

## *In vitro* Assessment of the Antioxidant Effect of *Cunila microcephala* Benth Infusion (Poejo) on Erythrocytes from Individuals with HIV/AIDS

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### Abstract

The aim of the present study is to investigate *in vitro* antioxidant activity of *Cunila microcephala* Benth infusion (Poejo) in erythrocytes from individuals with HIV/AIDS. Erythrocytes were used from seventeen patients of both sexes, with HIV/AIDS, receiving antiretroviral therapy and the control group consisted of erythrocytes of HIV/AIDS free individuals belonging to both sexes. The erythrocytes were treated *in vitro* for an hour with infusions of Poejo at the following concentrations: 1, 5, 10, 25 and 50 g/L. After treatment, the levels of thiobarbituric acid reactive substances (TBARS), carbonylated proteins (CPs), and reduced glutathione (GSH) were measured. A reduction in lipoperoxidation and protein carbonylation was observed after treatment with poejo tea at the concentrations of 5, 10, 25 and 50g/L. In addition, a reduction of GSH at such concentrations was observed. Poejo infusion appears to perform an antioxidant activity in lipid oxidation and in the protein carbonylation found in individuals with HIV/AIDS.

**Keywords:** human immunodeficiency virus, oxidative stress, plants

### 1. Introduction

The human immunodeficiency virus (HIV) causes a chronic and progressive immune dysfunction in the body known as Acquired Immunodeficiency Syndrome (AIDS). This virus has infected more than 60 million people and caused approximately 30 million deaths worldwide due to the cytolytic infection of the CD4-like T lymphocytes (Cooper et al., 2013). Brazil has recorded 41,100 annual AIDS cases, on average, in the last five years, according to a recent report in the AIDS and STD Epidemiological Bulletin of the Ministry of Health (Brasil, 2016). Szwarcwald et al. (2008) and Martins et al. (2014) highlighted approximately 2% increase in the AIDS detection rate, mainly in Northeastern Brazil where it increased by 62.6%; however, the mortality rate increased by 33.3% in the same region, at the same period.

Although the HIV/AIDS infection outbreak emerged in the 1980s as a public health and social well-being issue and reached concerning proportions at that time, it remains worrisome (Carvalho et al., 2007). Despite the advances in HIV/AIDS diagnosis and treatment, reality presents itself as a challenge to health professionals due to the high incidence of the disease and to the impacts of it on carriers' lives (Springer et al., 2012).

Accordingly, reactive oxygen species (ROS) play an important role in T-cell proliferation and in immune defense under normal conditions. However, the excessive ROS production in HIV patients may boost the disease's progression, since it has the potential to cause significant damage to deoxyribonucleic acid (DNA), proteins and lipids by impairing the immune system's response to HIV and/or by inducing apoptosis. In addition, seropositive patients have low antioxidant compound levels, fact that neutralizes ROS and inhibits kappa nuclear

factor (NF- $\kappa$ B), which is a transcription factor accelerating the viral replication process (Stephenson et al., 2006; Van Gaalen & Wahl, 2009; Amador-Licona et al., 2016).

Multivitamin supplements and antioxidant minerals have appeared as a therapeutic adjunct used in recent decades to reduce the oxidative stress caused by HIV/AIDS. However, there is no evidence of the positive effects of the antioxidant therapy, since sometimes mortality increases due to the intake of these synthetic compounds (Bjelakovic et al., 2012, Poljsak, et al., 2013). Therefore, it is worth investigating role of possible natural compounds capable of minimizing oxidative cellular damages.

The grasshopper species *Cunila microcephala Benth* belongs to family Lamiaceae and is popularly known as poejinho or poejo. The species is native to Southern Brazil, Argentina and Uruguay. Its antitussive, stimulant and antispasmodic actions stand out among its main popular applications. Phytochemical studies applied to *Cunila microcephala Benth* have identified the presence of phenolic compounds such as tannins, coumarins and flavonoids—originating from the intermediate metabolic pathway of the shikimic acid—in the production of secondary plant metabolites. The presence of an aromatic ring and of hydroxyls is common in these compounds; this structure is responsible for the antioxidant potential of *Cunila microcephala Benth* (Angelo et al., 2007; Morais et al., 2009). Therefore, the aim of the present study is to investigate *in vitro* antioxidant activity of *Cunila microcephala Benth* infusion in erythrocytes from individuals with HIV/AIDS.

## 2. Method

### 2.1 Ethical Aspects

The present research was approved by the Research Ethics Committee of Cruz Alta University (UNICRUZ), protocol n. 15510413.3.0000.5322. The participants were consulted about the viability of their participation and signed the Free Consent Form.

### 2.2 Preparation of *Cunila microcephala Benth* Infusion

The aerial parts of the poejo of the UNICRUZ garden, Rio Grande do Sul, were used, free of flowers at the time of collection. After the collection, the aerial parts were dried in an oven at 30 °C for four days. The preparation of the infusions was obtained by pouring 150 ml of boiling water (100 °C) into 10 g of the dry aerial parts of both plants in a glass bottle which remained closed for 10 minutes (Brasil, 2011). The other concentration stocks used in this study (1, 5, 10 and 25 g/L) were prepared from infusion of poejo at 50 g/L.

### 2.3 Characterizing the Phytochemical Components

#### 2.3.1 Total Polyphenols

The sample was diluted to a concentration of 0.150 mg/mL and in this solution was added 20% sodium carbonate and Folin-Ciocalteu 2N reagent. The solution was incubated for 10 minutes and the absorbance was measured, in triplicate, in spectrophotometer at 730 nm wavelength, according to Chandra and Mejia (2004). The total content of polyphenols was expressed through the equivalent Gallic acid mg per ml of infusion, according to the gallic acid calibration curve

#### 2.3.2 Flavonoids

The total content of flavonoids was determined according to the method described by Woisky and Salatino (1998). The sample was diluted to a concentration of 1 mg/L and in this solution was added aluminum chloride and methanol. The absorbance was read at 420 nm. The tests were done in triplicate and the quercetin standard curve was used to calculate the dose. The content of flavonoids was expressed through milligrams of quercetin per infusion mL.

#### 2.3.3 Condensate Tannins

Condensate tannins were determined through the method described by Morrison et al. (1995), with some modifications. The sample was diluted in 25 mg/mL ethanol; subsequently, vanillin (1 g vanillin diluted in 100 mL ethanol) and hydrochloric acid 8% in methanol solutions were added to the sample. The absorbance was set at 500 nm. The analysis was conducted in triplicate and the total content of tannins was expressed through the equivalent milligrams of catechin per infusion mL.

### 2.4 Sample Collection and Processing

Biological material from seventeen patients belonging to both sexes from random, with HIV/AIDS, receiving antiretroviral therapy, was collected through venipuncture by using vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). The biological material was homogenized and packed in thermal boxes after collection. Subsequently, the samples were centrifuged at 3000 rpm for 10 minutes and the plasma was

removed. The erythrocytes were washed three times in isotonic saline solution and diluted with saline solution 0.9% until reaching 5% hematocrit (Horn et al., 2015). After dilution, the supernatant was discarded and the erythrocytes of each participant were divided in six treatment groups:

Basal group (poejo infusion free): Erythrocytes from HIV/AIDS individuals treated with saline solution;

Group 1: Erythrocytes from HIV/AIDS individuals treated with poejo infusion at 1 g/L;

Group 5: Erythrocytes from HIV/AIDS individuals treated with poejo infusion at 5 g/L;

Group 10: Erythrocytes from HIV/AIDS individuals treated with poejo infusion at 10 g/L;

Group 25: Erythrocytes from HIV/AIDS individuals treated with poejo infusion at 25 g/L;

Group 50: Erythrocytes from HIV/AIDS individuals treated with poejo infusion at 50 g/L.

Erythrocytes from seventeen HIV/AIDS-free individuals belonging to both sexes were used to compose the control group. The erythrocytes were treated *in vitro* for one hour in water bath at 37 °C. Subsequently, the samples were vortexed for erythrocyte hemolysis and centrifuged at 3600 rpm for 15 minutes for supernatant removal, which allowed performing the analytical determinations.

### 2.5 Analytical Determinations

#### 2.5.1 Determining the Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS were measured according to the protocol described by Stocks and Dormandy (1971). The supernatant was added with the reaction mixture of trichloroacetic acid (TCA) to 28% (v/v) thiobarbituric acid (TBA) at 0.1 mol/L, at 95 °C. The readings were taken at 532 nm. The results were expressed as  $\eta\text{mol MDA/g Hb}$ . The hemoglobin level analysis was conducted according to the recommendations in the Labtest<sup>®</sup> commercial kit.

#### 2.5.2 Determining the Levels of Carbonyl Proteins (CPs)

The CPs level analyses were performed through the technique described by Levine (1990), adapted to erythrocytes, wherein the trichloroacetic acid (TCA) is used at 10% (v/v), 2N hydrochloric acid; 2,4-Dinitrofenilhidrazina (DNPH) in 10mM and sodium dodecyl sulfate (SDS) 3% (m/v) in the reaction mixture. The readings were carried out in visible spectrophotometer, at 370 nm. The results were expressed in  $\eta\text{mol carbonyl/mg protein}$ .

#### 2.5.3 Determining the Levels of Reduced Glutathione (GSH)

The GSH levels were determined through the technique described by Ellman (1959), adapted for erythrocytes, which uses a potassium phosphate buffer (TFK) at 1 M, pH 7.4, and acid 5,5'-ditiobis-(2-nitrobenzoic) (DTBN). The procedure was performed in ice bath and the readings were realized in visible spectrophotometer, at 412 nm. The results were expressed as  $\mu\text{mol GSH/Hb}$ . The hemoglobin level analysis was conducted according to the recommendations in the Labtest<sup>®</sup> commercial kit.

### 2.6 Statistical Analysis

The phytochemical extract and the lemongrass infusion featuring was carried out in triplicate and the results were expressed through mean  $\pm$  standard deviation. The results were subjected to Student t-test for parametric data collection, by taking the significantly different rates ( $p < 0.001$ ) into consideration.

The analytical determinations of all samples were performed in triplicate and the results were expressed through  $\pm$  SEM (standard error of the mean). The distribution of variables was tested through the Kolmogorov-Smirnov test, Shapiro-Wilk normality test and D'Agostino-Person omnibus normality test. Data concerning all studied groups, for the same parameters, were subjected to the one-way variance analysis (ANOVA), followed by Tukey-Kramer test. Significantly different rates, at  $p < 0.05$ , were considered.

## 3. Results

There was an increase in the levels of TBARS and CPs in HIV-positive individuals when compared to the control group (Figures 1A and 2A). After *in vitro* treatment with poejo infusion there was a reduction of 20.75%, 67.13%, 46.55% and 66.57% in TBARS levels at concentrations of 5, 10, 25 and 50 g/L respectively when compared to basal (Figure 1B). Regarding CPs levels, there was a reduction of 56.33%, 47.37%, 52.71% and 34.36% in the concentrations of 5, 10, 25 and 50 g/L respectively when compared to basal (Figure 2 B).

Regarding GSH, a reduction of this antioxidant was observed in HIV-positive individuals when compared to the control group (Figure 3A). There was a reduction of this biomarker after the *in vitro* treatment with the infusion

of poejo in the concentrations of 1, 5, 10, 25 and 50 g/L. This reduction was 97.38%, 78.81%, 88.24%, 61.94% and 71.82%, respectively, when compared to basal (Figure 3B).

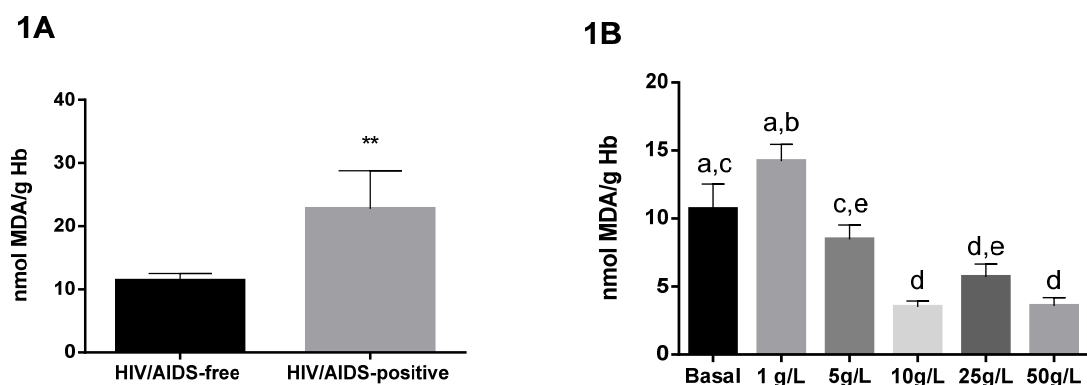


Figure 1. TBARS levels ( $\eta$ mol MDA/g Hb) in erythrocytes from HIV/AIDS-free patients and from HIV/AIDS-positive patients (1A). TBARS levels ( $\eta$ mol of MDA/g Hb) in erythrocytes from HIV/AIDS-positive patients after the exposure to *Cunila microcephala Benth* infusion at concentrations 1, 5, 10, 25 and 50 g/L (1B).

Values expressed as Mean  $\pm$  Standard error. Different letters represent significantly different statistics, at  $p < 0.05$

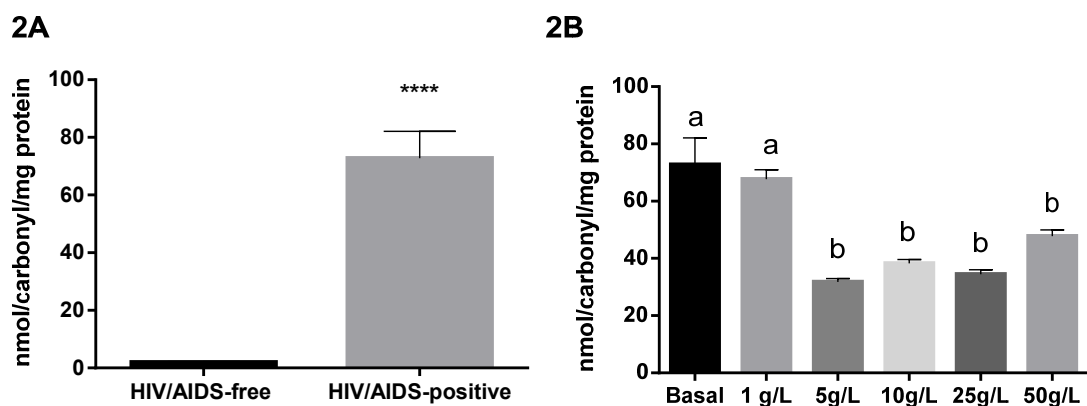


Figure 2. CPs levels ( $\eta$ mol carbonyl/mg protein) in erythrocytes from HIV/AIDS-free and HIV/AIDS-positive patients (2A). CPs levels ( $\eta$ mol carbonyl/mg protein) in erythrocytes from HIV/AIDS-positive patients after the exposure to *Cunila microcephala Benth* infusion at concentrations 1, 5, 10, 25 and 50 g/L (2B). Values expressed as Mean  $\pm$  Standard error. Different letters represent significantly different statistics at  $p < 0.05$

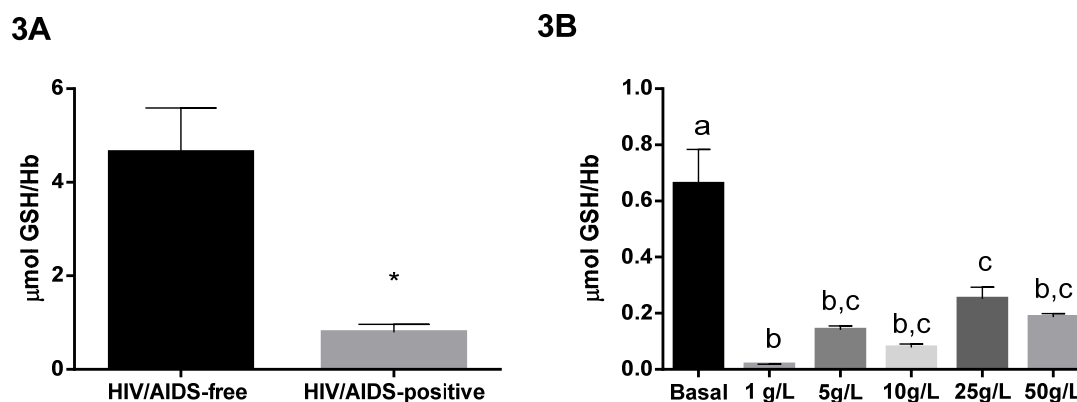


Figure 3. GSH levels ( $\mu\text{mol GSH/Hb}$ ) in erythrocytes from HIV/AIDS-free and HIV/AIDS-positive patients (3A). GSH levels ( $\mu\text{mol GSH/Hb}$ ) in human erythrocytes from HIV/AIDS-positive patients after the exposure to *Cunila microcephala Benth* infusion at concentrations 1, 5, 10, 25 and 50 g/L (3B). Values expressed as Mean  $\pm$  Standard error. Different letters represent significantly different statistics, at  $p < 0.05$

#### 4. Discussion

Human blood is an excellent source of oxidative stress markers *in vivo*, since it transports and redistributes antioxidants and endobiotics modified by the action of reactive species. Erythrocytes, particularly, are anucleated cells, lacking the ability to repair damages caused by oxidation. In addition, the plasma membrane in this cell is one of the main damage targets, fact that turns it into a widely used experimental oxidative stress model (Begum & Terao, 2002; Schmitz, 2008). Therefore, oxidative damages, as well as the possible antioxidant activity observed in the erythrocytes, slightly demonstrate individuals' cellular behaviors.

Accordingly, it is possible quantifying the lipid peroxidation levels, which are set by the attack of species reactive to the lipids found in the cell membrane. Ogunro et al. (2005) have shown that patients with type I HIV have malondialdehyde (MDA) levels—which is a byproduct of lipid peroxidation—much higher than those in the control group. It corroborates the increased levels of TBARS found in the present study (Figure 1A), in which the lipoperoxidation levels in HIV/AIDS patients are comparable to the same level of these biomarkers in healthy patients. Figure 1B, on the other hand, shows the TBARS levels after the erythrocytes' treatment with poejo infusion; thus revealing that the lipoperoxidation was lower after the treatment using poejo tea in the highest tested concentrations, 5, 10, 25 and 50 g/L.

Felisbino et al. (2014) identified the presence of antioxidant phytochemicals, such as phenolic compounds, tannins, flavonoids and coumarins—which could justify the lipid oxidation reduction (these substances are potent ER sequestrers)—in *Cunila microcephala Benth* leaves. The features of the herein used mother infusion (50g/L) also corroborate the findings of the aforementioned author, by considering that polyphenols ( $70.96 \pm 1.02$  mg gallic acid/mL infusion), flavonoids ( $34.50 \pm 0.14$  mg quercetin/mL infusion) and tannins ( $6.22 \pm 1.01$  mg catechin/mL infusion) antioxidant effect. Overall, the antioxidant activity of phenolic compounds concerns their oxidation properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Guerra, 2001). According to Anila and Vijayalakshmi (2003), flavonoids and tannins are active in inhibiting membrane phospholipid peroxidation, microsomal and mitochondrial lipid peroxidation and erythrocyte peroxidation.

Figure 2A shows that the CP and TBARS levels in erythrocytes from HIV/AIDS-free individuals are significantly lower than the protein damage observed in HIV/AIDS carriers. Such finding was expected since lipid hydroperoxides, formed from lipoperoxidation, can attack proteins and result in the formation of these carbonyl compounds (Griffiths et al., 2014). Figure 2B, on the other hand, shows the CP levels after the erythrocytes' treatment with poejo infusion and reveals lower rates of carbonyl compounds in the highest tested concentrations 5, 10, 25 and 50 g/L. According to Höhn, König, and Grune (2013), carbonyl compound formations are irreversible; however, there are proteolytic systems removing damaged proteins and recycling amino acids for protein synthesis purposes. Therefore, it is possible suggesting that the herein tested poejo infusions may have stimulated the functioning of these proteolytic mechanisms.

The proteolytic mechanisms mentioned above provide a last protection line against cellular oxidation and complete the performance of the endogenous antioxidant system (Höhn et al., 2013), in which GSH plays a key role. This intracellular thiol acts as cofactor for the glutathione peroxidase enzymes family, which is essential to the protection against oxidative stress.

A few years ago, Staal, Roederer, and Herzenberg (1990) reported decreased erythrocyte and plasma GSH levels in HIV-1 carriers and correlated them to AIDS progression and to the treatment with oxidant drugs. These findings were partially confirmed in the present study, if one considers that the GSH levels from HIV/AIDS patients were significantly lower than in the studied HIV/AIDS-free individuals (Figure 3A). It is worth noticing that the erythrocytes GSH levels maintain the cysteine residues of hemoglobin and other proteins. Thus, when this function fails, meta-hemoglobin are formed and, consequently, there is oxygen carrying capacity loss. Such loss leads to tissue hypoxia (Sies, 1999) and can induce the generation of more reactive species, since the non-transported oxygen tends to lose two electrons from its last layer and produce superoxide radicals or also by additional enzymatic and metabolic actions, can form other types of unpaired oxygen molecules, which are generically known as ROS (Dröge, 2002; Gottlieb et al., 2011).

After the erythrocytes from HIV/AIDS carriers were treated with poejo infusions, the GSH levels showed further reduction at all tested concentrations. Accordingly, the reduced GSH levels can be explained by the decreased TBARS and PCs levels, since the enzymatic antioxidants are responsible for the decreased GSH-dependent lipid and protein damages.

## 5. Conclusion

Poejo infusion appears to perform an antioxidant activity in lipid oxidation and in the protein carbonylation found in individuals with HIV/AIDS. Therefore, in the future, this plant may become a natural therapeutic adjuvant used to reduce oxidative stress in seropositive patients.

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