In Vitro Antioxidant Potential of *Baccharis trimera* and *Baccharis articulata* Infusions in Postmenopausal Women

Gabriela Tassotti Gelatti, Ana Caroline Tissiani, Mariana Spanamberg Mayer, Tamiris Felippin, Daiana Meggiolaro Gewehr, Jana Koefender, Evelise Moraes Berlezi, Diego Pascoal Golle, Roberta Cattaneo Horn*

Laboratory of Oxidative Stress and Medicinal Plants, Postgraduate Program in Integral Health Care, University of Cruz Alta (UNICRUZ), Rio Grande do Sul, Brazil

Abstract

Considering that postmenopausal women generally have high levels of oxidative markers and low levels of antioxidant defense markers due to the reduction of estrogen levels, and because of the vast popular use of several species of carja in the form of infusion, this study aimed to evaluate in vitro if infusions of B. trimera and B. articulata have antioxidant potential in erythrocytes of postmenopausal women and which are the most effective. The erythrocytes from 40 postmenopausal women were treated in vitro for 1 hour with infusions of B. trimera and B. articulata at the following concentrations: 4, 8, 16, 33, 66 g/L. The negative control consisted of erythrocytes from postmenopausal women without treatment with the plants. After treatment, the levels of thiobarbituric acid reactive substances (TBARS), carbonylated proteins (CP), and reduced glutathione (GSH) were measured. The infusions of B. trimera and B. articulata at concentrations of 33 ($p<0.001$) and 66 g/L ($p<0.001$) reduced the level of TBARS in comparison to the negative control, and the effect size (ES) for this reduction was small. The levels of GSH increased after treating with B. trimera infusion at a concentration of 66 ($p<0.001$) and with B. articulata at concentrations of 33 ($p<0.001$) and 66 g/L ($p<0.001$), when compared with the negative control, and the ES for this increase was average. The infusions of B. trimera and B. articulata show antioxidant potential in vitro, thus showing a similar effect with regards to the reduction of oxidative damage to lipids and increased endogenous antioxidant protection.

Keywords: Postmenopausal; Oxidative Stress; Carqueja; Antioxidant.

1. Introduction

Hypoestrogenism triggers significant neurological, psychogenic, and metabolic changes in postmenopausal women (NAMS 2013). Some of the symptoms that are characteristic of this period of life, such as heat waves, osteoporosis, and cardiovascular diseases (CD), are related to oxidative stress (Doshi and Agarwal 2013). Oxidative stress occurs whenever there is excess or insufficient removal of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can cause damage to macromolecules and cellular structures, such as lipid peroxidation, protein carbonylation, and damage to DNA. In order to neutralize

these harmful effects, the body is equipped with a variety of antioxidant molecules, in addition to those absorbed through the diet (Sies 2015).

Hormone replacement therapy (HRT) has a beneficial effect on oxidative stress by strengthening antioxidant defense mechanisms in postmenopausal women (Doshi and Agarwal 2013). However, owing to possible serious adverse effects, such as breast cancer (Lasserre and Fournier 2016) and venous thromboembolism (Lee et al. 2015), there has been a reduction in the long-term use of HRT. Moreover, this therapy is contraindicated for use in women with breast and endometrial cancers, abnormal uterine bleeding, liver disease, clot history, and CD (NAMS, 2013). There are also women who reject hormonal treatment due to cultural and socioeconomic factors. In such cases, non-medicated alternatives are available, including acupuncture, homeopathy, and use of medicinal plants (Borrelli and Edzard 2010).

The use of medicinal plants contributes significantly to addressing the need for primary health care. In Brazil, its use derives from the difficult access of the population to medical and pharmaceutical assistance, the cost of industrialized medicine, disappointment with the results obtained from conventional treatments, side effects, the damage caused by the abuse and/or incorrect use of medicine, and a consumer tendency to use products of natural origin (Rates 2001).

The genus *Baccharis* belongs to the Asteraceae family and comprises approximately 500 species distributed across Brazil, Argentina, Colombia, Chile, and Mexico (Alonso and Desmarchelier 2006). *Baccharis trimera* (Less.) DC. and *Baccharis articulata* (Lam.) Pers., popularly known as "carqueja" and widely used primarily for dyspepsia, are among the most pharmacologically studied species (Campos et al. 2016).

Some authors reported the antioxidant activity of n-butanolic (Oliveira et al. 2004), aqueous (Simões-Pires et al. 2005), hydro-alcoholic (Mendes et al. 2007; Pádua et al. 2014), chloroformic, ethyl acetate, absolute ethanol, ethanol 50% (Dias et al. 2009), and phenolic (De Oliveira et al. 2012) extracts (obtained by maceration) from the aerial parts of *B. trimera*, as well as the n-butanolic (De Oliveira et al. 2003) and ethyl acetate (Borgo et al. 2010) fractions of *B. articulata* extracts (also obtained by maceration). However, there is a lack of information regarding the activity of those species as infusions, which according to the Brazilian Herbal Pharmacopeia Form is the main preparation for oral administration (Brazil 2011).

Therefore, taking into consideration that postmenopausal women feature high levels of oxidative markers and low levels of antioxidant defense markers due to reduced estrogen levels and also the popularity of several species of "carqueja", the objective of this study was to analyze *in vitro* whether the infusions of *B. trimera* and *B. articulata* have antioxidant potential in erythrocytes of postmenopausal women and determine which of the species is the most effective.

2. Materials and Methods

2.1 Ethical aspects

This research is a subproject of the research projects: "Study of the Antioxidant Effect of Different Active Principles" developed in the University of Cruz Alta (UNICRUZ, approved by Research Ethics Committee (REC) of this university under Consolidated Opinion No. 273,167; and "Female Aging Study"

developed in the Regional University of Northwestern Rio Grande do Sul State (UNIJUÍ) approved by the REC of this university under Consolidated Opinion No. 864,988. All participants signed a free and clarified consent form.

2.2 Samples

Forty blood samples from postmenopausal women were included in this study. The negative control consisted of blood samples from 40 postmenopausal women without treatment with the plants infusion and the positive control consisted of 19 blood samples from women with regular menstrual cycle also without treatment with the plants infusion.

- Criteria for the inclusion of postmenopausal women: blood samples from women with an active record in primary health care in the urban area of the municipality of Ijuí/RS; participants in the Female Aging Study research project with at least 12 months of amenorrhea;
- Criteria for exclusion of postmenopausal women: use of HRT, antioxidant medicines, or vitamin supplements.
- Criteria for inclusion in the positive control: blood samples from women with regular menstrual cycles;
- Criteria for exclusion in the positive control: use of antioxidant medicines or vitamin supplements.

2.3 Preparation of *B. trimera* and *B. articulata* infusions

Aerial parts of *B. trimera* and *B. articulata* from the garden of UNICRUZ, Rio Grande do Sul, and the exsiccates of the botanical materials were stored in the herbarium of this university, with registration number 1107 and 1106, respectively. After collection, the aerial parts were dried in an oven at 30º C for four days. The preparation of the infusions involved the following: 150 mL of boiling water (100°C) was poured on 10 g of the dried aerial parts of both plants, in a glass bottle that remained closed and was held still for 10 minutes (Brazil 2011). The concentration of the infusions of the two species of carquejas used in this study was 66.67 g/L, whereas the other concentrations of the infusions were: 4.17, 8.34, 16.67 and 33.34 g/L which were prepared by dilution.

The phytochemical characterization was performed at the concentration of 66.67 g/L, after freeze-drying.

2.4 Preparation of hydroethanolic extracts of *B. trimera* and *B. articulata*

The hydroethanolic extracts of *B. trimera* and *B. articulata* were prepared in order to compare the concentrations of the phytochemicals present in the respective infusions. The vegetable matter was dried in an oven at 30ºC, grinded using a knife grinder and 66.67 g was weighed before extraction was performed. Maceration was performed with absolute ethanol and water (70:30). Both solutions were subjected to daily manual agitations for seven days followed by re-maceration. After this period of 14 days, the extracts were filtered, concentrated in a rotary evaporator, and freeze-dried, thus producing the hydroethanolic extracts (Simões et al. 2017).

2.5 Phytochemical characterization of the infusions and hydroethanolic extracts of *B. trimera* and *B. articulata*

The total phenolic compounds were determined according to the method described by Chandra and Meija (2004). The results are expressed in milligrams of gallic acid/g dry mass. The total content of flavonoids was determined according to the method described by Woisky and Salatino (1998). The results are expressed in milligrams quercetin/g dry mass. Condensed tannins were determined using the method described by Morrison et al. (1995). The results are expressed in milligrams catechin/g dry mass.

2.6 Blood collection and sample preparation

Blood was collected after 12 hours of fasting, with a vacutainer containing ethylenediamine tetra-acetic acid (EDTA) to obtain the erythrocytes. Samples were centrifuged at 3,000 rpm for 10 minutes. Erythrocytes were then separated and washed three times with an isotonic saline solution (0.9% NaCl) and centrifuged. After a final wash, erythrocytes were resuspended in saline solution (0.9% NaCl), and then diluted to a hematocrit of 10%, according to the technique described by Catagol, Ozden, and Alpertunga (2007). Each group (positive and negative controls) consisted of 1500µL of erythrocytes at 10% hematocrit.

2.7 *In vitro* treatments with *B. trimera* and *B. articulata* infusions

Positive control: erythrocytes of women with regular menstrual cycles treated with the vehicle (0.9% NaCl).

Postmenopausal group – *B. trimera*: erythrocytes of postmenopausal women treated with *B. trimera*:
Negative control: erythrocytes of postmenopausal women treated with the vehicle (0.9% NaCl);
4.17 g/L: erythrocytes of postmenopausal women treated with *B. trimera* infusion at 4.17 g/L;
8.34 g/L: erythrocytes of postmenopausal women treated with *B. trimera* infusion at 8.34 g/L;
16.67 g/L: erythrocytes of postmenopausal women treated with *B. trimera* infusion at 16.67 g/L;
33.34 g/L: erythrocytes of postmenopausal women treated with *B. trimera* infusion at 33.34 g/L;
66.67 g/L: erythrocytes of postmenopausal women treated with *B. trimera* infusion at 66.67 g/L.

Postmenopausal group – *B. articulata*: erythrocytes of postmenopausal women treated with *B. articulata*:
Negative control: erythrocytes of postmenopausal women treated with the vehicle (0.9% NaCl);
4.17 g/L: erythrocytes of postmenopausal women treated with *B. articulata* infusion at 4.17 g/L;
8.34 g/L: erythrocytes of postmenopausal women treated with *B. articulata* infusion at 8.34 g/L;
16.67 g/L: erythrocytes of postmenopausal women treated with *B. articulata* infusion at 16.67 g/L;
33.34 g/L: erythrocytes of postmenopausal women treated with *B. articulata* infusion at 33.34 g/L;
66.67 g/L: erythrocytes of postmenopausal women treated with *B. articulata* infusion at 66.67 g/L.
The treatments were carried out *in vitro* for an hour in a hot water bath at 37ºC and after this period, the samples were hemolyzed by vortex agitation for 10 seconds and centrifuged at 3600 rpm for 15 minutes,

resulting in the separation of the supernatant, which was stored in a freezer at -20ºC until laboratory analysis was performed.

2.8 Laboratory analyses

The levels of lipid peroxidation were determined using the formation of TBARS, according to the protocol described by Stock and Dormandy (1971). The results were expressed as ηmol MDA/g hemoglobin (Hb). Hb levels were determined according to the methodology described by the manufacturers of the Labtest® Kit.

The levels of carbonylated protein (CP) were determined according to the technique described by Levine (1990), adapted for use in erythrocytes. The results were expressed as ηmol of CP/mg of total protein (TP). The TP content was quantified in erythrocytes diluted with Hepes, according to the protocol for the Labtest® commercial kit.

The reduced glutathione (GSH) levels were measured according to the technique described by Ellman (1959), adapted for use in erythrocytes. The results were expressed as µmol GSH/mL of supernatant.

3. Statistical analysis

The Kolmogorov-Smirnov test was performed to test the distribution of the sample. Quantitative variables were described as mean, standard deviation or standard error, 95% confidence interval (CI), and relative frequency. T-test for independent samples was used to compare the phytochemicals components of hydroethanolic extracts and respective infusions, in addition to the comparison between infusions.

For the comparison between the positive control and negative control, the Mann-Whitney test was used. For the analysis of the five concentrations of each plant species, a One-Way Analysis of Variance test (ANOVA) was performed, followed by a Tukey test. In addition to the statistical significance test, the intra- and inter-group Effect Size (ES) was calculated from Cohen's d test. Values of $r<0.19$ are considered insignificant, values from 0.20 to 0.49 represent a small ES, values between 0.50 and 0.79 are considered average, and values greater ≥ 0.80 represent a high ES (Cohen 1988). A value of $p \leq 0.05$ was considered significant for all tests.

4. Results

The redox profiles of the study participants are described in Table 1. Postmenopausal women without infusion treatment (negative control) showed an increase of 212% ($p<0.001$) and 126% ($p<0.001$) in TBARS and CP levels, respectively, when compared to women with a regular menstrual cycle (positive control). The negative control showed a decrease of 41.7% ($p<0.001$) in GSH levels in comparison with the positive control.

Table 1: Oxidative stress markers in women with regular menstrual cycles (positive control) and in postmenopausal women without treatment with infusions (negative control).

Oxidative Stress Markers	Positive Control	Negative Control	*p**
	Mean ± SD CI 95%	Mean ± SD CI 95%	
TBARS (nmol/g Hb)	51.10 ± 36.14 33.68 – 68.52	159.44 ± 83.67 131.94 – 186.95	<0.000
CP (nmol/mg PT)	5.51 ± 1.5 4.78 – 6.22	12.49 ± 4.05 10.49 – 14.40	<0.000
GSH (µmol/mL)	0.36 ± 0.11 0.31 – 0.42	0.21 ± 0.09 0.18 – 0.24	<0.000

* Mann-Whitney

TBARS - Thiobarbituric Acid Reactive Substances, CP – Carbonylated Proteins, GSH – Reduced Glutathione.

Regarding phytochemical characterization, the infusions of *B. trimera* and *B. articulata* showed total phenolic compounds, total flavonoids, and condensed tannins in their compositions, although they contained smaller quantities than their respective hydroethanolic extracts (Table 2). The comparison of these components between the infusions showed a higher content of total phenolic compounds in *B. articulata* (p=0.023).

Table 2: Quantification of total phenolic compounds, total flavonoids, and condensed tannins present in hydroethanolic extracts (HE) and the infusions of *B. trimera* and *B. articulata*.

Phytochemicals (mg/g)	H.E. *B. trimera* Mean ± SD	Infusion *B. trimera* Mean ± SD	*p** *B. trimera*	H.E. *B. articulata* Mean ± SD	Infusion *B. articulata* Mean ± SD	*p** *B. articulata*
Total Phenolic Compounds	326.64 ± 6.8	103.25 ± 3.6	<0.000	339.3 ± 8.4	117.45 ± 5.9	<0.000
Total Flavonoids	21.51 ± 2.2	15.63 ± 1.8	0.023	23.73 ± 2.1	16.37 ± 0.7	0.004
Condensed Tannins	13.32 ± 1.3	9.10 ± 0.5	0.006	11.87 ± 0.9	8.23 ± 0.3	0.001

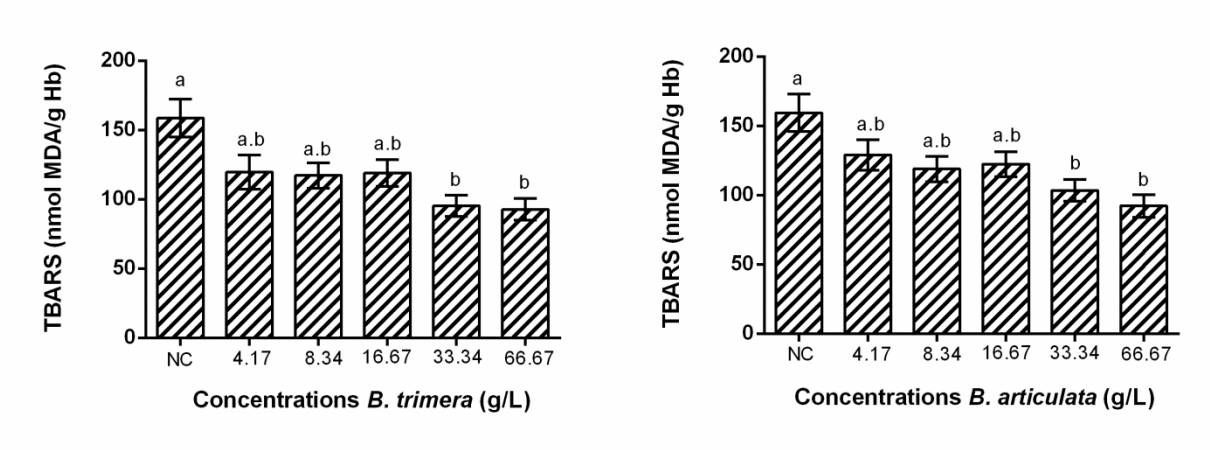
* T test for independent samples

H.E. – Hydroethanolic Extracts

The infusion of *B. trimera* at concentrations of 33.34 g/L and 66.67 g/L reduced the TBARS levels by 40.2% (p<0.001) and 41.9% (p<0.001), respectively, when compared to the negative control (Figure 1).

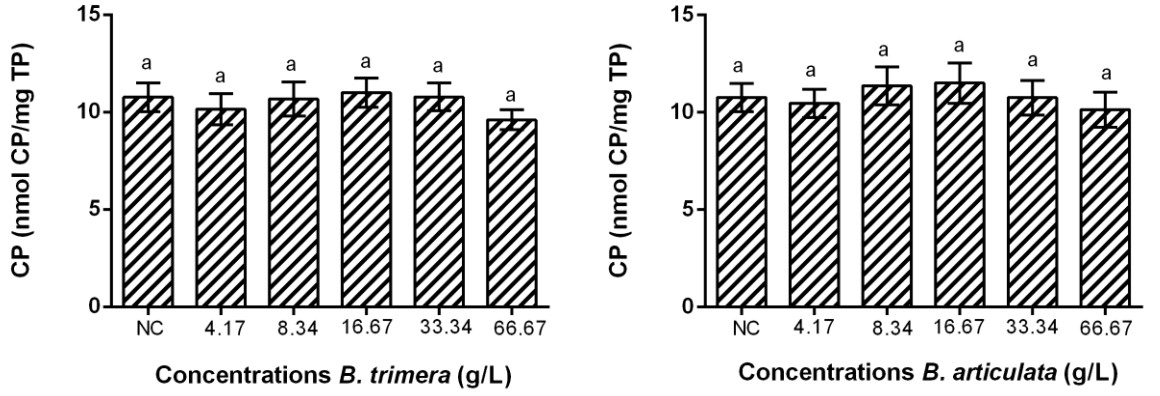
Moreover, the concentrations of 33.34 g/L (r=0.42, p<0.00) and 66.67 g/L (r=0.49, p<0.00) showed a small ES on lipid peroxidation reduction. The infusion of *B. articulata* at concentrations of 33.34 g/L and 66.67 g/L also decreased the TBARS levels by 35.1% (p<0.001) and 42.2% (p<0.001), respectively, in comparison with the negative control (Figure 1). The ES observed for this reduction was small for the concentrations of 33.34 g/L (r=0.31, p<0.00) and 66.67 g/L (r=0.39, p<0.00). After the comparison between species regarding lipid peroxidation decrease, we found no difference in ES, considering that both *B. trimera* and *B. articulata* both showed a small ES.

Figure 1: Levels of Thiobarbituric Acid Reactive Ssubstances (TBARS) (ηmol MDA/g Hb) in erythrocytes of postmenopausal women treated *in vitro* with different concentrations of *B. trimera* and *B. articulata* infusions.



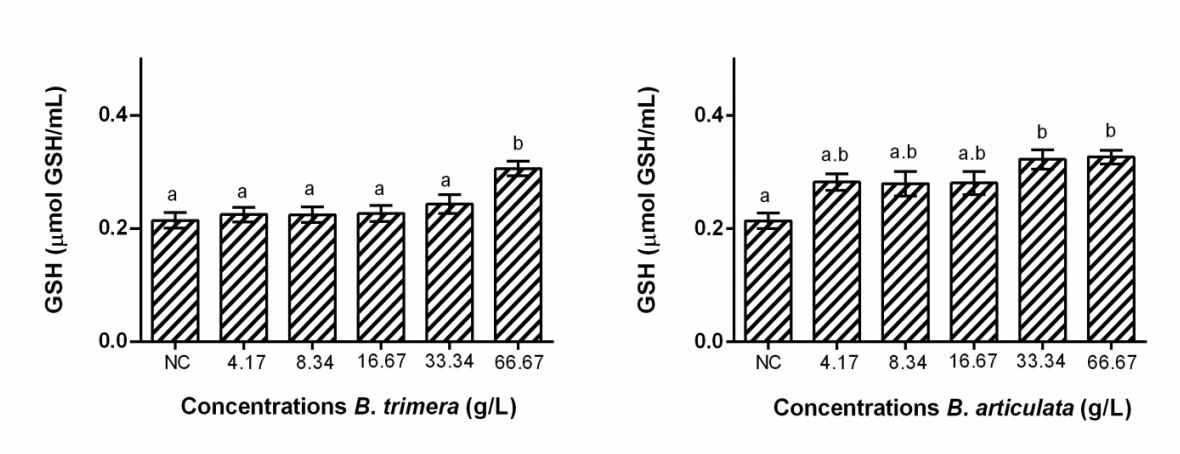
There was no decrease in CP levels after erythrocyte treatment with both infusions (Figure 2), and both the intra- and inter-group ES were insignificant.

Figure 2: Levels of Carbonylated Proteins (CP) (ηmol CP/mg TP) in erythrocytes of postmenopausal women treated *in vitro* with different concentrations of *B. trimera* and *B. articulata* infusions.



In Figure 3 we observed a 42.9% (p<0.001) increase in GSH levels after the treatment with *B. trimera* infusion at a concentration of 66.67 g/L when compared to the negative control.

Figure 3: Levels of Reduced Glutathione (GSH) (µmol GSH/mL) in erythrocytes of postmenopausal women treated *in vitro* with different concentrations of *B. trimera* and *B. articulata* infusions.



The ES observed for this concentration was average (r=0.51, p<0.00). The levels of this antioxidant increased 50.5% (p<0.001) and 52.8% (p<0.001) for the concentrations of 33.34 g/L and 66.67 g/L, respectively, for *B. articulata* when compared to the negative control.

An average ES value was calculated for the concentrations of 33.34 g/L (r=0.51, p<0.00), and 66.67 g/L (r=0.59, p<0.00). Comparison between species regarding the increase of GSH showed a difference in the ES for the concentration of 33.34 g/L (p<0.00), i.e., *B. articulata* showed a greater effect on the increase of GSH at the concentration of 33.34 g/L when compared to *B. trimera* at the same concentration.

5. Discussion

This study shows that the highest concentrations of *B. trimera* and *B. articulata* infusions have a potential antioxidant effect in the *in vitro* treatment of erythrocytes of postmenopausal women. It should be noted that this is a low-cost, easily accessed and disseminated in the population, which is widely used by aging women.

This may be justified, first, because both species of carquejas in the infusion preparation mode have antioxidant phytochemicals. The low amount of these phytochemicals in the infusions is due to the time required for extraction, the infusions were in contact with the solvent extractor for 10 minutes, while the incubation for the extracts was 14 days. It is important to study the method of preparation of infusions because of the greater consumption and adherence by the population compared to extracts obtained by encapsulated maceration. This can be explained by the home cultivation of the plants, in addition to a lower cost compared to phytotherapy (Gelatti et al. 2016).

Among the quantified components, total phenolic compounds were the largest group of phytochemicals. Their antioxidant potential is due to their structure, particularly because hydroxyls can transfer electrons

and thus support their relocation around the aromatic system. Flavonoids have antioxidant properties through the chelation of transition metals, and can act directly on free radicals through hydrogen transfers. In addition to this, they have the ability to inhibit cyclooxygenase, lipoxygenase, NAPDH-oxidase, xanthine oxidase, and phospholipase, and to stimulate antioxidant enzymes, such as catalase and superoxide dismutase. The condensed tannins sequester free radicals and have the ability to complex with macromolecules, such as proteins and polysaccharides (Simões et al. 2017).

An experimental model was used based on the effects on erythrocytes, taking into consideration that it presents an adequate cellular system for the study the effects of ROS due to the structural simplicity, accessibility and vulnerability of its components to oxidation, as well as the presence of an antioxidant system. In addition, erythrocytes are incapable of repairing damaged components since they are anucleate cells (Maurya et al. 2015).

With this experimental model, it was possible to observe oxidative damages in lipids and proteins and decreased levels of the main endogenous antioxidant in the erythrocytes of postmenopausal women without treatment (negative control) than in women with regular menstrual cycles (positive control). These data are supported by literature, which indicates that oxidative damage occurs in cells and their membranes under normal metabolic conditions. However, the rate of lipid and protein damage increases during post-menopause (Signorelli et al. 2006), partly because of reduced levels of estrogen as well as the ability of the antioxidant defense system reduces (Ogunro et al. 2014).

Oxidative damage in lipids is highly detrimental to the body since it can alter membrane permeability, fluidity, and integrity. These alterations will eventually lead to severe cytotoxicity, resulting in uncontrolled cellular growth or cell death, facilitating the development of a variety of pathological conditions such as diabetes, metabolic syndrome, dyslipidemia, neural and vascular degeneration, liver and renal toxicity, cancer, and ischemia, in addition to accelerated aging (Circu and Aw 2010; Umesh and Ramana 2013). In this study, the infusions of *B. trimera* and *B. articulata* reduced the formation of lipid peroxidation products *in vitro*, mainly malondialdehyde (MDA), at treatment concentrations of 33.34 g/L and 66.67 g/L, when compared to the negative control. This is probably due to the higher content of phenolic components in these concentrations. When comparing species, both concentrations of 33.34 g/L and 66.67 g/L showed a similar effect on the reduction of lipid peroxidation.

In addition to lipids, ROS also causes oxidative damage in proteins. One of the consequences of this is the appearance of carbonyl groups, which can be caused by direct oxidation of amino acid residues and ROS. This is achieved through the formation of a reactive intermediary formed during lipid peroxidation, or produced during the reduction of sugars or their products from oxidation with lysine residues of proteins, leading to the formation of advanced glycation end products (Butterfield and Dalle-Donne 2012). After the *in vitro* treatment with different infusion concentrations, both species showed no decrease in protein carbonylation.

This result may be explained by the fact that the majority of oxidatively modified proteins is not repaired, but removed by proteolytic degradation. Therefore, in order to maintain protein integrity, it is essential to have systems capable of recognizing and degrading damaged or incorrectly folded proteins efficiently, in order to prevent aggregation and cross-linking. One of these is the proteasomal system, particularly

proteasome 20S (Davies 2001). However, the accumulation of CP is common during aging, due to a progressive decline in the activity of proteasomes 20S and 26S (Höhn et al. 2013), which explains the increase in carbonylation levels seen in the negative control when compared to the positive control.

In order to neutralize the harmful effects caused by the excessive production of ROS, the human body is equipped with a variety of antioxidant molecules, synergistically working through different action mechanisms: preventing the formation of ROS (prevention systems), eliminating ROS (sweeping systems), or even favoring the repair and reconstitution of damaged biological structures (repair systems) (Koury et al. 2003). Among the non-enzymatic antioxidants, GSH, a tripeptide formed from glutamic acid, cysteine, and glycine should be mentioned. The antioxidant defense mechanism of GSH is determined by redox active thiol (-SH) of cysteine which oxidizes when GSH reduces target molecules (Pompella et al. 2003). During aging, there is a decrease in the outflow of cysteine in erythrocytes. As a result, the ratio of GSH:oxidized glutathione decreases with age (Kumar and Maurya 2013), which explains the decrease in GSH levels observed in the negative control.

After treatment with the infusions, both species showed an increase in GSH, with *B. trimera* being more effective at a concentration of 66.67 g/L, whereas *B. articulata* was more effective at 33.34 g/L and 66.67 g/L. This increase in GSH was probably not caused directly by the "carquejas," since synthesis of this antioxidant agent is regulated by cysteine (Pompella et al. 2003). These findings indicate that the reason for the increase in GSH in treatments with 33.34 g/L and 66.67 g/L may have occurred as the "carquejas" decreased the TBARS levels through the antioxidant action of their phytochemical components (Simões et al. 2017), which consequently caused the accumulation of GSH.

6. Conclusion

What are the concentrations of B. trimera and B. increase the antioxidant protection in the body. It should be noted that B. articulatement has a better effect on GSH at a concentration of 33.34 g / L compared to B. trimera at the same concentration. The same negative effect when administered is 66.67 g / L. As well as infusions of B. trimera and B. articulata as antioxidant potential in vitro, demonstrating a similar effect in the reduction of lipid peroxidation in relation to the increase of the main endogenous antioxidant. of the organism.

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Conflict of Interests

The authors declare no conflict of interest.

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