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Original Article

Validation of a photostability indicating method for quantification of furanocoumarins from *Brosimum gaudichaudii* soft extract

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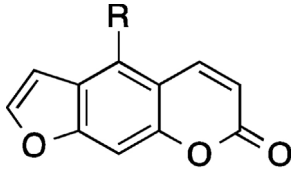
ABSTRACT

A validation study of a reverse-phase high-performance liquid chromatographic assay for the quantification of two furanocoumarins (psoralen and bergapten) in soft extract obtained from *Brosimum gaudichaudii* Trécul, Moraceae, roots was conducted. The developed method was sensitive, rapid, reproducible, easy and precise, and showed linearity ($r > 0.99$) in the range of 10–64 μg/ml for psoralen, and 9–56 μg/ml for bergapten. It also showed a good efficiency for the photodegradation analysis of psoralen and bergapten in the soft extract. The photostability results showed that the Higuchi model presented the best fitting to the obtained data. Both chemical markers showed stability over 2.6 days, suggesting potential applications of the extract in obtaining intermediate products from this plant material. Furanocoumarins take around 30 min to be activated by UV light, reaching the maximum biological potential. Thus, the results obtained to the Higuchi model, corresponding to 2.6 days of stability, shows feasibility with future applications of these chemical markers.

Introduction

Psoralen (**1**) and bergapten (**2**) are linear furanocoumarins mainly found in Apiaceae, Fabaceae, Moraceae, Rutaceae, and Thymelaeaceae families, present in Brazilian Cerrado. The specie *Brosimum gaudichaudii* Trécul belongs to the Moraceae family, and contains high amounts of psoralen and bergapten mainly in its roots. This plant is popularly known in Brazil as "mama-cadela" or "mamica-de-cadela", and it is widely used in folk medicine to treat vitiligo (Pozetti and Bernardi, 1971; Pozetti, 2005). The root bark tea is used in baths, and the juice of the triturated root is used as a component added to ointments and lotions (Varanda et al., 2002).



1 R=H
2 R=OCH_3

Vitiligo is a specific form of skin depigmentation that affects approximately 2% of the world's population. The word "vitiligo" has Latin origin, and comes from "vitium" and "vitelium" which means "default" and "white spots", respectively (Khovacs, 1998). The cause of vitiligo is still unknown; however, several theories have been proposed to explain the depigmentation process that occurs in this dermatological disease (Steiner et al., 2004; Ali et al., 2010). It is believed that this illness has an autoimmune origin that can be triggered after states of neurohumoral imbalance or oxidative stress (Rezaei et al., 2007).

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The first-line treatment against vitiligo is based on the use of topical corticosteroids, phototherapy using ultraviolet B (UVB) radiation (311–312 nm), and photochemotherapy. The photochemotherapy consists of topical application or ingestion of linear furanocoumarins and subsequent exposure to ultraviolet A (UVA) radiation (320–340 nm). A recent research has shown that the photochemotherapy with UVA radiation (PUVA) method has been the most effective and with lowest percentage of side effects compared to other forms of treatment for vitiligo and other skin diseases, such as psoriasis (skin rash) (Smith et al., 2011).

In silico studies showed that the furanocoumarins have photosensitizing action, due to the covalent bond with the melanocytes DNA (Rocha et al., 2009), and these substances could act by the absorption of photons and excitation of electrons. In the cellular environment, these electrons find substrates such as oxygen and nucleic acids, which act as its receptors. The photosensitizing agents, such as linear furanocoumarins returns to the ground state, giving rise to highly reactive species (Serrano-Pérez et al., 2009). These species assume an excited triplet state and can establish a covalent bond with a thymine base (T1), and this binding causes the molecule reposition between the nucleotide bases of the DNA, resulting in cellular replication blocking, whereby damaged cells are prevented of replicate, which leads to a regression of the disease. Another situation arises from the absorption of a second photon in the lactonic ring and subsequent interaction with another thymine base (T2), generating a cross-link in DNA (Silva et al., 2009).

The photostability of furanocoumarins is an essential test, once the treatment using the extract or its formulation depends on UV radiation (PUVA therapy) to result in biological effect of these secondary metabolites. Photostability studies also can be used to establish the shelf life of furanocoumarins products and the efficiency of their packaging (Brazil, 2014). The photostability testing of plant extracts is very uncommon, despite being very important, since secondary metabolites may be unstable and degraded by light (Tonnensen, 2001; Costa, 2001).

There are few analytical methods available in the literature for simultaneous quantification of psoralen (**1**) and bergapten (**2**). Thus, the method proposed in this work is rapid and efficient for quantification of these markers, and have been co-validated for the parameters of selectivity, linearity, precision and accuracy (Martins et al., 2015).

Therefore, the present work aimed to develop and validate a simple and specific HPLC method for quantification of psoralen and bergapten, and to evaluate its photostability profile in the *B. gaudichaudii* soft extract.

Material and methods

Reagents and chemicals

Psoralen (**1**, ≥99%) and bergapten (**2**, 99%) were purchased from Sigma Aldrich® Co (Steinheim,Germany). Acetonitrile and methanol of HPLC grade (J.T. Becker®, Center Valley, U.S.A.) and ultrapure water from a Mili-Q-System (Milipore®, Bedford, MA) was used.

Herbal material

Samples of *Brosimum gaudichaudii* Trécul, Moraceae, roots were collected from specimens located at Jussara-Goiás (13°43′08.04″S 50°31′44.98″W, 332 m). Once identified, a voucher specimen was prepared and deposited in the Universidade Federal de Goiás Herbarium under identification number – 45517. The roots were dried at room temperature (about 28 °C) and crushed in a knife mill (TE 625). The powdered material was stored sheltered from light and humidity.

Standardized soft extract from Brosimum gaudichaudii

According to the Brazilian Pharmacopeia 5th ed (2010), a soft extract it is a preparation with doughy appearance with a minimum of 70% of solid content (w/w). The extract was obtained by percolation method using 1 kg of the powdered material (particle size 710 ± 0.5 μm) and 1 l of a solvent mixture of ethanol:water 45:55 (v/v). The powdered material was maintained in percolation for about 14 days, and ten re-percolations, with controlled flow, were made with the same solvent mixture until the exhaustion of the markers. The obtained extract was first evaporated at 40 °C ± 2 using rotary evaporator Buchi® (SP, Brazil) until 10% of solid content. Then the extract was concentrated for ten consecutive days at room temperature in propeller concentrator until reaching solid content higher than 75% (w/w). This extract was characterized for pH, solid content, apparently density, and viscosity.

Instrumental and chromatographic conditions

Isocratic UV-HPLC method was performed using HPLC Waters® e2695 (Milford, Massachusetts, USA), comprising a quaternary pump, an online degasser, an auto sampler and a photodiode array detector model 2998. The treatment of data and control of HPLC equipment were performed using Empower® 2.0 software. An isocratic elution was performed on an Agilent Technologies® (USA) C8 (250 mm × 46 mm, 5 μm) column. The temperature was maintained at 30 °C. Absorbance was monitored at λ = 244 nm for psoralen and λ = 360 nm for bergapten (Martins et al., 2015). The mobile phase was a binary mixture of acetonitrile:water 45:55 (v/v). The flow rate was 0.6 ml/min. Samples were previously filtered (0.45 μm membranes) and degassed by sonication in an ultrasound bath before the analysis.

Sample preparation

Amounts of 100 mg of the soft extract from *B. gaudichaudii* were transferred into a 10 ml volumetric flask, dissolved in methanol and submitted to sonication for 30 min. The samples were filtered using a 0.45 μm cellulose acetate membranes (Merck® Brazil, São Paulo) prior to injection.

Co-validation of analytical method

The method was co-validated according to the Brazilian legislation (Anvisa, 2003) and the International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005). The analytical parameters of selectivity, linearity, range, precision (repeatability and intermediate precision), and accuracy were evaluated.

The system suitability was evaluated daily to verify its capacity of providing reproducible results. This evaluation was carried out with a set of parameters to ensure that the equipment used is capable of generating results of acceptable accuracy and precision. These parameters are the tailing factor (*T*), resolution (*Rs*), capacity factor (*K*) and number of theoretical plates (*N*), that were evaluated using the Empower 2.0 software (Shabir, 2003).

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms of a blank (methanol) solution, the samples solution and the standards. The UV spectral similarities of psoralen,

and bergapten peaks in the standard and in the samples were also compared at 220 and 360 nm (Anvisa, 2003).

Linearity

The linearity was determined using the standards calibration curves at five concentration levels of psoralen (10, 21.33, 32, 42.66 and 64 μg/ml) and bergapten (9, 18.93, 28.4, 37.86, 56 μg/ml) diluted in methanol and obtained from HPLC analysis. Each point was analyzed in triplicate and the resulting data was plotted as peak area (μAU.s) *versus* concentrations of the chemical markers, and studied by linear regression analysis. The linear equation for each marker was obtained by the least squares method and expressed by $y = ax + b$, where the angular coefficient (a) is the slope of the line in relation to the axes and the linear coefficient (b) is the intersection of the line with the y-axis. The linear range was determined using the Pearson correlation coefficient (r). The linear equations were used to quantify psoralen and bergapten in the *B. gaudichaudii* soft extract samples.

Limit of quantification (LOQ) and Limit of detection (LOD)

The LOQ and LOD for psoralen and bergapten were calculated by the standard deviation between the linear coefficients (SDb) and the slope of the calibration curves (S), according to the equations:

$$LOQ = SDb.10/S \quad LOD = SDb.3/S$$

Precision

Precision was evaluated at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision), and were expressed by the relative standard deviation (RSD), calculated according to equation:

$$RSD = Standarddeviation/Mediumconcentration \times 100$$

Repeatability

To evaluate the repeatability, six solutions containing 100 mg/ml of the soft extract diluted in methanol, were extracted in an ultrasonic bath for 30 min. The samples were filtered on a 0.45 μm membrane and injected in triplicate.

Intermediate precision

The intermediate precision was performed by different analysts, on different days, with the preparation of the samples and the analysis was performed as described for repeatability.

Accuracy

Accuracy was calculated by the standard addition method (Farmacopeia Brasileira, 2010), where a known amount of the psoralen and bergapten standards were added to a known amount of plant drug (*B. gaudichaudii* + standard of psoralen + standard of bergapten) in triplicate. Both the standard and the plant drug were individually analyzed and, after addition, the resulting solution was also analyzed. Nine determinations were performed at three different concentrations (low, medium and high, in triplicate), contemplating the method interval. The accuracy value was obtained by the recovery rate of the concentration of the standard added in the sample and the concentration of the standard before addition, in percentage.

Photostability studies

The soft extract of *B. gaudichaudii* was submitted to an accelerated photostability assay in a 424 CF photo-stability chamber (Nova Etica, Brazil) equipped with a near-UV fluorescent lamp (15 W) with a spectral distribution of 320 to 400 nm and several cool white fluorescent lamps (15 W). Samples were exposed to radiation at room temperature (25 ± 0.5 °C), which provided integrated UVA energies of 98.4, 196.8, 295.2 and 393.6 W/m^2 corresponding to exposure periods of 4, 8, 12, 24 and 48 h, respectively. The experiment was performed according to International Conference on Harmonization (ICH, 1996). A parallel experiment was run in the dark as a negative control for the effects of light on the degradation. All experiments were conducted in triplicate. After light exposure, samples were immediately analyzed by HPLC for the quantitative determination of psoralen and bergapten content.

Results and discussions

Extract characterization

The results for the extract characterization are shown in Table 1.

The physicochemical properties of the soft extract can provide useful information on the standardization of this material, since these parameters may affect the drying process, and guarantee its safety conditions.

Co-validation of HPLC-PDA method for psoralen and bergapten quantification

The method employed to quantify psoralen (**1**) and bergapten (**2**) was described by Martins et al. (2015). It can be observed that all the system suitability parameters were in accordance with the Food and Drug Administration (US-FDA, 2001) recommendations (Table 2). The analytical methods were designed to work in a range of 10–64 μg/ml of psoralen and 9–56 μg/ml of bergaptenin the samples, and five standard solutions were used to calculate the capacity factor (k%) and tailing factor (T) for psoralen and bergapten.

The selectivity of the method can be proved by Fig. 1, which shows the chromatographic profiles of the standards (Fig. 1a), the soft extract sample (Fig. 1b) and the blank solution (Fig. 1d). The peak purity can be seen by the UV spectra of psoralen and bergapten, showing no interfering substances.

The described method was linear for psoralen and bergapten in the range of 10–64 μg/ml and 9–56 μg/ml, respectively. The linearity plots for psoralen and bergapten presented correlation

Table 1
Results for the characterization of *Brosimum gaudichaudii* soft extract.

Test description	Results
pH	5.77 ± 0.2
Solids content	76.53 ± 0.5 (%, w/w)
Density	1.25 g/ml
Viscosity	299.9 mPa s

Table 2
System suitability data of the analytical HPLC method for quantification of bergapten and psoralen in *Brosimum gaudichaudii* soft extract.

Parameter	Results for psoralen	Results for bergapten	Literature recommendations[a]
Capacity factor (k')	4.29	5.58	>2
Resolution (Rs)	4.92	3.8	≥2
Tailing factor (T)	1.49	1.35	<2
Theoretical plates (N)	4.290	4.800	>2000

[a] Shabir (2003).

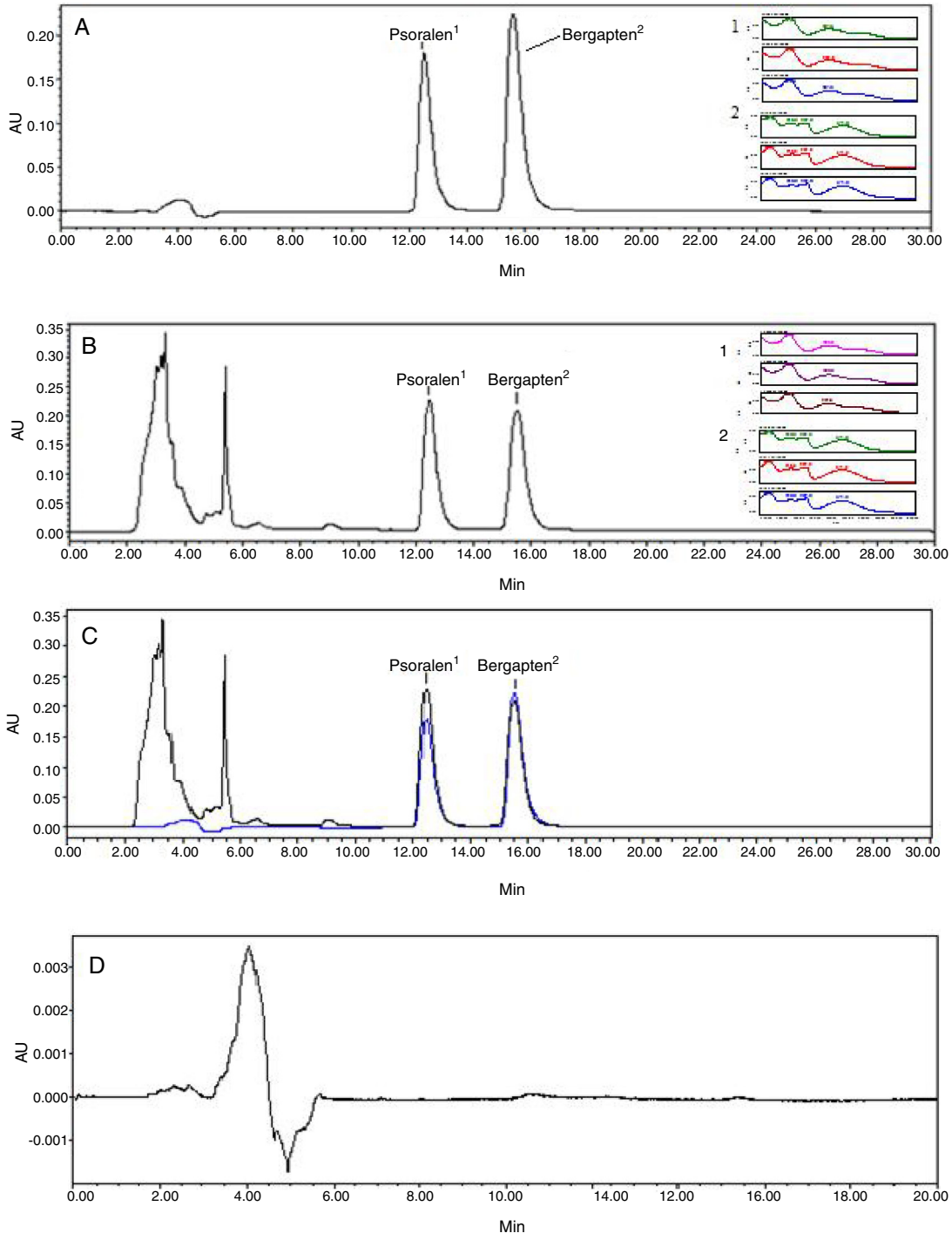

Fig. 1. HPLC-PDA chromatograms of (a) psoralen and bergapten standard; (b) *Brosimum gaudichaudii* soft extract; (c) overlap A and B; and (d) blank solution (methanol). Chromatographic conditions: column C8, 250 × 4.6 mm, 5 μm, 30 °C/ACN:H_2O 45:55 flow rate: 0.6 ml min^{-1}/injection vol: 20 μl.

coefficients of 0.9983 and 0.9992, respectively, proving the linearity of the method in the evaluated concentration range. The ANOVA test for psoralen and bergapten linearity is shown in Table 3, considering a confidence level of 95% ($p \leq 0.001$).

The results for repeatability and intermediate precision are presented in Table 3. The RSD value of repeatability was 4.23% for psoralen and 2.26% for bergapten, and the RSD value of intermediate precision was 4.15% for psoralen and 5.06% for bergapten. The precision values were less than 5%, in accordance with Anvisa recommendation, and less than 15% in accordance with the Guide for herbal medicines as the soft extract is a complex matrix, admitting a standard deviation of up to 15% (Anvisa, 2014).

The mean recovery rate calculated to evaluate the accuracy of the method were 105.11% for psoralen and 100.21% for bergapten. The values calculated for each concentration are presented in Table 3. According to Ribani et al. (2004) the acceptable recovery intervals depends on the complexity of the sample, and they admit values ranging from 50% to 120% for accuracy with a standard deviation of ±15%. The results and deviations for accuracy are presented in Table 3.

Table 3
Validation parameters values obtained from HPLC-PDA method for the quantification of psoralen and bergapten in soft extract from *Brosimum gaudichaudii* roots.

Parameter	Results for Psoralen	Results for Bergapten
Linearity		
Linearity range (μg/ml)	10–64	9–56
Intercept	−1.9704	0.9701
Standard error of estimated	1.6455	0.9182
Slope (a)	234,951	252,276
Intercept (b)	510,234	231,090
Standard error	0.9408	0.4881
$Y = ax + b$	$y = 234{,}951x + 510{,}234$	$y = 252{,}276x - 231{,}090$
R	0.9963	0.9984
Sensitivity		
Limit detection μg/ml	0.15499 μg/ml	1.13 μg/ml
Limit quantification μg/ml	1.1317	3.772631
Precision		
Repeatability μg/ml	54.84 ± 0.02	13.46 ± 0.01
Intermediate precision μg/ml	51.97 ± 0.01	12.42 ± 0.003
Accuracy		
Recovery 80%	102.49 ± 2.24	102.41 ± 2.18
Recovery 100%	106.74 ± 0.42	105.89 ± 0.58
Recovery 120%	106.06 ± 0.35	6.34 ± 0.38

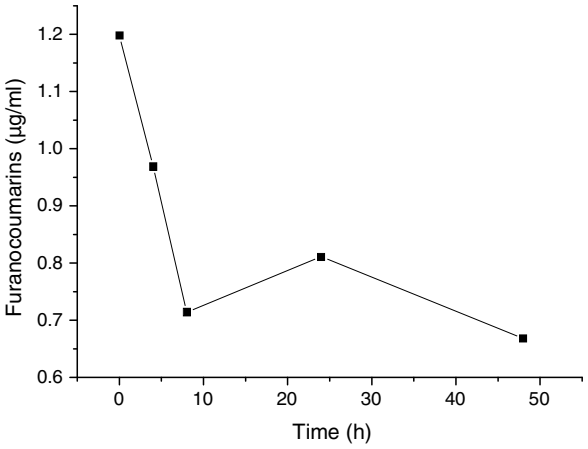

Fig. 2. Plot showing the decreasing in the contents (mean values) of total furanocoumarins (%) in the soft extract of *Brosimum gaudichaudii* under storage conditions.

Chunyan et al., 2009 and Zheng (2011) also described methods for quantification of furanocoumarins. However, the time of analysis is slower; they use gradient methods and more expensive reagents. The method developed in this work, besides being an isocratic, fast and simple method, it can also be used for quality control of photostability tests in future formulations.

Photostability and photodegradation tests

The photostability and photodegradation of the content of the total furanocoumarions (psoralen and bergapten) under storage conditions are presented in Fig. 2. The photodegradation rate of the two chemical markers was evaluated together, because they present synergic effect and the activity can be increased when the two compounds are together in higher levels (Wagner, 2011).

The Higuchi model presented the best fitting to the data obtained from the photostability assays (Fig. 2). Following this model, it was found that $k = 0.0689$ and $R^2 = 0.73$. The obtained equation was: $C = 1.102 - 0.06895\, T^{1/2}$.

According to this model, the predicted time to decrease 10% of the total furanocoumarins in the investigated extract corresponds to approximately 2.6 days. If it was considered only the furanocoumarins to the stability profile of the extract, the degradation time could be the predicted by the shelf-life ($t_{10\%}$). The potential use of the soft extract would be topical, so the photostability rate of the active compounds against vitiligo is suitable for this application.

The results obtained from the photostability test suggest the potential application of the soft extract in topical administration for the vitiligo patients, because in approximately 30 min in contact, the maximum concentration of the furanocoumarins would be reached (Danno et al., 1983). Thus, a time corresponding to 2.6 days of stability could keep the active compounds available for the affected regions in the skin (mainly in face, hands, and genital area) of the patients with vitiligo (Pan and Sarkany, 2011; Taieb et al., 2013)

The standardized soft extract of *B. gaudichaudii* could be used in the preparation of formulations for topical and oral use for the treatment of vitiligo and in the development of phytotherapic product. Therefore, the current Brazilian legislation (RE n. 88/04, RE n. 89/04, RE n. 90/04, RE n. 91/04) requires for the registration of herbal medicines, a test that assesses the efficacy and safety of medicines.

Safety studies of cellular toxicity, genotoxicity, and mutagenicity are critical for pre-clinical and to support in clinical studies (Anvisa, 2014). The profiles of the photostability and photodegradation are very useful to provide information for further studies aiming the development of intermediated and final products from herbal material, such as *B. gaudichaudii*. These findings highlight the need to future insight in the study of a feasibly formulation, which could be used by vitiligo's patients. Thus, the knowledge obtained from this work should be useful for further exploitation and application of the standardized soft extract.

Authors' contributions

MCMR (PhD student) contributed in all parts of work: running the laboratory work, analysis of the data and drafted the paper. PHGA (undergraduate student) contributed in running the laboratory work. NLOF (MSc Student) contributed in drafted the paper. RLA contributed in drafted the paper. LLBcontributed in analysis of the data and drafted the paper. OF and ECC supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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