Homeopatila 100[®] in the diet of Nile tilapia reduces stress during production and improves the well-being of fish. The objective of this study was to develop nuggets of tilapia fed with Homeopatila 100[®] and to assess their quality. Physical, chemical, microbiological and sensory analyses were performed on three formulations with 25, 50 and 75% mechanically separated meat (MSM) for each of the treatments. The nuggets with 75% MSM revealed a higher pH (5.89 ± 0.02), the tissue was softer ($1.29 \text{ N} \pm 0.04$), and they had a higher lipid value ($15.96\% \pm 0.05$). With 50 and 75%, the color (L^*) was darker ($60.76\% \pm 0.91$ and $60.03\% \pm 0.78$), and there were lower protein amounts ($15.54\% \pm 0.31$ and $13.55\% \pm 0.35$). Nuggets had an acceptable value of lipid oxidation (0.672 ± 0.007 mg MDA/kg). The microbiological analyses demonstrated that the product met the requirements of legislation. Nuggets with 25 and 50% MSM were deemed acceptable. There was no difference ($p > 0.05$) between the control treatment group and the Homeopatila 100[®] group for the analysis undertaken. The results indicated that the use of Homeopatila 100[®] in the diet of the Nile tilapia did not change the physical, chemical, microbiological and sensorial quality characteristics of the nuggets, ensuring consumer acceptability.

Key words: Co-product, aquaculture, homeopathy, fish, technology.

INTRODUCTION

Aquaculture provides one-third of the world's fishery products (Yarnpakdee et al., 2014). Tilapia is the second most popular fish cultivated globally (85 countries) and features low cost, firm white meat, mild flavor, high protein content and low lipid and energy content. It is a

common item on the menu in Europe, Asia and the Americas. With the increase in the production of tilapia, it is desirable to develop products that enable the use of processing waste for human consumption (Zhang et al., 2011).

*Corresponding author. E-mail: lvargas@uem.br. Tel: +55 44 3011-8919

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

The filleting residue has 60 to 70% of the total weight of tilapia. Part of this residue is discarded, causing waste of natural resources (Taskaya and Jaczynski, 2009). The use of mechanically separated meat (MSM) enables the use of 14% of the total weight of the fish, reducing the cost, and presenting nutritional value equivalent to entire muscle. The MSM is little used as raw material for the production of fishburger, nuggets and sausages (Ninan et al., 2010; Gehring et al., 2011).

The nuggets are restructured, breaded and prepared from the disintegration of the flesh by mechanical methods (Marengoni et al., 2009). The manufacture of these products with MSM uses the processing residues, avoids health and environmental problems and adds commercial value to final product (Nunes et al., 2006). The products of homeopathy, a complementary and alternative medicine, are produced by dinamization, a process that involves sequentially stirred dilutions in small volumes applied to human and animal diets (Adler et al., 2011). The use of population homeopathy reduces stress to animals, especially in intensive systems that are very different from the natural environment, and increases the production potential and survival. It is a non-toxic product. The use of extremely diluted active ingredients ensures that there are no residues in meat and contamination in water and soil (Andretto et al., 2014).

Homeopatila 100[®] is a homeopathic complex designed to decrease stress during production and guarantee the well-being of Nile tilapia (*Oreochromis niloticus*) in the productive cycle of the commercial fish fry. Several studies have been undertaken with Nile tilapia and Homeopatila 100[®] (Siena et al., 2010; Braccini et al., 2013; Merlini et al., 2014). Because there are few reports on the products prepared from tilapia fillets and MSM treated with homeopathy, the current investigation provides the physical, chemical, microbiological and sensory evaluation of nuggets from MSM of Nile tilapia prepared with Homeopatila 100[®].

MATERIALS AND METHODS

Prime matter and ingredients

The use of animals was approved by the Committee for Ethical Behavior in Animal Usage in Experiments of the State University of Maringá, Maringá PR Brazil (Protocol 019/2013). After sexual reversion, Nile tilapias (*O. niloticus*), variety Supreme, with a mean initial weight of 101.12 ± 17.73 g and mean initial length of 18.52 ± 6.00 cm for the control treatment and an initial mean weight of 99.73 ± 19.85 g and initial mean length of 18.05 ± 1.11 cm for the Homeopatila 100[®] treatment were randomly distributed in 16 fiber glass water boxes, with 20 animals per box. Homeopatila 100[®] and control treatments were analyzed with eight replications each by a totally randomized experimental design, with 160 fish per treatment. Fish were fed on commercial extruded meals with 32% crude protein and a diameter of 5 mm twice a day (10 and 16 h).

At the end of the experiment, when the fish weighed approximately 300 g, to make filleting easy, they were deprived of food for 24 h. All of the fish were captured by nets, desensitized

with water and ice at 0°C (Scherer et al., 2006) and killed by breaking the spinal marrow. Two treatments were evaluated: a control treatment with a hydro-alcohol solution 30% v/v (addition of 40 ml/kg meal) and an experimental treatment with the homeopathic product Homeopatila 100[®] (addition of 40 ml/kg meal). We used this concentration because studies on Nile tilapia fed on the same product with the same concentration in the diet provided better results than those with other concentrations (Siena et al., 2010).

Homeopatila 100[®] was prepared by REALH, Campo Grande MS, Brazil, with registration of product for veterinary use number 024/05736-3 Ministry of Agriculture, Livestock and Food Supply, Brazil. The composition and respective Hahnemannian centesimal dilution of the homeopathic product Homeopatila 100[®]: Iodum (12 CH); Sulphur (30 CH); Natrum muriaticum (200 CH); Streptococcinum (30 CH) and vehicle - ethyl alcohol 30% v/v (sufficient amount). Fish were beheaded, eviscerated, filleted and skinned (Souza, 2002). The fillets were immediately vacuum-conditioned (Microvac CV 8, Selovac, BR) in smooth transparent nylon bags (thickness 0.280 mm) and frozen at -18°C until nugget preparation. MSM was obtained from the fillet wastes of the two treatments by the (HT C100, High Tech, BR), wrapped in polyethylene bags, frozen at -86°C in an ultrafreezer (IULT 90D, INDEL, BR) and stored at -18°C until the preparation of nuggets.

Preparation of nuggets

Three formulations were prepared for the Homeopatila 100[®] and control treatments with different concentrations as follows: 75% (F / 75), 50% (F / 50) and 25% (F / 25) with MSM, replacing the tilapia fillet. The F / 50 was chosen as the best formulation through the result of sensory analysis. Therefore, this formulation was prepared again, named F2 / 50, to verify the sensory acceptance and purchase intent among Homeopatila 100[®] and control. Further, 1200 g of emulsion was used for each assay, featuring the following formulation: 16% cold water; 10% hydrogenated fat; 4% soybean-concentrated protein; 2% corn starch; 2% salt; 0.15% dehydrated onion; 0.10% dehydrated garlic; 0.08% dehydrated parsley and chive; 0.07% dehydrated salvia; 0.03% dehydrated rosemary; 0.07% white pepper; 0.15% sodium tripolyphosphate; 0.05% sodium erythorbate; 0.25% citric acid solution 0.05%; and 65% meat portion (tilapia fillet and MSM).

Fillets and MSM were previously thawed at $4 \pm 1^\circ\text{C}$ for approximately 24 h, ground in an electric grinder (PCP-10 L, Poli, BR) and placed with MSM in a mini-cutter (Sire, Filizola, BR). Hydrated tripolyphosphate, the other ingredients and, finally, fat were added. After being homogenized for 2 min, the mass was placed on polyethylene and covered with a polyethylene film and stored for 24 h in a freezer at $-18 \pm 2^\circ\text{C}$. The frozen mass was cut in four parts (2.25 cm long and 2 cm wide), made breaded and then frozen at -18°C. For pre-frying, they were kept at $4 \pm 1^\circ\text{C}$ for 3 h and pre-fried in soybean oil for 1 min at $180 \pm 1^\circ\text{C}$. For each formulation, 60 pieces were prepared with approximately 20 g not breaded, 24.5 g breaded and 23.5g breaded and pre-fried. The nuggets were prepared in triplicate, wrapped in polyethylene wrappers and frozen at $-18 \pm 2^\circ\text{C}$ for 120 days.

Physical and chemical composition

The moisture, fixed mineral residue, lipid and crude protein amounts were determined on the 10th day after preparation, according to a protocol by the Association of Official Analytical Chemists (AOAC, 2006). Carbohydrate values were calculated by the difference: $100 - (\% \text{Moisture} + \% \text{fixed mineral residue} + \% \text{crude protein} + \% \text{total lipids})$. Further, pH analysis was performed at room temperature with a pH-meter (pH 21, Hanna[®], Romania) on

10 g of the sample homogenized with 50 ml of distilled water. The evaluation of color is an important quality parameter used to observe natural changes of fresh food or changes during industrial processing (Sato and Cunha, 2005). The absorption was measured by a colorimeter (CR 400, Minolta, Japan) using D65 light and a vision angle of 10°. The absorption was measured at three different sites in the internal part of the product (Perlo et al., 2006), at approximately $45 \pm 1^\circ\text{C}$. The luminosity (L^*), red-green component (a^*) and yellow-blue component (b^*) values were presented according to the color system by the Commission Internationale de L'Eclairage (CIELAB) (Minolta, 1998).

Water activity (A_w) was evaluated at 25°C by a water activity apparatus (4TE, Aqualab, USA), a very important parameter to determine the preservation and shelf life of food. Microorganisms require different minimum A_w levels for growth. Usually, bacteria are more sensitive and almost all have their growth inhibited at A_w values between 0.90 to 0.91. Yeast and molds are more tolerant to lower A_w values, growing in bands ranging from 0.87 to 0.94 and 0.70 to 0.80, respectively (Abbas et al., 2009). To evaluate the shear force, the nuggets were cut into pieces measuring 1.5 cm height \times 1.0 cm width \times 2 cm length. Analyses were performed using a texture analyzer (TA.HD plus, Stable Micro Systems, UK) equipped with a Warner-Bratzler Blade and 5 g charge cell at a speed of 5.0 mm/s and a distance of 20 mm with a 0.001 mm resolution. The results of the minimum force needed for cutting are given in Newton (N). The samples of each formulation were used for the analyses. The chemical analyses were performed in triplicate. The color parameters, shear force, pH and A_w analyses were performed with 10 repetitions.

Microbiological evaluation

Twenty-four hours after preparation, the nuggets were evaluated for the presence of *Staphylococcus coagulase* and *Bacillus cereus*. The results were reported in colony forming units (CFU/g); coliforms were reported at 45°C and *Escherichia coli* in most probable number per gram (MPN/g). *Salmonella* sp. was determined by absence in 25 g and counting viable aerobic mesophilic and psychrotrophic expressed in Log_{10} CFU/g (ICMSF, 1982).

Lipid stability

After the preparation of the nuggets and pre-frying to an internal temperature of $75 \pm 1^\circ\text{C}$, they were stored at -18°C . The products were then thawed at $4 \pm 1^\circ\text{C}$ for three hours prior to the lipid oxidation analysis at times 0 (24 h after preparation), 30, 60, 90 and 120 days by the thiobarbituric acid reactive substances (TBARS) method following Tarladgis et al. (1964), modified by Crackel et al. (1988) to evaluate the difference between means in storage days and between different treatments.

Sensory evaluation

The research was approved by the Research Ethics Committee and involvement of Human Beings of the State University of Maringá (297.336/2013). The 9-point hedonic acceptance test for nuggets were applied, ranging between 9 = I liked it very much and 1 = I definitely did not like it (Dutcosky, 2007). The attributes of color, aroma, tenderness, taste and overall impression were evaluated. It also approved the 5-point buying intention test ranging between 5 = I will surely buy it and 1 = I will surely not buy it (Ferreira et al., 2000). Sensory evaluations were undertaken in three stages. Each step used an untrained panel of 120 teachers and students, between 19 and 50 years of age, who represented consumers at a higher education level from the Federal Technology University of

Paraná. In the first stage, the formulations F / 75, F / 50 and F / 25 of the control treatment were used; in the second stage, the same formulations with the Homeopatila 100® treatment were used. The formulation with the best overall evaluation and best buying intention was used in the third stage (50% MSM, F2 / 50) for the sensory analysis of nuggets made of fillet and MSM treated with Homeopatila 100®. The nuggets were thawed and baked until they reach a minimum temperature of $75 \pm 1^\circ\text{C}$, cooled to approximately 45°C and served. The samples from both treatments were coded with random three digit numbers; therefore, the volunteer participant did not know which sample contained the homeopathic product. The equation $IA (\%) = (A \times 100) / B$, where A is the mean score for overall evaluation and B is the maximum score observed for overall evaluation, was employed to calculate the acceptability index of the formulations under analysis (Dutcosky, 2007; Monteiro, 1984).

Statistical analysis

Physical, chemical, microbiological and sensory evaluations for the formulations F / 75, F / 50 and F / 25 were evaluated by analysis of variance (ANOVA) and Tukey's test ($p < 0.05$), and the results of the analysis for the F2 / 50 formulations underwent an analysis of variance at a 5% probability and Student's t test using the statistical analysis system (SAS) 9.0 (SAS, 2009).

RESULTS AND DISCUSSION

Physical and chemical composition

Whereas the greatest difference ($p < 0.05$) in the instrumental analysis of color occurred in L^* (luminosity) when a low value (25%) of MSM was added to the nuggets, no significant difference was reported in the a^* (red/green) and b^* (yellow/blue) of the other formulations and between the control and the Homeopatila 100® treatments (Table 1). A significant difference was reported in the L^* , a^* and b^* values in chicken nuggets with MSM (Perlo et al., 2006). A 75% increase in MSM in the formulation of Nile tilapia nuggets significantly affects ($p < 0.05$) the shear force and pH. There was no significant difference ($p > 0.05$) in A_w in the various MSM additions (Table 1). Nuggets with 75% MSM had a more tender texture than nuggets with 25% MSM due to the MSM process that ruptures the muscle fiber. A similar effect has been reported in fish sausages, where MSM replacing the fillet, increased its softness (Oliveira Filho et al., 2010). The increase in pH due to MSM was probably due to a higher amount of phosphate caused by the fragmentation of the tilapias' spines during processing (Gomide et al., 1997). The pH results meet Brazil's legislation for fresh fish (Brasil, 2001a). Higher values of pH were obtained by Oliveira Filho et al. (2010) and Dallabona et al. (2013) in sausages prepared with the addition of MSM at time 0 storage. The mean A_w value did not reveal any significant differences ($p > 0.05$) between the formulations and treatments. The value remained within the high A_w food range for microbial growth and was similar to the value for fresh fish (0.98). The cold storage was required (Oliveira Filho et al., 2010).

Table 1. Color (L*: luminosity a*: red/green; b*: yellow/blue), shear force, pH and Aw (water activity) of nuggets of Nile tilapia treated with Homeopatila 100®.

Parameter	L*	a*	b*	L*	a*	b*	Shear force (N) ¹		pH		Aw	
	Control			Homeopatila 100®			Control	Homeopatila 100®	Control	Homeopatila 100®	Control	Homeopatila 100®
F / 75 ^{2,3,5}	60.27±0.43 ^{bc}	0.85±0.04 ^a	31.56±0.14 ^a	60.03±0.78 ^c	0.90±0.03 ^a	32.05±0.24 ^a	1.45±0.08 ^b	1.29±0.04 ^b	5.88±0.03 ^a	5.89±0.02 ^a	0.975±0.002 ^a	0.972±0.003 ^a
F / 50 ^{2,3,5}	60.34±0.28 ^{bc}	0.96±0.03 ^a	30.64±0.74 ^a	60.76±0.91 ^{bc}	0.89±0.04 ^a	32.02±3.14 ^a	2.57±0.10 ^{ab}	2.46±0.06 ^{ab}	5.86±0.02 ^{ab}	5.80±0.09 ^{ab}	0.962±0.007 ^a	0.963±0.001 ^a
F / 25 ^{2,3,5}	64.42±0.21 ^a	0.99±0.06 ^a	28.58±0.41 ^a	62.66±0.14 ^{ab}	0.86±0.05 ^a	30.89±0.38 ^a	3.33±0.06 ^a	3.43±0.08 ^a	5.74±0.04 ^b	5.74±0.03 ^b	0.972±0.006 ^a	0.972±0.006 ^a
F2 / 50 ^{2,4,5}	60.97±0.30 ^a	1.34±0.03 ^a	30.41±0.06 ^a	60.09±0.61 ^a	1.31±0.05 ^a	31.42±0.26 ^a	2.85±0.01 ^a	2.82±0.06 ^a	5.97±0.01 ^a	5.85±0.01 ^a	0.975±0.002 ^a	0.975±0.002 ^a

¹N: Newton. ²F / 75; F / 50; F / 25; F2 / 50 with 75%, 50%, 25% and 50% of MSM. ³Value with different letters (a-b-c) on the same line and column differ significantly by Tukey's test (p<0.05). ⁴Line are not significantly different at 5% significance level by Student's t test (p<0.05). ⁵The results are given by the means ± standard error (n=10).

Table 2. Centesimal composition (%) of nugget from fillet and MSM of Nile tilapia treated with Homeopatila 100®.

Parameter	Moisture		Crude protein		Fixed mineral residue		Total lipids		Carbohydrate ⁴	
	Control	Homeopatila 100®	Control	Homeopatila 100®	Control	Homeopatila 100®	Control	Homeopatila 100®	Control	Homeopatila 100®
F / 75 ^{1,2,5}	55.50±0.39 ^a	55.67±0.31 ^a	12.85±0.18 ^b	13.55±0.35 ^b	2.75±0.07 ^a	2.77±0.07 ^a	16.07±0.22 ^a	15.96±0.05 ^a	12.79±0.81 ^a	12.05±0.17 ^a
F / 50 ^{1,2,5}	55.13±0.63 ^a	55.49±0.32 ^a	15.32±0.24 ^b	15.54±0.31 ^b	2.72±0.04 ^a	2.65±0.06 ^a	14.34±0.07 ^b	14.52±0.03 ^b	12.49±0.86 ^a	11.80±0.62 ^a
F / 25 ^{1,2,5}	54.59±0.47 ^a	54.46±0.60 ^a	16.04±0.02 ^a	16.12±0.33 ^a	2.86±0.06 ^a	2.73±0.11 ^a	13.98±0.38 ^b	13.62±0.20 ^b	12.53±0.80 ^a	13.07±1.03 ^a
F2 / 50 ^{1,3}	52.04±0.65 ^a	52.84±0.50 ^a	15.71±0.21 ^a	15.14±0.24 ^a	2.54±0.05 ^a	2.59±0.04 ^a	16.12±0.27 ^a	15.63±0.25 ^a	13.59±0.36 ^a	13.80±0.77 ^a

¹F / 75; F / 50; F / 25; F2 / 50 with 75%, 50%, 25% and 50% of MSM. ²Value with different letters (a-b-c) on the same line and column differ significantly by Tukey's test (p<0.05). ³Line are not significantly different at 5% significance level by Student's t test (p<0.05). ⁴Total carbohydrates were calculated by difference: 100 - (% moisture + % fixed mineral residue + % crude protein + % total lipids). ⁵The results are given by the means ± standard error (n=3).

2010).

There was no significant difference between treatments in the selected formulation (F2 / 50) in the pH measurement value, shear force, Aw and color (Table 1). The mean values for the moisture, fixed mineral residue and carbohydrates did not differ ($p > 0.05$) between the various MSM percentages between treatments (Table 2). The protein results were similar to those of the control group, but they were lower when the MSM was 50 and 75%. In the latter MSM proportion, the lipid values were higher in both treatment groups. There was no significant difference between the control and Homeopatila 100® in the 50% MSM (F2 / 50) formulation when the centesimal composition was taken into account. When the

amount of MSM in sausages was increased, the protein value decreased, the fat increased and the moisture content and mineral residue were not changed (Oliveira Filho et al., 2010). The results were similar to those obtained for nuggets.

The high fat quantity in the nuggets when the MSM was increased may be related to its high lipid value because the ventral muscle parts in the carcass normally have a higher fat content (Oliveira Filho et al., 2010). The minimum protein content and the maximum level of total carbohydrates required by law in breaded products are 10 and 30%, respectively (Brasil, 2001b). The nuggets were developed following these requirements. Results similar to this research in lipids were obtained in croquettes of Nile

tilapia MSM (Bordignon et al., 2010).

Microbiological quality

In the enumeration of coliforms at 45°C, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus coagulase* were not found and the presence of *Salmonella* sp. was not detected in the samples of nuggets for the two treatments. The counts of viable mesophilic and psychrotrophic aerobic bacteria ranged from 1.54 ± 0.27 to 3.68 ± 0.11 and showed no significant difference between the products and treatments. The analyses were in agreement with those established by legislation (ICMSF, 1982). Results

Table 3. Sensorial evaluation of nugget from fillet and MSM of Nile tilapia treated with Homeopatila 100®.

Parameter	Stage 1			Stage 2			Stage 3	
	Control			Homeopatila 100®			Control	Homeopatila 100®
	F / 75 ^{3,4,6}	F / 50 ^{3,4,6}	F / 25 ^{3,4,6}	F / 75 ^{3,4,6}	F / 50 ^{3,4,6}	F / 25 ^{3,4,6}	F2 / 50 ^{3,5,6}	F2 / 50 ^{3,5,6}
Color ¹	6.77±0.37 ^b	7.24±0.38 ^a	7.53±0.38 ^a	7.02±0.15 ^b	7.44±0.16 ^a	7.20±0.15 ^{ab}	6.96 ± 0.15 ^a	7.08 ± 0.16 ^a
Aroma ¹	7.06±0.36 ^b	7.76±0.37 ^a	7.89±0.37 ^a	6.75±0.14 ^a	7.34±0.15 ^a	7.11±0.15 ^a	7.04 ± 0.16 ^a	7.24 ± 0.16 ^a
Tenderness ¹	7.18±0.35 ^b	7.54±0.35 ^{ab}	7.66±0.35 ^a	6.68±0.13 ^b	7.70±0.15 ^a	7.69±0.15 ^a	7.53 ± 0.13 ^a	7.64 ± 0.14 ^a
Taste ¹	6.49±0.47 ^b	7.39±0.48 ^a	7.94±0.49 ^a	6.64±0.16 ^b	7.21±0.18 ^a	6.99±0.17 ^a	6.72 ± 0.19 ^a	7.06 ± 0.20 ^a
Overall evaluation ¹	5.75±0.33 ^c	6.47±0.33 ^b	7.10±0.33 ^a	6.41±0.13 ^b	7.28±0.15 ^a	7.31±0.15 ^a	6.94 ± 0.14 ^a	7.17 ± 0.15 ^a
Buying intention ²	4.08±0.45 ^b	4.79±0.46 ^a	4.16±0.46 ^a	2.91±0.11 ^b	3.72±0.14 ^a	3.75±0.14 ^a	3.59 ± 0.12 ^a	3.77 ± 0.13 ^a

¹Hedonic scale between 1 and 9 (1 I disliked it very much; 2 I disliked it; 3 I disliked it fairly; 4 I disliked it a little; 5 I didn't like it nor disliked it/ I didn't dislike it; 6 I liked it a little; 7 I liked it fairly; 8 I liked it; 9 I liked it very much). ²Hedonic scale between 1 and 5 (1 I will certainly not buy it; 2 I would possibly not buy it; 3 Perhaps I will buy it, perhaps I will not; 4 I may buy it; 5 I will certainly buy it). ³F / 75; F / 50; F / 25 and F2 / 50 with 75, 50, 25 and 50% of MSM. ⁴Value with different letters (a-b-c) on the same line differ significantly by Tukey's test (p<0.05). ⁵Lines are not significantly different at 5% significance level by Student's t test (p<0.05). ⁶The results are given as the means ± standard error (n=120).

similar to this experiment were obtained in lyophilized mixtures of fish croquettes at time zero with the same main ingredient as the MSM of the Nile tilapia (Fuchs et al., 2013) and in croquettes of the MSM of tilapia after being pre-fried, (Bordignon et al., 2010) for *Salmonella* sp., Coliforms at 45°C, *B. cereus* and *S. coagulase*. However, higher values were found in 4 types of prepared fishburger with the MSM of the Nile tilapia for *E. coli* and coliforms at 45°C (Marengoni et al., 2009) and lower values for viable mesophilic and psychotrophic aerobic bacteria (Bordignon et al., 2010). The pre-frying process (180°C/1') helped in obtaining breaded nuggets with low microbial counts, agreeing with the results obtained in croquettes of MSM of Nile tilapia (Bordignon et al., 2010).

Stability of lipid oxidation

The TBARS values for the Homeopatila 100® treatment increased from 0.007 ± 0.003 mg MDA/kg to 0.672 ± 0.007 mg MDA/kg. In the case

of the control, treatment increased the value of 0.004 ± 0.001 mg MDA/kg to 0.758 ± 0.007 mg MDA/kg. There was a gradual increase in lipid oxidation up to 120 days of storage (Figure 1), although no significant difference (p > 0.05) was found between 60 and 90 days. TBARS was not different (p < 0.05) for either treatment. Different values are cited as the mg MDA/kg limits in foods that might indicate rancidity by sensory evaluators. At 0.576, the oxidation value is low and there is no rancidity. Values greater than 1.51 are classified as unacceptable (Ke et al., 1984). In stored fishburger tilapia (-18°C/180 days), the values were lower than in nuggets (Tokur et al., 2004), and similar values were found in quenelles prepared with Nile tilapia (Angelini et al., 2013), ranging from 0.72 ± 0.50 to 0.88 ± 0.63 mg MDA/kg storage (-18°C/180 days). In the current assay, each formulation received an addition of the same amount of antioxidant (0.05% sodium erythorbate), which may have contributed to inhibiting oxidation. Therefore, the evaluated nuggets have an acceptable level of lipid oxidation.

Sensory evaluation

The first stage of sensory evaluation (Table 3) revealed a significant difference (p < 0.05) in formulation F / 75 for color, aroma, tenderness and taste. The three formulations showed differences (p < 0.05) in overall acceptability. Formulations with 50 and 25% MSM were preferred, whereas the sample with 75% MSM had the lowest acceptance. Sample F / 75 in the second stage of the sensorial test (Table 3) had a lower acceptability for color, tenderness, taste, overall evaluation and buying intention, but no significant difference for aroma. When the two sensorial evaluations were analyzed, acceptance tests showed results between 5.75 and 7.94 and 6.41 and 7.70, respectively, for the control and Homeopatila 100®. Buying intentions were close to 4 and 5 for the control and close to 3 and 4 for Homeopatila 100®. Five formulations provided acceptability values above 70% (Table 4). If these products were for sale, they would be accepted by consumers (Dutcosky, 2007). Formulation F / 50 for the control and Homeopatila 100® was within

Table 4. Acceptability index (%) by attribute and buying intention of fillet and MSM nugget.

Parameter	F / 75 ¹	F / 75 ¹	F / 50 ¹	F / 50 ¹	F / 25 ¹	F / 25 ¹	F2 / 50 ¹	F2 / 50 ¹
	Control	Homeopatila 100 [®]	Control	Homeopatila 100 [®]	Control	Homeopatila 100 [®]	Control	Homeopatila 100 [®]
Color	75.22	78.00	80.44	82.67	83.67	80.00	77.33	78,67
Aroma	78.44	75.00	86.22	81.56	87.67	79.00	78.22	80.44
Tenderness	79.78	74.22	83.78	85.56	85.11	85.44	83.67	84.89
Taste	72.11	73.78	82.11	80.11	88.22	77.67	74.67	78.44
Total evaluation	63.89	71.22	71.89	80.89	78.89	81.22	77.11	79.67
Buying intention	81.56	58.22	95.78	74.44	83.22	75.00	71.80	75.40

¹F / 75; F / 50; F / 25; F2 / 50 with 75, 50, 25 and 50% of MSM.

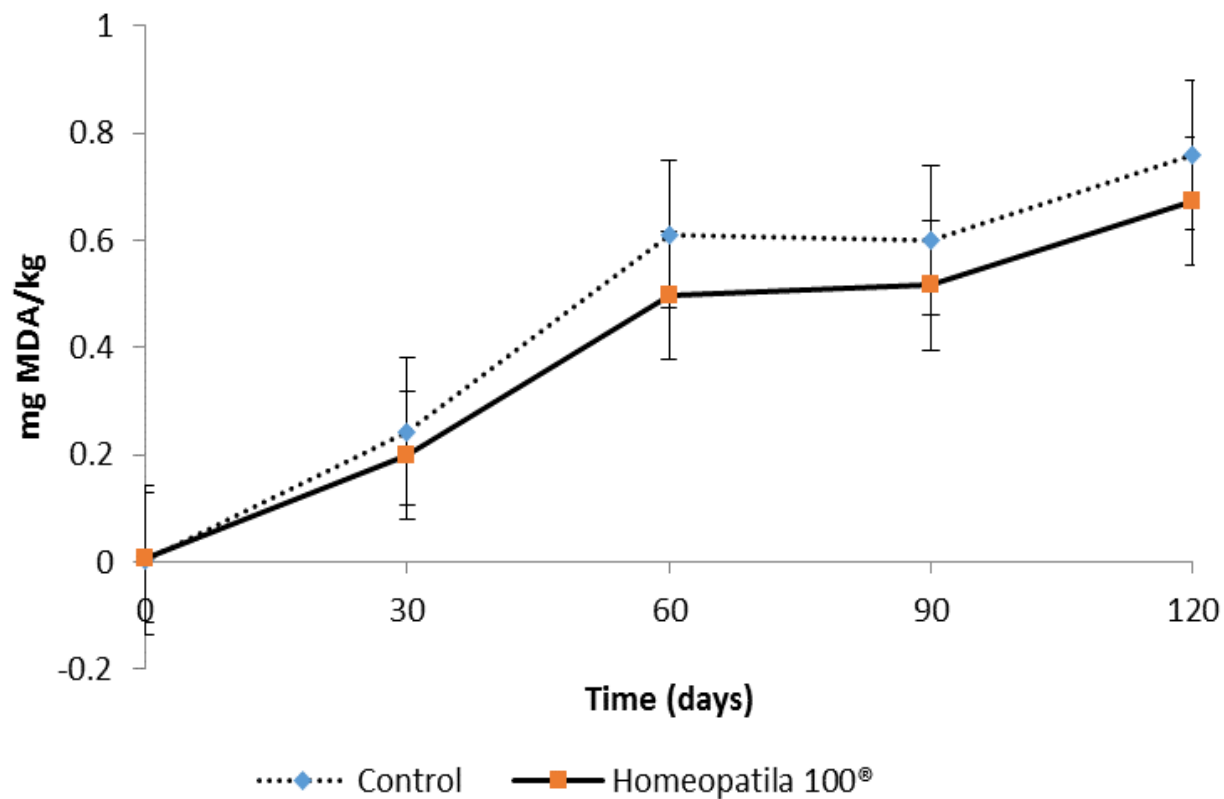


Figure 1. TBARS (thiobarbituric acid reactive substances) values of fillet and MSM 50% (F2 / 50) nuggets of Nile tilapia treated with *Homeopatila 100[®]* during storage of 120 days.

the range of “Surely I will buy it” and “Possibly I would buy it” because the acceptability indexes had values of 95.78 and 74.44%, respectively (Table 4), whereas the results were different only in the overall evaluation between F / 50 and F / 25. In Stage 1, the formulation with 50% MSM (F2 / 50) was chosen. The third stage of sensory analysis evaluated the consumer’s acceptability of nuggets with Homeopatila 100[®]. In the case of sausages prepared with fillet and MSM, the highest acceptance occurred between sausages prepared with 40 and 60% MSM, as in the current research (Oliveira Filho et al., 2010).

With regard to the sensory evaluation of F2 / 50 (Stage 3), the acceptability and buying intention did not have any significant difference between treatments. The acceptance test varied between 6.93 and 7.64, and tenderness had the highest score in each of the treatments (Table 3). Scores close to 4 for buying intention for the two treatments were reported, and the values for the acceptability index were 71.80 and 75.40% for the control and Homeopatila 100[®], respectively (Table 4), with good acceptability (Dutcosky, 2007). In other research, tilapia MSM-based products were reasonably well accepted. In the case of tilapia breaded, scores were over 7.0 (Cortez Netto et al., 2010). Fishburgers with tilapia MSM had means between 7.14 and 7.46 for all attributes, and the mean scores for buying intention varied between 3.86 and 3.98 (Marengoni et al., 2009). Overall acceptance of sausages with 64% MSM of tilapia, smoked and pasteurized, ranged between 7.7 and 7.5, respectively (Dallabona et al., 2013).

In the instrumental evaluation of color (Table 1), nuggets with 25% MSM had a lighter color than those with 50% and 75%. In the qualitative analysis, the volunteer participants noted color differences with 75% MSM in both treatments. The shear force value was lower in formulation F / 75 (Table 1), which was also detected by the volunteers in the sensory analysis. In agreement with the results of this study, evaluators reported that the texture of sausages made with the highest percentage of MSM (80 and 100%) were softer than the other treatments (Oliveira Filho et al., 2010). The results (step 3) indicated no differences in color, aroma, tenderness, taste, overall evaluation and buying intention in the sensory evaluation with tilapia nuggets treated with Homeopatila 100[®]. F2 / 50 had the highest score with regard to tenderness. The same was reported in an assay with 40 and 60% MSM in sausages (Oliveira Filho et al., 2010). Buying intention varied for F2 / 50 between 71.80 and 75.40% for the control and Homeopatila 100[®] groups, respectively (Table 4), and 83.76% declared they had never eaten fish nuggets. With the results observed in the sensory analysis (steps 1 and 2), nuggets can be produced with 75% of filleting waste as a replacement for fillets of Nile tilapia without changing the physical, chemical, microbiological and sensory characteristics. However, to maintain a better acceptability and purchase

intent for nuggets, the maximum amount of MSM as a substitute for the fillet can be 50%, reducing waste in fish processing and preventing negative environmental impact.

In the sensory evaluation of nuggets of Nile tilapia treated with Homeopatila 100[®] and control (stage 3), the results showed no significant differences in the color attributes, aroma, tenderness, taste, overall evaluation and purchase intent. The use of homeopathic product in the diet of tilapia did not affect the sensory quality of the nuggets. This effect is important because the homeopathic product has provided significant results in performance parameters in the cultivation of Nile tilapia.

Conclusion

The results provide no evidence that adjuvant treatment with Homeopatila 100[®] for fish growth improves the quality of meat and products made from Nile tilapia.

Conflict of interest

The authors declared no conflict of interest.

REFERENCES

- Abbas KA, Saleh AM, Mohamed A, Lasekan O (2009). The relationship between water activity and fish spoilage during cold storage: A review. *J. Food Agric. Environ.* 7:86-90.
- Adler UC, Paiva NMP, Cesar AT, Adler MS, Molina A, Padula AE, Calil HM (2011). Homeopathic Individualized Q-Potencies versus Fluoxetine for Moderate to Severe Depression: Double-Blind, Randomized Non-Inferiority Trial. *Evid. Based Complement. Altern. Med.* Article ID 520182, p.7.
- Andretto AP, Fuzinato MM, Bonafe EG, Braccini, GL, Mori RH, Pereira RR, Oliveira CAL, Visentainer JV, Vargas L (2014). Effect of an homeopathic complex on fatty acids in muscle and performance of the Nile tilapia (*Oreochromis niloticus*). *J Homop.* 103:178-185.
- Angelini MFC, Galvão JA, Vieira AF, Savay-da-Silva LK, Shirahigue LD, Cabral ISR, Modesta RCD, Gallo CR, Oetterer M (2013). Shelf life and sensory assessment of tilapia quenelle during frozen storage. *Pesq. agropec. bras.* Brasília 48(8):1080-1087.
- AOAC (2006). Association Methods of Analysis of AOAC International, Volume I and II, 18th Edition, Rev. 1.
- Bordignon AC, Souza BE, Bohnenberger L, Hilbig CC, Feiden A, Boscolo WR (2010). Elaboração de croquete de tilápia do Nilo (*Oreochromis niloticus*) a partir de CMS e aparas do corte em ‘V’ do filé e sua avaliação físico-química, microbiológica e sensorial. *Acta Sci. Anim. Sci.* 32:109-116.
- Braccini GL, Natali MRM, Ribeiro RP, Mori RH, Riggo R, Oliveira CA, Hildebrandt JF, Vargas L (2013). Morpho-functional response of Nile tilapia (*Oreochromis niloticus*) to a homeopathic complex. *Homeopathy* 102(4):233-241.
- Brasil (2001a). Ministério da Agricultura, Pecuária e Abastecimento. Departamento Nacional de Inspeção de Produtos de Origem Animal (DIPOA). Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal – RIISPOA, Pescados e derivados, Capítulo VII. Brasília, DF.
- Brasil (2001b). Ministério da Agricultura, Pecuária e Abastecimento, Secretaria de Defesa Agropecuária. Instrução Normativa nº 6 de 15 de fevereiro de 2001. Regulamento técnico de identidade e qualidade de empanados. Diário Oficial da União, Brasília, DF, 19

- fev, Seção 1, p 60.
- Cortez Netto JP, Boscolo WR, Feiden A, Maluf MLF, Freitas JMA, Simões MR, (2010). Formulação, análises microbiológicas, composição centesimal e aceitabilidade de empanados de jundia (*Rhamdia quelen*), pacu (*Piaractus mesopotamicus*) e tilápia (*Oreochromis niloticus*). Rev. Inst. Adolfo Lutz. 69(2):181-187.
- Crackel RL, Gray JI, Booren AM, Buckely DJ (1988). Effect of antioxidants on lipid stability in restructured beef steaks. J. Food Sci. 53:656-657.
- Dallabona BR, Karam LB, Wagner R, Bartolomeu DAFS, Mikos JD, Francisco JGP, Macedo REF, Kirschnik PG (2013). Effect of heat treatment and packaging systems on the stability of fish Sausage. R. Bras. Zootec. 42:835-843.
- Dutcosky SD (2007). Análise sensorial de alimentos. 2.ed., rev. e ampl. Curitiba: Champagnat p 239.
- Ferreira VLP, Almeida TCA, Pettinelli MLC, Silva MAAP, Chaves JBP, Barbosa EMM (2000). Análise sensorial: testes discriminativos e afetivos. Campinas: SBCTA (Manual: Série Qualidade) p 127.
- Fuchs RH, Ribeiro RP, Bona E, Matsushita MD (2013). Development of a freeze-dried mixture of Nile tilapia (*Oreochromis niloticus*) croquette using a GA-based multiobjective optimization J. Sci. Food Agric. 93(5):1042-1048.
- Gehring CK, Gigliotti JC, Moritz JS, Tou JC, Jaczynski J (2011). Functional and nutritional characteristics of proteins and lipids recovered by isoelectric processing of fish by-products and low-value fish: A review. Food Chem. 124:422-431.
- Gomide LAM, Garcia AM, Pereira ASO, Mendonça RCS (1997). Avaliação físico-química e microbiológica da adição de carne de frango mecanicamente separada em embutido fermentado. Cienc. Tecnol. Aliment. 17:125-131.
- ICMSF (1982). International Commission of Microbiological Specifications for Foods. Microorganisms in foods. Their significance and methods of enumeration. 2nd ed. University of Toronto Press, Toronto p 436.
- Ke PJ, Cervantes E, Roblemartinez C (1984). Determination of thiobarbituric acid reactive substances (TBARS) in fish tissue by an improved distillations spectrophotometric method. J. Sci. Food Agric. 35(440):1248-1254.
- Marengoni NG, Pozza MSS, Braga GC, Lazzeri DB, Castilha LD, Bueno GW, Pasquetti TJ, Polese C (2009). Caracterização microbiológica, sensorial e centesimal de *fishburgers* de carne de tilápia mecanicamente separada. Rev. Bras. J. Saúde Prod. Anim. 10:168-176.
- Merlini LS, Vargas L, Piau R, Ribeiro RP, Merlini NB (2014). Effects of a homeopathic complex on the performance and cortisol levels in Nile tilapia (*Oreochromis niloticus*). Homeopathy 103(2):139-142.
- Minolta (1998). Precise color communication - color control from perception to instrumentation. Japan: Minolta Co., Ltd. p 59.
- Monteiro CLB (1984). Técnicas de avaliação sensorial. 2.ed. Curitiba: CEPPA-UFPR p 101.
- Ninan G, Bindu J, Joseph J (2010). Frozen storage studies of value-added mince-based products from tilapia (*Oreochromis mossambicus*, Peters 1852). J. Food Process. Preserv. 34: 255-271.
- Nunes TP, Trindade MA, Ortega EMM, Castilho CJC (2006). Aceitação sensorial de reestruturados empanados elaborados com filé de peito de galinhas matrizes de corte e poedeiras comerciais. Cienc. Tecnol. Aliment. Campinas 26(4):841-846.
- Oliveira Filho PRC, Netto FM, Ramos KK, Trindade MA, Viegas EMM (2010). Elaboration of sausage using minced fish of Nile tilapia filleting waste. Bras. Arch. Biol. Technol. 53:1383-1391.
- Perlo F, Bonato P, Teira G, Fabre R, Kueider S (2006). Physicochemical and sensory properties of chicken nuggets with washed mechanically deboned chicken meat: Research note. Meat Sci. 72:785-788.
- SAS (2009). Institute Inc. SAS/STAT 9.2 user's guide. 2nd ed. Cary, N.C.: SAS Institute Inc., c2009. The Genmod procedure p 37.
- Sato ACK, Cunha RL (2005). Avaliação da cor, textura e transferência de massa durante o processamento de goiabas em calda. Bras. J. Food Technol. 8(2):149-156.
- Scherer R, Augusti PR, Bochi VC, Steffens C, Fries LLM, Daniel AP, Kubota EH, Radünz Neto J, Emanuelli T (2006). Chemical and microbiological quality of grass carp (*Ctenopharyngodon idella*) slaughtered by different methods. Food Chem. 99(1):136-142.
- Siena CE, Natali MRM, Braccini GL, Oliveira AC, Ribeiro RP, Vargas L (2010). Efeito do núcleo homeopático *Homeopatila 100*® na eficiência produtiva em alevinos revertidos de tilápia do Nilo (*Oreochromis niloticus*). Semina. Cien. Agrar. 31(4):985-994.
- Souza MLR (2002). Comparação de Seis Métodos de Filetagem, em Relação ao Rendimento de Filé e de Subprodutos do Processamento da Tilápia-do-Nilo (*Oreochromis niloticus*). Rev. Bras. Zootec. 31(3):1076-1084.
- Tarladgis BG, Pearson AM, Dugan LR (1964). Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods - II. Formation of the TBA-malonaldehyde complex without acid-heat treatment. J. Sci. Food Agric. 15:602-604.
- Taskaya L, Jaczynski J (2009). Flocculation-enhanced protein recovery from fish processing by-products by isoelectric solubilization/precipitation. LWT - Food Sci. Technol. 42:570-575.
- Tokur B, Polat A, Beklevik G, Özkütük S (2004). Changes in the quality of fishburger produced from tilapia (*Oreochromis niloticus*) during frozen storage (18°C). Eur. Food Res. Technol. 218:420-423.
- Yarnpakdee S, Benjakul S, Penjamras P, Kristinsson HG (2014). Chemical compositions and muddy flavour/odour of protein hydrolysate from Nile tilapia and broadhead catfish mince and protein isolate. Food Chem. 142:210-216.
- Zhang X, Feng J, Xu M, Hu J (2011). Modeling traceability information and functionality requirement in export-oriented tilapia chain. J. Sci. Food Agric. 91:1316-1325.



African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

- *Journal of Medicinal Plant Research*
- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals