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 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.

**Keywords:** Glutathione; Metallothionein; Oxidative stress; Hypoglycemic plant; Chrysobalanus icaco; Regionalization

**Abbreviations:** GSH: Glutathione; MT: Metallothionein; ROS, Reactive 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 sulphydryl 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•), 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 sulphydryl groups (-SH) due to their high cysteine amounts, which facilitates interactions with bivalent metals such as Hg²⁺, Cu²⁺, Cd²⁺, Zn²⁺, 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; Macié (MA; -9.6360524, -35.6979559) and Marechal Deodoro (AL; -9.7823233, -35.82364), located in the Northeast; Praia Grande (PG; -22.9696606, -42.0302859), Praia do Fogueu (PF; -22.908997, -42.034936) and Restinga de Massambamba (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.

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.Na₂ (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 (γ=0.0145x, R²=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 resurgences 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 restigas, 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 Fogo;e; RMA: Restinga de Massambaba. Values are expressed in median, mean, standard deviation, maximum and minimum. N = 3.](image)

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, R²= 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 – Abacetuba, 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.](image)

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 physiological importance of the binding of Zn⁺ 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].
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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