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Glutathione and metallothionein as oxidative stress biomarkers in the medicinal plant *Chrysobalanus icaco* L. from different Brazilian regions

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Abstract

Abiotic stresses usually result in the overproduction of Reactive Oxygen Species (ROS), which cause oxidative damage. ROS regulation is vital to improve plant resistance to stress and is implemented by a defense system. Higher levels of glutathione (GSH) and Metallothionein (MT) indicate a defense mechanism in response to oxidative stress, participating in ROS detoxification. The abajerú (*Chrysobalanus icaco* L.) leaf extract is used in folk medicine to control glucose levels in diabetic individuals. This effect may be altered due to abiotic factors that differ according to the region where *C. icaco* occurs, reflected in oxidative stress biomarker levels. In this context, the aim of this study was to evaluate and compare GSH and MT levels in *C. icaco* specimens from different Brazilian regions. A statistically significant difference in GSH and MT levels was observed between Southeastern and Northeastern samples, indicating that regionalization may interfere in the biochemistry of *C. icaco* leaf extracts. Furthermore, a statistically significant correlation between GSH and MT levels was noted, which may be linked to metal exposure, which induces MT expression which then acts against ROS, and corroborates that GSH expression may also be related to metal contamination.

Abbreviations: GSH: Glutathione; MT: Metallothionein; ROS, Reactvie Oxygen Species; AB, Abaetetuba; MA: Maceió; AL, Marechal Deodoro; JBRJ: Botanical Garden Of Rio De Janeiro; PG: Praia Grande; PF: Praia Do Foguete; RMA: Restinga De Massambaba

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Introduction

Abiotic stresses usually result in the overproduction of Reactive Oxygen Species (ROS), which cause oxidative damages, leading to lipid peroxidation, protein oxidation, enzyme inhibition and DNA/RNA impairments. ROS regulation is vital to improve plant resistance to stress and is implemented by a defense system composed of a series of antioxidant enzymes and non-enzymatic antioxidants [1,2].

Among non-enzymatic antioxidants, Glutathione (GSH) is noteworthy. This is a tripeptide (γ-glutamyl-L-cysteinyl-glycine) containing a sulfhydryl group (-SH) present in cysteine, conferring a high reducing capacity, allowing for ROS detoxification, either directly or indirectly [1]. Higher GSH levels indicate a defense mechanism in response to oxidative stress, with the removal of the hydroxyl radical ($OH^{\cdot}$), whereas decreased GSH levels can occur by conjugation with the toxic substance with the aid of glutathione S-transferase. This reduces the cellular ability to destroy free radicals and reactive oxygen species, increasing the oxidative potential of the cell [2]. The various biochemical GSH properties contribute to plant growth and development, even under different stress conditions. GSH also plays a key role in leaf senescence by the modulation of hydrogen peroxide, observed in plants belonging to the *Arabidopsis* genus [3].

The relationship between cellular redox imbalances leading to oxidative stress and metal toxicity in plants has been assessed [1]. Generally, metals bind and interfere with target proteins, thereby altering their functions, leading to changes in cellular metabolism. Repair of the damaged macromolecules, strengthening of the antioxidant defense system and decreases in metal levels in the plasma compartments are some of the plant protection mechanisms, resulting in metal tolerance [4].

In order to maintain metal homeostasis and to control the production and the harmful effects of free radicals, plants and other organisms have developed numerous ways to mitigate effects, such as the synthesis of certain metalloproteins, such as Metallothioneins (MT) [5]. MTs are a family of low molecular weight proteins (6-7 kDa) with 30% of their amino acid chains composed of Cysteine (Cys), which make them capable of binding to metals [5]. MTs possess radical thiols or sulfhydryl groups (-SH) due to their high cysteine amounts, which facilitates interactions with bivalent metals such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, among others, preventing toxic and cell damage [6]. MTs play important roles in cellular processes, including detoxification of toxic metals, such as cadmium and mercury, regulation of essential metal, such as zinc and copper, regulation of cell growth and proliferation, DNA damage repair, and protection against ROS [7,8].

The leaf extract of the plant *Chrysobalanus icaco* L., a restinga species commonly known as abajerú, is used in folk medicine in Brazil due to its biological activities, such as decreasing blood sugar levels, and, thus, indicated for the treatment of diabetes, as well as diuretic and antioxidant properties [9]. These effects are associated to the presence of terpenoids (diterpenes and triterpenes), flavonoids, steroids and tannins [10]. In addition, data also indicate that phytochemical and mineral compounds in the abajerú fruit protect against DNA damage, associated to antioxidant properties [9]. Abajerú fruits are rich in anthocyanins, natural pigments displaying antioxidant capacity, and are responsible for many beneficial effects, such as protection against oxidative stress [9].

As different parts of this plant species are used for the treatment of chronic diseases, such as diabetes, there is a need to evaluate if environmental factors interfere in its oxidative stress biomarkers. In this context, the aim of the present study was to evaluate glutathione and metallothionein levels in *Chrysobalanus icaco* L. specimens from different regions of Brazil.

Material and methods

Plant sampling

Abajerú (*Chrysobalanus icaco* L.) leaves were collected from the Rio de Janeiro Botanical Garden (JBRJ – cultivated), and from natural habitats: Abaetetuba (AB; -1.373041, - 48.485532), located in Northern Brazil; Maceió (MA; -9.6360524, -35.6979559) and Marechal Deodoro (AL; -9.7823233, -35.852364), located in the Northeast; Praia Grande (PG; -22.9696606, -42.0302859), Praia do Foguete (PF; -22.908997, -42.034936) and Restinga de Massambaba (RMA; -22.9337727, -42.4267012), located in the Southeast, as displayed in the Figure 1. It was decided to divide samples from Praia Grande, to verify a possible variation according to leaves' age. They were classified according to size and leaf color, as young leaves (PG1), adult leaves (PG2) and branches (PG*), containing both types of leaves, as well as fruits (PG3). Samples collected from Praia Grande (PG) and Abaetetuba (AB) were deposited at the Rio de Janeiro Botanical Garden herbarium (accession numbers RB761269 and RB774051, respectively). All samples were collected during wet season, according to each region, and pressed in newspaper sheets to transport to the laboratory, where plants were then frozen at -80 °C and lyophilized.

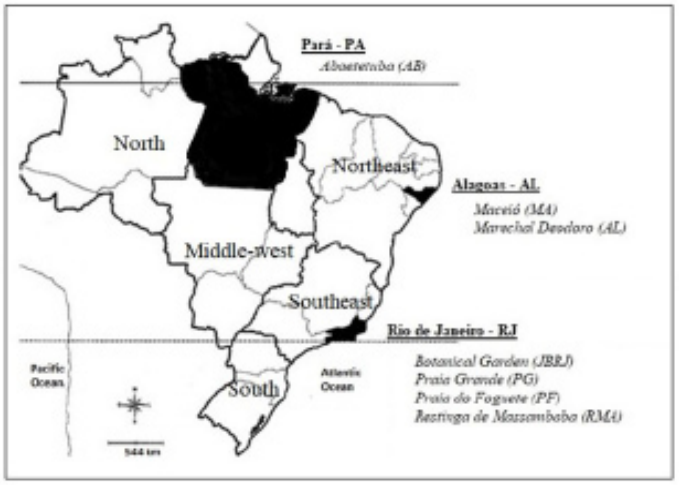

Figure 1: *Chrysobalanus icaco* sampling sites. Samples cultivated in botanical garden of Rio de Janeiro and from natural habitats – restinga and beaches.

Glutathione (GSH) extraction and quantification

Glutathione (GSH) extraction followed a previously described method [11]. Briefly, samples (50 mg of each lyophilized plant), in triplicate (N=27, leaves mixed from different branches), were homogenized in a glass dounce homogenizer, in a 0.1 M sodium phosphate buffer solution (pH 6.5), containing 0.25 mol L^{-1} sucrose and 1 mmol L^{-1} EDTA and centrifuged at 11,000 x g for 30 minutes at 4 °C. The supernatant were used for GSH quantification.

Quantification was performed according to the method described by Beutler [12], using Ellman's reaction [13]. A 10 mmol L^{-1} GSH stock solution was used to prepare the standard curve points at 0, 30, 60, 90, 120, 180, 240 and 300 µmol L^{-1}, prepared

in a 0.1 M sodium phosphate buffer solution (pH 7.0). Solutions with the curve aliquots were subsequently prepared (1:1, v/v) with 0.25 mmol L^{-1} DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) in 0.1 mol L^{-1} sodium phosphate buffer (pH 8.0) and incubated in the dark for 15 minutes. Samples were diluted (1:1, v/v) with ultrapure water (Milli-Q® Direct - Merck, Darmstadt, Germany), followed by preparation (1:1, v/v) with 0.25 mmol L^{-1} DTNB, followed by incubation in the dark for 15 minutes. Curve points and samples were then transferred to a 96-well plate and absorbances were determined at 412 nm on an Asys Expert Plus ELISA microplate reader (Biochrom, Cambridge, UK). All analytical curves presented correlation coefficients of over 0.98.

Metallothionein (MT) extraction and quantification

MT extraction was performed by the method described by Erk et al. [14]. Briefly, samples (50 mg of each lyophilized plant), in triplicate (N=27, leaves mixed from different branches), were homogenized in a glass dounce homogenizer with 0.02 M Tris-HCl buffer (pH 8.6) containing phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol as anti-oxidant. The extracts were then centrifuged at 20,000 x g for 60 minutes at 4 °C, the supernatants were incubated at 70 °C for 10 minutes and centrifuged again at 20,000 x g for 30 minutes at 4 °C. The purified supernatants were then used for MT quantification.

Quantification followed the method proposed by Viarengo et al. [15], using a GSH 10 mmol L^{-1} stock solution prepared in 0.1 mol L^{-1} sodium phosphate buffer (pH 7.0). The analytical curve was prepared at the following concentrations: 0, 60, 90, 120, 180, 240, 300, 500, 700, 1000 and 1500 µmol L^{-1}. A 4 mmol L^{-1} EDTA.Na_2 (disodium ethylenediaminetetraacetic acid) solution acidified with HCl 32% was then added to the samples and curve points. Then, a 0.43 mmol L^{-1} DTNB solution prepared in 0.2 mol L^{-1} sodium phosphate buffer (pH 8.0) containing 2 mol L^{-1} sodium chloride was added to the samples and curve, followed by incubation in the dark for 30 minutes. The curve points and samples were then transferred to a 96-well plate and absorbances were determined at 412 nm on an Asys Expert Plus ELISA microplate reader (Biochrom, Cambridge, UK). MT concentrations were estimated according to a ratio of 1 mol of MT corresponding to 20 moles of GSH. All analytical curves presented correlation coefficients of over 0.98.

Statistical analyses

Non-parametric tests, namely the Kruskal-Wallis (for multiple independent groups) and Mann-Whitney test (for two independent groups) were chosen to assess data medians. Possible outliers were assessed by Grubbs' test. Correlation coefficients were calculated to determine possible associations among concentration values. The results were considered significant when $p < 0.05$. All results were analyzed using the STATISTICA® 12 software package (StatSoft Inc, Tulsa OK, USA).

Results & discussion

Glutathione (GSH)

The samples were submitted to the extraction and quantification of metallothionein (MT), in triplicate. With the absorbance values obtained and the analytical curve (y=0.0145x, R^2=0.9978), the concentrations of GSH in µmol g^{-1} were calculated. GSH levels ranged from 1.10 to 8.09 µmol g^{-1}. Samples of *Chrysobalanus icaco* from Praia Grande (PG) showed higher levels than the others (Figure 2). Young leaves (PG2) samples presented the highest GSH concentrations. Another noteworthy sample is the Restinga de Massambaba (RMA), also located in the Lakes Region, East of Rio de Janeiro State. The *C. icaco* samples cultivated at JBRJ did not present high GSH levels as well as other samples in Southeast Brazil, from natural environments (PF, PG*, PG1, PG2 and RMA). Northeast samples (MA and AL), collected from natural habitats, showed lower levels of GSH than Southeast samples (PF, PG*, PG1, PG2 and RMA). GSH levels found in North sample (AB) were similar to those from Southeast.

Apparently, regionalization (land classification according to climatic factors) may interfere with the expression of GSH, perhaps by the wind regime, and is higher in the Lakes Region [16], where specimens from PF, PG and RMA were collected. This region presents a rainfall contrast with the rest of the State of Rio de Janeiro, with low rainfall [16]. Lake Region is characterized by the presence of different water bodies, including cold and nutrient-rich waters of the Central Atlantic South Atlantic (ACAS), which appear on the continental shelf during the spring-summer resurgence periods, when there are pre-dominant NE winds, resulting in differential climate and ecology [17]. The nutrient content of the soil may also be responsible for GSH expression, according to sampling site. In restingas, the soil is generally poor in nutrients [18], but due to the peculiar environment of the Lakes Region, this presents a different geomorphology.

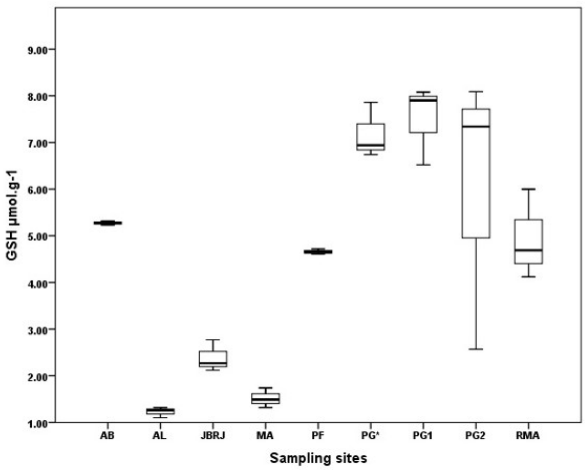

Figure 2: GSH levels variation in samples from different sites. AB: Abaetetuba; AL: Marechal Deodoro; JBRJ: Botanical Garden; MA: Maceió; PG: Praia Grande (PG*: branch; PG1: young leaves; PG2: adult leaves), PF: Praia do Foguete; RMA: Restinga de Massambaba. Values are expressed in median, mean, standard deviation, maximum and minimum. N = 3.

Higher GSH concentrations indicate a defense mechanism against oxidative stress or cellular protection against external agents. Abiotic stresses usually result in the overproduction of Reactive Oxygen Species (ROS), whose regulation is vital in improving plant stress resistance, implemented by an antioxidant defense system composed of a series of antioxidant enzymes and non-enzymatic antioxidants [19,1]. Glutathione (GSH) participates in ROS detoxification of ROS both directly and indirectly. GSH also plays a role in phytochelatin formation, which bind metals for safe transport and sequestration into the vacuole. Thus, it is clear that GSH plays a vital role in the detoxification of toxic metals/metalloids and xenobiotics [4,20]. In many plant species, tolerance to toxic metals is highly dependent on GSH. The higher GSH levels found in the samples described above may, thus, be related to metal contamination.

GSH regulates early signaling events, stress-related gene expression and defense mechanisms [21], increasing plant tol-

erance to different abiotic stresses, including salinity, dryness, high and low temperatures, and stress caused by toxic metals [1]. GSH, its redox pair (GSH/GSSG) and related enzymes (GPXs, GSTs, GR), for example, have been shown to lead to plant protection against oxidative stress induced by water deficit. Thus, the GSH system is considered as a useful marker in plant ecophysiological studies [2]. The high GSH levels observed in the samples described above may also be related to drought and salinity conditions, especially in restinga sites, such as Praia Grande (PG) and Restinga de Massambaba (RMA).

da Silva et al. [22] evaluated glutathione metabolism responses to arsenite (AsO^-) in *Salvinia molesta*, an aquatic fern displaying phytoremediation potential. AsO^- caused damage to the cell membrane of submerged leaves, indicating oxidative stress. Increases in GSH content and glutathione peroxidase, glutathione sulfotransferase and glutathione reductase enzymatic activities were also noted. The authors, thus, suggest that these findings suggest an important GSH role in the protection of *S. molesta* against toxic AsO^- effects.

No significant difference ($p>0.05$, Kruskal-Wallis) was observed for GSH levels among *C. icaco* samples, only when grouping them as Southeast (PF, PG and RMA) and Northeast sampling sites (MA and AL), $p<0.001$, according to the Mann-Whitney test. This seems to indicate that regionalization may influence protein expression and oxidative stress responses.

Metallothionein (MT)

The samples were submitted to the extraction and quantification of metallothionein (MT) in triplicate. With the absorbance values obtained and the analytical curve ($y=0.0123x$, $R^2= 0.9983$), the MT concentrations in µmol g^{-1} were calculated. The MT levels ranged from 0.105 to 1.65 µmol g^{-1}. Samples of *Chrysobalanus icaco* from Praia Grande (PG) showed high levels of MT (Figure 3), followed by the Restinga de Massambaba (RMA) sample, also located in the Região dos Lagos - RJ.

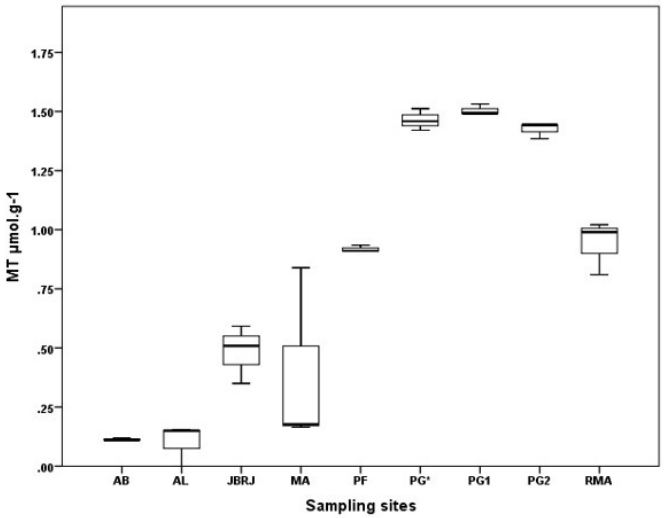

Figure 3: MT levels variation in samples from different sites. AB – Abaetetuba, MA – Maceió, AL: Marechal Deodoro; JBRJ: Botanical Garden; PG: Praia Grande (PG*: branch; PG1: young leaves; PG2: Adult leaves): PF: Praia do Foguete; RMA: Restinga de Massambaba. Values are expressed in median, mean, standard deviation, maximum and minimum. N = 3.

Apparently, MT protected cells from exposure to oxidants and electrophiles, through theirs reaction with sulfhydryl groups. The MT activation has several stimuli, such as metal ions, cytokines, growth factors and radiation [8]. Metallothionein determinations are routinely used to evaluate metal toxicity and bioaccumulation. However, MT can be induced in response to factors that promote oxidative stress, as these metalloproteins play an important role in cellular processes, including cell growth regulation, DNA and cell damage repairs and ROS elimination [23]. Experiments *in vitro* have shown that hydroxyl radical can be scavenged by the cysteinyl thiolate groups presents on MT. In fact, the rate constant of the reaction of hydroxyl radical with MT is about 340-fold higher than that with GSH [8]. Observing the MT results obtained herein, it is likely that Praia Grande samples are under oxidative stress, possibly due to a conjunction of factors.

MTs are capable of binding to a variety of metals by the formation of mercaptan bonds between the numerous Cys residues present in these metalloproteins and metals. The arrangement of those residues partially determines the metal-binding properties of these proteins [23,6].

The underlying premise for certain stoichiometries is that for each MT there is an "ideal" number of metal ions, which results in a well-structured protein, with all Cys thiolates bound to at least one metal ion. The expected bonds for plant metallothionein are Zn^{2+}, Cd^{2+} and Cu^{1+} ions, although others have been reported [6]. Yang et al. [5], for example, reported that *Ziziphus jujuba* accumulates Cd^{2+} in leaves and the toxicity of this metal in roots through MT binding, detoxifying this metal.

The physiological importance of the biding of Zn^{2+} by MT is been proved. In such cases, MTs function as zinc chaperones for the regulation of gene expression and activity of proteins, such as metalloproteins and metal-dependent transcription factors. It has been shown that under stress conditions, intracellular zinc release mediated by MT occurs when the levels of reactive oxygen species increase [8,24].

No significant difference ($p> 0.05$, Kruskal-Wallis) was observed between *C. icaco* samples considering all sampling sites. A significant difference is noted, however, between Southeast (PG and RMA) and Northeast (MA and AL) samples, $p <0.05$, according to the Mann-Whitney test. This seems to suggest that regionalization may lead to different metal sensitivities for this species, and may also interfere in MT levels for the same reasons explained for GSH. MT levels found in AB samples were not high as for GSH levels; it may indicate that only oxidative stress is interfering in GSH levels. For this reason, the North region was not considered to differ from other regions.

A statistically significant correlation between GSH and MT levels, $p <0.05$, considering all samples ($r = 0.765$) (Figure 4). This indicates that MT is also detoxifying ROS, and not only metals. According to Bryman and Cramer [25], statistical correlations are very weak when $0,00 <r <0,19$; weak when $0,20 <r <0,39$; moderate when $0,40 <r <0,69$; strong when $0,70 <r <0,89$; and very strong when $0,90 <r <1,00$. Considering only the *C. icaco* samples collected at Praia Grande (PG), the correlation between GSH and MT is even higher ($r = 0.826$). This means that these samples, in particular, are under definite oxidative stress, probably caused by metal exposure, inducing MT accumulation. In addition, GSH may also complex with certain metals, such as Cd, as reported previously in the literature, further aiding in metal detoxification [26].

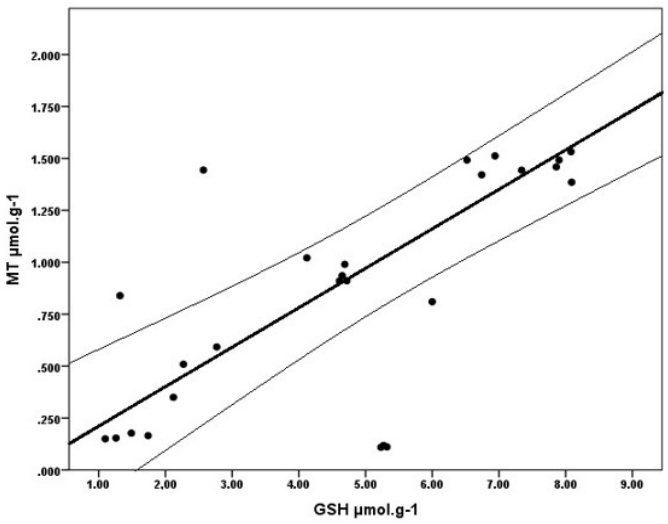

Figure 4: Correlation between GSH and MT levels in *C. icaco* samples.

Conclusions

GSH and MT levels found in *Chrysobalanus icaco* samples may be influenced by metal contamination, sampling site and regional characteristics. A statistically significant difference in GSH and MT levels was observed between Southeastern and Northeastern samples, indicating that regionalization may interfere in the biochemistry of *C. icaco* leaf extracts. GSH levels may also be linked to metal exposure due to correlation with MT levels, for Southeast and Northeast samples. Metal analyses in these samples will be carried out in future studies. Metal analyses may contribute to elucidate these relationships and are a further step in the assessment and characterization of medicinal plants. For the North sample, GSH was related only to oxidative stress.

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References

1. Hasanuzzaman M, Nahar K, Anee TI, Fujita M. Glutathione in plants: Biosynthesis and physiological role in environmental stress tolerance. Physiol Mol Biol Plants. 2017; 23: 249–268.
2. Labudda M, Azam FMS. Glutathione-dependent responses of plants to drought: A review. Acta Soc Bot Pol. 2014; 83: 3–12.
3. Ding S, Wang L, Yang Z, Lu Q, Wen X, Lu C. Decreased glutathione reductase2 leads to early leaf senescence in *Arabidopsis*. J Integr Plant Biol. 2016; 58: 29–47
4. Sharma SS, Dietz K. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J Exp Bot. 2016; 57: 711–726.
5. Yang M, Zhang F, Wang F, Dong Z, Cao Q, Chen M. Characterization of a type 1 metallothionein gene from the stresses-tolerant plant Ziziphus jujuba. Int J Mol Sci. 2015; 16: 16750–16762.
6. Leszczyszyn OI, Imam HT, Blindauer CA. Diversity and distribution of plant metallothioneins: A review of structure, properties and functions. Metallomics. 2013; 5: 1146–1169.
7. Domènech J, Mir G, Huguet G, Capdevila M, Molinas M, Atrian S. Plant metallothionein domains: functional insight into physiological metal binding and protein folding. Biochimie. 2016; 88: 583–593.
8. Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T, Stiborova M, Adam V, Kizek R. The Role of Metallothionein in Oxidative Stress. Int J Mol Sci. 2013; 14: 6044-6066.
9. Venancio VP, Almeida MR, Antunes LMG. Cocoplum (*Chrysobalanus icaco* L.) decreases doxorubicin-induced DNA damage and downregulates Gadd45a, Il-1β, and Tnf-α in vivo. Food Res Int. 2018; 105: 996–1002.
10. White PAS, Araújo JM, Cercato LM, Souza LA, Barbosa AP, Quintans-Junior LJ, Machado UF, Camargo EA, Brito LC, Santos MR. Chrysobalanus icaco L. leaves normalizes insulin sensitivity and blood glucose and inhibits weight gain in high-fat diet-induced obese mice. J Med Food. 2016; 19: 155–160.
11. Wilhelm-Filho D, Torres MA, Zaniboni-Filho E, Pedrosa RC. Effect of different oxygen tensions on weight gain, feed conversion, and antioxidant status in piapara, *Leporinus elongatus* (Valenciennes, 1847). Aquaculture. 2015; 244: 349–357.
12. Beutler E. The preparation of red cells for assay, in: (ed). Red Cell Metabolism: A Manual of Biochemical Methods. Grune and Straton Company, New York. 1975 ; 8-18.
13. Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7: 88–95.
14. Erk M, Ivanković D, Raspor B, Pavicić J. Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins. Talanta. 2002; 57: 1211–1218.
15. Viarengo A, Ponzano E, Dondero F, Fabbri R. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Mar Environ Res. 1997; 44: 69–84.
16. Coe HHG, de Carvalho CN, de Souza LOF, Soares A. Peculiaridades ecológicas da região de Cabo Frio, RJ. TAMOIOS, Julho/Dezembro-Ano IV. 2007; 1980- 4490.
17. da Silva ACC, Albuquerque ALS, Oliveira CM, Correa EC, Andrade MM, et al. Variabilidade da ressurgência de cabo frio ao longo do holoceno com base nas associações de foraminíferos planctônicos. XI Congresso da Associação Brasileira de Estudos do Quaternário. 2005.
18. Junior JL, Cuzzuol GRF. Caracterização de solos de duas formações de restinga e sua influência na constituição química foliar de Passiflora mucronata Lam. (Passifloraceae) e Canavalia rosea (Sw.) DC. (Fabaceae). Acta Bot Bras. 2009; 23: 239-246.
19. Cogo AJ, Siqueira AF, Ramos AC, Cruz ZMA, Silva AG. Utilização de enzimas do estresse oxidativo como biomarcadoras de impactos ambientais. Natureza on Line. 2009; 7: 37–42.
20. Sharma SS, Dietz K. The relationship between metal toxicity and cellular redox imbalance. Cell Press. 2008; 14: 43–50.
21. Dubreuil-Maurizi C, Poinssot B. Role of glutathione in plant signaling under biotic stress. Plant Signal Behav. 7: 210–212.
22. da Silva AA, de Oliveira JA, de Campos FV, Ribeiro C, Farnese, FS. Role of glutathione in tolerance to arsenite in *Salvinia molesta*, an aquatic fern. Acta Bot Bras. 2017; 31: 657–664.
23. Grennan A. Metallothioneins, a diverse protein family. Physiol Plant. 2011; 155:1750–1751.
24. Baltaci AK, Yuce K, Mogulkoc, R. Zinc Metabolism and Metallothioneins. Biol Trace Elem Res. 2018; 183: 22–31.

25. Bryman A, Cramer D. Data analysis with IBM SPSS 17, 18 & 19 - A guide for social scientists. Routledge, New York. 2011.

26. Mah V, Jalilehvand F. Cadmium(II) complex formation with glutathione. J Biol Inorg Chem. 2010; 15: 441-458.

27. Dutra RC, Campos MM, Santos ARS, Calixto JB. Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol Res. 2016; 112: 4–29.