Redox alterations in pregnant women: Antioxidant effect of lemongrass
(Cymbopogon citratus (DC.) Stapf)

Alterações redox em gestantes: Efeito antioxidante do capim-limão
(Cymbopogon citratus (DC.) Stapf)

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Cristieli Carine Braun Rubin
Mestranda em Atenção Integral à Saúde (Unicruz/Unijuí, RS, Brasil). Bacharel em Farmácia pela Universidade de Cruz Alta (Unicruz, RS, Brasil). Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: cristi.braun@hotmail.com

Roberta Cattaneo Horn
Doutora e Mestre em Ciências Biológica-Bioquímica Toxicológica pela Universidade Federal de Santa Maria (UFSM, RS, Brasil). Bacharel em Farmácia/Bioquímica e Especialista em Toxicologia Aplicada pela Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS, Brasil). Professora Titular II da Universidade de Cruz Alta (Unicruz, RS, Brasil). Docente permanente do Programa de Mestrado em Atenção Integral à Saúde (Unicruz/Unijuí, RS, Brasil) e colaboradora do Mestrado Profissional em Desenvolvimento Rural da Unicruz. Líder do Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: rcattaneo@unicruz.edu.br

Gabriela Hirsch
Pós-doutoranda no Programa de Pós-graduação em Atenção Integral à Saúde (PPGAIS; Mestrado) pela Universidade Regional do Noroeste do Estado do Rio Grande do Sul. Doutorado em Ciências Biológicas - Bioquímica (Laboratório de Proliferação e Viabilidade Celular e Metabolismo de Lipídeos) pela Universidade Federal do Rio Grande do Sul. Mestrado em Ciência e Tecnologia dos Alimentos (Linha de Pesquisa em Qualidade dos Alimentos) pela Universidade Federal de Santa Maria. Graduação em Farmácia pela Universidade Federal de Santa Maria.
E-mail: ehgabis@yahoo.com.br

Gabriela Tassotti Gellatti
Mestranda em Atenção Integral à Saúde (Unicruz/Unijuí, RS, Brasil). Bacharel em Farmácia pela Universidade de Cruz Alta (Unicruz, RS, Brasil). Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: gabriela.gelatti@hotmail.com

Ana Carolina Tissiani
Mestranda em Atenção Integral à Saúde (Unicruz/Unijuí, RS, Brasil). Bacharel em Farmácia pela Universidade de Cruz Alta (Unicruz, RS, Brasil). Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: ana.c.t@hotmail.com
João Pedro Cunha Calçada
Obstetrícia e Ginecologia pelo Hospital Universitário da PUC, pelo Hospital São Lucas Rio Grande do Sul.
E-mail: jpccalcada@gmail.com

Tiago Heringer
Acadêmico do curso de Biomedicina (Unicruz, RS, Brasil). Bolsista de Iniciação Tecnológica pela Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), atuando no Laboratório de Plantas Medicinais e Estresse Oxidativo da UNICRUZ (LAMOX). Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: antoniother408@gmail.com

Aimê Cunha
Mestranda e bolsista CAPES do Programa de Mestrado em Atenção Integral a Saúde (Unicruz/Unijuí, RS, Brasil). Bacharel em Fisioterapia pela Universidade de Cruz Alta (Unicruz, RS, Brasil). Equitadora do Centro de Equoterapia Unicruz, licenciada pela Associação Nacional de Equoterapia (Ande, Brasil). Grupo Interdisciplinar de Pesquisa em Saúde da Unicruz (GIPS-Unicruz).
E-mail: aimecunha4@gmail.com
ABSTRACT

**Purpose:** This study aimed to verify the redox profile of different gestational phases of pregnant women, in addition to the antioxidant potential of the *Cymbopogon citratus* leaves infusion. 

**Methods:** The infusion characterization was performed by measuring total phenolics, flavonoids and condensed tannins levels. The oxidative damage markers to lipids and proteins in plasma and erythrocytes were evaluated by measuring the TBARS and carbonylated proteins (CP) levels and antioxidant activity by GSH levels. The antioxidant effects of the infusion (20 g/L) were evaluated by measuring the same markers after the pregnant women erythrocytes incubation with the same infusion. The phytochemicals with the highest concentration founded in infusion were flavonoids, followed by tannins and phenolics. 

**Results:** It was observed an increase in TBARS levels in the 2nd gestational phase in plasma, concomitant with a decrease in GSH levels in the 2nd and 3rd phases, and CP in the 1st and 4th gestational phases in relation to control. It was observed a decrease in all markers levels in erythrocytes in the 1st and 2nd phases. Analyzing the antioxidant potential of the infusion on erythrocytes, it was observed a decrease in TBARS concomitant with an increase in GSH levels, being little effective in reversing protein damage. 

**Conclusion:** Thus, it is suggested that the infusion could be used as adjuvant to vitamin supplements recommended during pregnancy.

**Keywords:** gestation, oxidative stress, bioactive compounds, medicinal plants.

RESUMO

**Objetivo:** Este estudo teve como objetivo verificar o perfil redox de diferentes fases gestacionais de mulheres, além do potencial antioxidante da infusão das folhas de *Cymbopogon citratus*. 

**Métodos:** A caracterização da infusão foi realizada medindo os níveis de fenólicos totais, flavonóides e taninos condensados. Os marcadores de dano oxidativo a lipídios e proteínas no plasma e eritrócitos foram avaliados medindo os níveis de TBARS e proteínas carboniladas (CP) e a atividade antioxidante pelos níveis de GSH. Os efeitos antioxidantes da infusão (20 g / L) foram avaliados medindo-se os mesmos marcadores após a incubação dos eritrócitos das gestantes com a mesma infusão. Os fitoquímicos com maior concentração encontrada na infusão foram os flavonóides, seguidos dos taninos e dos fenólicos. 

**Resultados:** Observou-se aumento dos níveis de TBARS na 2a fase gestacional no plasma, concomitante com diminuição dos níveis de GSH nas 2a e 3a fases, e PC na 1a e 4a fases gestacionais em relação ao controle. Observou-se diminuição dos níveis de todos os marcadores nos eritrócitos na 1a e 2a fases. Analisando o potencial antioxidante da infusão nos eritrócitos, observou-se diminuição do TBARS concomitante ao aumento dos níveis de GSH, sendo pouco eficaz em reverter o dano proteico. 

**Conclusão:** Dessa forma, sugere-se que a infusão possa ser utilizada como adjuvante de suplementos vitamínicos recomendados durante a gestação.

**Palavras-chave:** gestação, estresse oxidativo, compostos bioativos, plantas medicinais.
1 INTRODUCTION

Gestation is a period marked by anatomical and metabolic changes in woman's body. Thus, this phase, even under physiological conditions, requires many physical and psychological adaptations with the purpose of preparing the woman for the pregnancy \(^1\).

Normal gestation is considered as a state of controlled oxidative stress, where the balance between the pro-oxidants and the defense mechanisms of the organism is maintained \(^2\). Situations of imbalance may pre-dispose the appearance of pathologies such as pre-eclampsia, gestational diabetes mellitus, preterm delivery, maternal obesity and generate many maternal-fetal complications \(^3,4\).

Oxidation is a fundamental part of the aerobic pathway of metabolism, where free radicals are produced under physiological conditions, but in excess they can lead to the development of oxidative damage in numerous, which when unrepaired, can trigger mutations and cytotoxicity \(^5\). In contrast, antioxidants are a natural defense system of the body, and they act by preventing or controlling the formation of reactive species, protecting the cell from oxidative damage \(^6\).

The consumption of antioxidants from fruits, vegetables and medicinal plants, especially in the form of teas and supplements, has shown beneficial effects on oxidative stress, strengthening antioxidant defense mechanisms \(^7\). This beneficial activity is related to the presence of bioactive compounds such as flavonoids, polyphenols and tannins, which act to stabilize and eliminate free radicals, and also reducing oxidized compounds, among other actions \(^8,9\).

In this sense, \textit{Cymbopogon citratus} is a plant popularly known as lemongrass, widely used in alternative medicine in the form of infusion or decoction \(^10,11\), with many pharmacological activities as antibacterial, antidiarrheal, antifungal, diuretic and sedative action \(^12\); however, its antioxidant activity remains unclear.

Therefore, considering that pregnancy is a period of many changes and adaptations in the woman's body, including redox balance, due to the different stages of formation and growth of the baby \(^1\), the use of antioxidants to maintain the oxidative balance of the body seems to be important, resulting in a healthy gestation. In addition, the lemongrass not present risks to the gestation \(^13\). Thus, the objective of this study was to evaluate the oxidative profile of the different gestational phases of pregnant women, in addition to evaluating the in vitro antioxidant potential of \textit{Cymbopogon citratus} infusion in erythrocytes of pregnant women at different gestational phases.
2 METHODS

2.1 STUDY POPULATION AND ETHICAL CONSIDERATIONS

This was an experimental in vitro study carried out with biological material of pregnant women at different gestational stages. The collection of biological material (blood) was carried out between August 2016 and December 2017. A total of 17 pregnant and 13 non-pregnant women (Control Group) participated in the study, who met the Eligibility Criteria and agreed to participate in the study by signing the informed consent. Only fourteen pregnant participants completed the study. They had ages ranging from 18 to 46 years.

The present study is the result of a subproject of the research project titled "Study of the Antioxidant Effect of Different Active Principles" of University of Cruz Alta (UNICRUZ), and it was reviewed and approved by the Research Ethics Committee (CEP) of the University of Cruz Alta under Constituted Opinion number 273.167. All participants declared their voluntary participation by signing the Free and Informed Consent form and answered an informative questionnaire with questions regarding the Inclusion and Exclusion Criteria.

2.2 INCLUSION AND EXCLUSION CRITERIA

The inclusion criteria were: being female, be between 18 and 46 years old, have remained pregnant for more than 28 weeks or have a regular menstrual cycle (Control Group) and do not have chronic conditions. The exclusion criteria were: to have development some pathology during pregnancy, such as intrauterine growth restriction, gestational hypertension, gestational diabetes, spontaneous abortions or who were under the age of 18 or older than 47 years.

Preparation of the lemongrass (Cymbopogon citratus (DC.) Stapf) infusion

The leaves of the C. citratus plant (Poaceae) were harvested at the University of Cruz Alta (RS, 28°38'19" S, 53°36'23" O, 452 m) in May 2016. The plant material was identified by the Botanist Dr. Gabriela S. Preto and has its exsiccate deposited at the Herbarium of University of Cruz Alta (UNICRUZ) under Voucher number 1.120. The leaves were dried in a stove at 30° C for 4 days. For the preparation of the infusion, 150 ml of boiling water (100° C) was used and poured into 12 g of the dried plant into a glass becker which remained closed for 10 min. The preparation and concentration (20 g/L; leaves/water) of the infusion was according to the Brazilian Pharmacopoeia (13).
2.3 COLLECTION OF BIOLOGICAL MATERIAL

Blood collection was performed using vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). Subsequently, the samples were divided into 2 groups to obtain plasma and erythrocytes. Thereafter, they were centrifuged at 3000 rpm for 10 min. The plasma was stored in a freezer at -20º C until the analytical procedures. The erythrocytes were separated and washed 3 times with 0.9% isotonic saline solution and centrifuged. After the final wash, the erythrocytes were resuspended in saline solution, and then diluted to a hematocrit value of 10% (14).

2.4 EXPERIMENTAL DESIGN

Plasma and erythrocyte samples were divided in the following treatment groups: Control Group (CG, n=13) formed by non-pregnant women treated with saline solution 0.9%; Group 1 (G1, n=14) formed by 1st Gestational Phase (women with 0-12 weeks of gestation); Group 2 (G2, n=14) formed by 2nd Gestational Phase (women with 13 to 20 weeks of gestation); Group 3 (G3, n=14) formed 3rd Gestational Phase (women with 21 to 28 weeks of gestation); and Group 4 (G4, n=14) formed by 4th Gestational Phase (women with more than 28 weeks of gestation). The groups (G1, G2, G3 and G4) or Basal Group (BG) were treated with infusion of C. citratus at 20 g/L or with 0.9% saline solution, respectively. Finally, the redox profile was evaluated in all the groups studied.

3 REDOX PROFILE EVALUATION

3.1 DETERMINATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) LEVELS

TBARS levels in the plasma samples were determined by the colorimetric reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) 15. The results were expressed as $\eta$mol MDA/ml plasma. TBARS levels in erythrocytes followed the protocol 16. The results were expressed as $\eta$mol MDA/g of hemoglobin (Hb). Labtest® kits were used to determine the hemoglobin (Hb) levels.

3.2 DETERMINATION OF CARBONYLATED PROTEINS (CP) LEVELS

CP levels were determined in plasma and erythrocytes 17. The results were expressed as $\eta$mol/mg of total protein (TP). Labtest® kits were used for the determination of TP levels.
3.3 DETERMINATION OF REDUCED GLUTATHIONE (GSH) LEVELS

GSH levels were determined in plasma and erythrocytes. The results were expressed in μmol GSH/mL of plasma or supernatant (erythrocytes).

Phytochemical characterization of the C. citratus leaves infusion

Total polyphenols levels present in the lemongrass leaves infusion was performed by the Folin-Ciocalteau method. And the values were expressed as mg of gallic acid (GA) per ml of infusion (mg AG/mL). Flavonoid levels was evaluated according to the method, and expressed as mg quercetin (QT) per ml of infusion (mg QT/mL). Tannins levels were evaluated using the method described, and the results were expressed as mg of catechin (CT) per mL of infusion (mg CT/mL).

3.4 DETERMINATION OF ANTIOXIDANT ACTIVITY IN VITRO BY THE DPPH METHOD

The ability to eliminate the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was performed according to described, and the results were expressed as μmol of butylated hydroxytoluene (BHT) per ml of infusion (mg BHT/mL).

3.5 STATISTICAL ANALYSIS

The homogeneity of the samples was verified by Kolmogorov-Smirnov test. One-way ANOVA followed by Tukey's test was performed to verify the difference between the groups and t test was performed to verify the difference between the treated and untreated samples (basal) with the C. citratus infusion. All results were expressed as mean ± standard deviation (SD) and the difference was considered significant when p<0.05.

4 RESULTS

4.1 ANALYSIS REDOX STATUS IN PLASMA OF THE DIFFERENT GESTATIONAL PHASES

In order to analyze the lipidic peroxidation (LPO) in the different gestational periods, TBARS levels were evaluated in the plasma of the participants. It was possible to observe an increase of 28.96% (p<0.0001) in LPO levels in the 2nd gestational phase in relation to control group (CG; Figure 1A). In the other gestational phases, no differences were observed in LPO. Regarding protein damage, a decrease of CP levels in the 1st (55.41%) and in the 4th (59.62%) gestational phases in relation to CG (Figure 1B, p<0.0001) was observed. In the other phases, there were no significant differences.
In the evaluation of antioxidant defenses status in the different gestational phases, verified through the analysis of GSH levels in plasma, a decrease of 49.94% and 48.26% in the 2nd and 3rd gestational phases, respectively, can be observed in relation to CG (Figure 1C, p<0.0001). In the other groups, no significant changes were observed.

4.2 ANALYSIS OF REDOX STATUS OF ERYTHROCYTES NOT TREATED WITH C. CITRATUS (BASAL) IN THE DIFFERENT GESTATIONAL PHASES

In the evaluation of LPO levels in the erythrocytes of the pregnant participants before treatment with C. citratus, a decrease in TBARS levels was observed during the 1st and 2nd gestational phases (58.90% and 49.43%, respectively; p=0.0008) when compared to CG. However, in the 3rd and 4th gestational phases there were an increase of more than 60% in LPO levels (97.38% and 61.44%, respectively; p=0.0008) when compared to CG (Figure 2A). In the analysis of protein oxidation levels, it was also possible to observe a decrease in the levels of CP in the 1st and 2nd gestational phases (49.92% and 64.51%, respectively; p<0.0001) when compared to the CG, without differences between them. However, CP levels in the 3rd and 4th gestational phases (80.55% and 89.47%, respectively; p<0.0001) decreased in relation to the CG (Figure 2B), differently from observed in the LPO assessment.

Similar to the CP levels, in the evaluation of GSH levels in untreated erythrocytes, there was a decrease in the 1st and 2nd gestational phases (57.07% and 63.74%, respectively; p<0.0001) when compared to CG (86.46% and 81.98%, respectively; p<0.0001) (Figure 2C).

4.3 ANALYSIS OF C. CITRATUS INFUSION ANTIOXIDANT POTENTIAL IN ERYTHROCYTES AT DIFFERENT GESTATIONAL STAGES: IN VITRO TESTS

To analyze the antioxidant potential of C. citratus infusion, TBARS, CP and GSH levels were analyzed in erythrocytes treated with infusion that were compared with the levels of these same markers in untreated erythrocytes (Basal).

Analysis of LPO levels in erythrocytes treated with C. citratus infusion demonstrated that there was a decrease in lipid damage at almost all gestational phases (2nd: p=0.0067; 3rd: p<0.0001; 4th: p<0.0001), except the 1st (Figure 3A). However, the infusion was not able to reverse the protein damage in all gestational phases, except in the 3rd phase (p<0.0001), where there was a decrease of 35.30% in CP levels (Figure 3B).

Moreover, it can be observed that the infusion caused an increase in GSH levels in the 3rd (p=0.0004) and 4th (p<0.0001) gestational phase, as observed in Figure 3C.
4.4 PHYTOCHEMICAL CHARACTERIZATION OF THE INFUSION OF C. CITRATUS LEAVES

According to Table 1, it was verified that the class of phytochemicals found in the highest concentration in the C. citratus infusion were the flavonoids, followed by tannins and total phenolic compounds.

4.5 DETERMINATION OF C. CITRATUS INFUSION ANTIOXIDANT ACTIVITY IN VITRO BY DPPH METHOD

The ability to remove the synthetic free radical DPPH exerted by the C. citratus infusion at 20 g/L (leaves/mL infusion) was 10.67 ± 0.0003 μmol BHT/mL.

4.6 USE OF VITAMIN COMPLEXES DURING THE GESTATIONAL PERIOD

All pregnant participants used folic acid (5 mg/day) until the 12th week of gestation. There were variations in the vitamin complexes used: 35.71% used ferrous sulfate, 14.28% used Ogestan®, and 7.14% used other supplements (Materna®, Femme®, Ultrafer® and Damater®). Only 21.42% of the participants did not use vitamins after the 12th week of gestation.

Normal gestation is considered as a state of controlled oxidative stress, marked by an increase in oxidizing agents, but it remains stable due to the action of antioxidants. However, in situations of extreme oxidative imbalance there may be a pre-disposition to the appearance of pathologies characteristic of gestation.

It is known that the increase of oxidizing agents negatively affects the lipid metabolism of pregnant women, and that the imbalance can pre-dispose the appearance of pathologies such as pre-eclampsia, gestational diabetes mellitus, preterm birth, maternal obesity. In addition to this, gestation is a process characterized by metabolic changes that evolve during the different gestational phases, and it is also known that there are variations between the levels of oxidizing agents and antioxidant defenses along of gestation, favoring the appearance of damages in certain gestational phases.

In this study, we found an increase of 28.96% in MDA levels in plasma of pregnant women during the 2nd gestational phase, concomitant with a decrease in GSH in the 2nd and 3rd gestational phases, which suggests that there must be an attempt to reverse oxidative damage at this phases. Corroborating our results, Barneo-Caragol et al., studied pregnant and non-pregnant women and also found an increase in LPO during pregnancy. In addition, several studies have shown a tendency to elevate lipid peroxidation levels during normal gestation. In an in vitro study where the plasma
of diabetic and healthy pregnant women were compared, an increase in TBARS levels was observed in diabetic pregnant women, demonstrating that oxidative damage may actually be related to the appearance of diseases in pregnancy. In other study which evaluated the redox balance in the non-pregnant and pregnant women plasma, it was observed that LPO levels increased during normal pregnancy progression.

The antioxidants levels are also extremely important to maintain redox balance during pregnancy and GSH plays a key role in this process, preventing oxidative damage to important cell components such as lipids. The main product of lipid peroxidation are lipid hydroperoxides, which if in reduced levels can inhibit oxidative damage. In this case, the main agents involved in this reducing are Glutathione Peroxidase (GPx) and GSH. This fact would explain the GSH levels reduction founded in the pregnant women plasma during the 2nd and 3rd phases, accompanied by the increase of TBARS levels.

Like lipids, proteins are also strong targets for oxidizing agents and damage to these molecules can have deleterious consequences for the protein structure and function. We observed a decrease in CP levels in the plasma during the 1st and 4th gestational phases when compared to the CG, indicating that there should be protection against damage to or elimination of these molecules. According to Höhn et al., cell proteins in young people, even if moderately oxidized, are not repaired, but recognized and degraded by the system known as proteasome and the decrease in CP levels may be being caused by their degradation and consequent elimination. Furthermore, GSH can be found in both the reduced and oxidized form, and their levels are maintained by de novo synthesis through enzymes that can also be inhibited by compounds produced in response to oxidative stress. In this case, excess of oxidative stress could be causing the antioxidant defenses consumption (GSH), which would no longer be sufficient to protect all molecules against damage, explaining the increase TBARS levels in plasma of pregnant women.

In the 3rd gestational phase, we observed that CP and TBARS levels in pregnant women plasma return to control levels (non-pregnant), but GSH levels remain lower. This fact may be because the protein is being used effectively in protecting the damage to those biomolecules. We also observed a decrease in the TBARS levels, as well as of CP and GSH levels, in the 1st and 2nd gestational phases in relation to the CG, in the erythrocytes of the pregnant participants. Again, decreased GSH levels are probably related to their consumption, in order to prevent oxidative damage to biomolecules.

However, TBARS levels increased in the 3rd and 4th gestational phases, suggesting an increase in oxidative stress. This result was also accompanied by an even more significant decrease.
in CP and GSH levels than the 1st and 2nd gestational phases. This finding may be associated with a higher susceptibility of lipid molecules to oxidative stress, together with the depletion of GSH, which demonstrates a probable increase of oxidative stress in these phases. GSH can be considered a cofactor in the hydrogen peroxide and lipid hydroperoxides reduction together with GPx.

Peuchant et al. evaluated the redox balance in diabetic and healthy pregnant women and they demonstrated that there was no difference between LPO levels among the groups, but there was a significantly higher LPO levels when compared to antioxidant defense systems. Evidence has shown the body seems to respond more rapidly to increased oxidative damage in early pregnancy, causing an increase in antioxidant defenses. In addition, although there is a balance between lipid peroxidation and antioxidant defenses in normal pregnancy, there is a tendency to oxidative stress at the end of pregnancy.

Oxidative stress can have serious deleterious effects during pregnancy and can be prevented by appropriate and effective antioxidant therapy. The use of medicinal plants to compensate a possible oxidative damage during the gestational period has been increasingly used. In this context, lemongrass stands out because it is widely used in this sense due to the presence of phytochemical compounds that guarantee its antioxidant activity.

Thus, our evaluation about the presence of phytochemicals in C. citratus infusion revealed that the bioactive compounds class found in the highest concentration were flavonoids, followed by tannins and phenolic compounds. Reinforcing our results, Soares et al. carried out a study to evaluate the therapeutic benefits of C. citratus and the main class of antioxidant compounds found in the characterization of the infusion were flavonoids, followed by tannins and phenolic compounds, which generally represented smaller groups.

Reinforcing the presence of the antioxidant compounds in the lemongrass infusion, it showed good ability to eliminate free radicals. A study with C. citratus leaves that also detected the presence of flavonoids, tannins and phenolic compounds in the plant verified that the tannins and flavonoids fractions were the main responsible for the antioxidant activity found in the lemongrass infusion. In the same study, infusion was the preparation that presented the best capacity to eliminate free radicals highlighting the importance of the studies regarding their potential use as an antioxidant in pregnancy.

The evaluation of the antioxidant activity of the infusion in erythrocytes further evidenced the potential of the lemongrass. It was verified that the C. citratus infusion was able to reduce LPO in almost all gestational phases, except in the 1st, thus confirming its antioxidant activity.
Lemongrass extract was able to prevent LPO and increase cell resistance to membrane damage, increasing cell survival in other studies.\textsuperscript{36,37}

In our study, lemongrass infusion was not able to reverse protein damage, except in the 3\textsuperscript{rd} gestational phase, where a small reduction in CP levels can be observed, supporting the previous hypothesis that the lipids are molecules more susceptible to the action of free radicals and also, of the antioxidants, like the GSH.\textsuperscript{29,38} On the other hand, the protein carbonylation process is irreversible, because there are proteolytic systems that eliminate damaged proteins and recycle amino acids with the purpose of performing protein synthesis.

Concomitantly with the reduction of oxidative damage observed in erythrocytes of pregnant women treated with infusion, GSH levels increased significantly in the 3\textsuperscript{rd} and 4\textsuperscript{th} gestational phase, indicating that this is probably one of the mechanisms involved in the antioxidant activity of the lemongrass infusion. This increase is satisfactory and shows that the infusion evaluated in this study has the capacity to revert oxidative damage during the gestational period, being able to protect the pregnant and the fetus.\textsuperscript{1}

During the several months of gestation, the woman needs a greater demand of nutrients for the fetus development. Thus, the use of vitamin supplements at different gestational stages aims to significantly reduce the risks of abnormalities to the baby.\textsuperscript{38} In this study, all participants took folic acid until 12\textsuperscript{th} week of gestation. This vitamin complex is very important at this stage, since its use is directly related to a significant reduction in the risk of fetal neural tube closure defects and other congenital anomalies.\textsuperscript{39} Still, the use of other vitamin complexes during pregnancy has the function of nourishing and complementing the pregnant woman's diet, and this nutritional contribution assists in the healthy development of the baby. According to Haider and Bhutta,\textsuperscript{40} micronutrient deficiencies are exacerbated in pregnancy due to the increased nutritional demands.

Thus, the association of micronutrients with iron and folic acid for the supplementation of pregnant women assists in the healthy development of pregnancy and the fetus, and helps prepare the pregnant woman for childbirth.

Therefore, the results found in this study were promising, since obstetricians indicate different gestational supplements during this period, and the use of medicinal plants with antioxidant action may be an option of choice for this function. Thus, as the lemongrass is a plant that is easy to obtain, low cost and does not present harmful effects to the pregnant woman,\textsuperscript{13} it can be considered a good option of antioxidant therapy in this concentration during the gestational period.
ETHICAL APPROVAL
The present study is the result of a subproject of the research project titled "Study of the Antioxidant Effect of Different Active Principles" of University of Cruz Alta (UNICRUZ), and it was reviewed and approved by the Research Ethics Committee (CEP) of the University of Cruz Alta under Constituted Opinion number 273.167.

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CONFLICT OF INTEREST
All authors have no conflicts of interest according the ICMJE.

INFORMED CONSENT
All authors report consent according the ICMJE
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