

Full Length Research Paper

Evaluation of biologic effects of an *Ilex paraguariensis* aqueous extract on the labeling of blood constituents with technetium-99m and on the morphology of red blood cells

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Ilex paraguariensis (mate-tea) has been used to treat type-2 diabetes and emphysema. This tea presents antioxidant activity and promotes satiety, body weight lowering and improves bone mineral density. Blood constituents have been labeled with technetium-99m (^{99m}Tc) and this labeling depends on the presence of a reducing agent. Stannous chloride has been widely utilized with this purpose. The influence of natural and synthetic drugs in this procedure has been reported. It was evaluated with effect of an aqueous mate-tea extract on the labeling of blood constituents with ^{99m}Tc and on the morphology of erythrocyte. Blood samples of Wistar rats were incubated with mate-tea extract, stannous chloride and ^{99m}Tc-sodium pertechnetate. Blood cells (BC) and plasma (P) were isolated. BC and P were also precipitated and soluble (SF) and insoluble (IF) fractions separated. The radioactivity was counted and percentage of incorporated radioactivity (%ATI) determined. Blood smears were performed for morphological evaluation. The data show a significant ($p < 0.05$) alteration of %ATI in BC, IF-C and IF-P as well as on the erythrocyte morphology. These findings could be related to the redox properties of the substances of the mate-tea extract, as well as due to the interference in the transport of stannous and/pertechnetate into the BC due to the morphological alterations of the erythrocytes.

Key words: *Ilex paraguariensis*, blood constituents, radiolabeling, morphology, technetium-99m, stannous ion.

INTRODUCTION

Ilex paraguariensis (mate-tea) is an herb whose leaves are utilized to prepare infusion. This infusion is a popular

drink known as mate-tea. It has been consumed under different forms. Various properties of this plant in the

form of tea have been already described and it has been popularly used to treat type-2 diabetes (Hussein et al., 2011) and emphysema (Lanzetti et al., 2011). In addition, data have demonstrated that this tea possesses antioxidant activity (Coentão et al., 2011) and promotes satiety, body weight lowering (Hussein et al., 2011) and improves bone mineral density (Confort et al., 2012). Consumption of mate powder would significantly contribute to antioxidant and stimulant intake, providing high amounts of phenolic acids, tannins and methylxanthines with biological effects potentially beneficial for human health (Vieira et al., 2010).

Blood constituents have been labeled with technetium-99m (^{99m}Tc) and used in several procedures in nuclear medicine. These radiopharmaceuticals can be used for important applications, including imaging of cardiovascular system (Niemeier et al, 1995; Crandall et al, 2008; Javadi et al, 2011), peripheral arterial blood flow (Blond and Madsen, 2000; Harel et al, 2005; Hsieh et al, 2009), evaluation of gastrointestinal bleeding (Zaman et al, 2004; Wong et al, 2004; Olds et al, 2005; Schillaci et al, 2009; Dolezal et al, 2011), measurement of red cell volume (Hladik III et al, 1987), hepatic hemangiomas (Artiko et al, 2004; Verdu et al, 2005; Yarlagaadda et al, 2008), renal carcinoma (Cortes et al, 2003), splenic reticuloendothelial system (Jin et al, 2004; Slart et al, 2004) and imaging infection (Stoeckli et al, 1996)..

The labeling process of red blood cells (RBC) is based on the transmembrane transport of the reducing agent (Sn^{+2}) and pertechnetate ($^{99m}\text{TcO}_4^-$) ions into the RBC, reduction of $^{99m}\text{TcO}_4^-$ by Sn^{+2} , and subsequent binding of the reduced ^{99m}Tc to internal structures (Early and Soddee, 1995; Dewanjee et al., 1982). The band-3 anion transport system and calcium channels may be involved in transport of $^{99m}\text{TcO}_4^-$ and Sn^{+2} , respectively (Callahan and Rabito, 1990; Gutfilen et al., 1992; Sampson, 1996). The fixation of ^{99m}Tc on plasma proteins also depends on the presence of a reducing agent (Saha, 2010).

Data have demonstrated the effects of synthetic and natural drugs on this radiolabeling process (Mousinho et al., 2008; Holanda et al., 2009; Bustani et al., 2009; Rocha et al., 2009; De et al., 2009; Cekic et al., 2011; Souza et al., 2011). In consequence, the labeling of blood constituents with ^{99m}Tc has been used as an *in vitro* assay to screening effects of synthetic or natural products that could have actions related to the band-3 and calcium channels or antioxidant/oxidant properties. Moreover, qualitative and quantitative morphological analysis of RBC has been used as a method to evaluate if the effects of drugs on this radiolabeling process could be related to changes on shape of RBC (Rocha et al., 2009; De et al., 2009).

The aim of this study was to evaluate biologic effects of an aqueous extract of mate-tea on the labeling of the blood constituents with ^{99m}Tc and on the morphology of

RBC.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3 to 4 months of age, body weight 250 to 350 g) were maintained in a controlled environment. The animals had free access to water and food and ambient temperature was kept at $25 \pm 2^\circ\text{C}$. Experiments were conducted in accordance with the Institutional Committee of Animal Care of the Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro that has approved the protocols used with the number CEA/213/2007.

Preparation of mate-tea extract

As the *I. paraguariensis* extract can be used in different forms of beverage, it is difficult to determine a specific concentration to be studied (Bastos et al., 2007; Miranda et al., 2008; Burris et al., 2012). In our investigation, to prepare the extracts, 10 g of dry leaves of commercial *I. paraguariensis* (Shapira, Brasil) were vortexed in 50 ml NaCl 0.9%. The crude extract was filtered, centrifuged (clinical centrifuge, 1500 rpm, 5 min) to obtain the final extract. The supernatant was considered to be in the concentration of 200 mg/ml.

In vitro labeling of blood constituents assay

Heparinized blood (500 μl), was withdrawn from Wistar rats and incubated with 100 μl of mate-tea extract at different concentrations (12.5, 25, 50, 100 and 200 mg/ml) or with a saline solution alone, as control, for 1 h (room temperature). Afterwards, 500 μl of stannous chloride (1.20 $\mu\text{g/ml}$) was added and the incubation continued for further 1 h. After this period of time, 100 μl of ^{99m}Tc (3.7 MBq) as sodium pertechnetate ($\text{Na}^{99m}\text{TcO}_4$), recently milked from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, São Paulo, Brasil) were added and the incubation was continued for 10 min. These samples were centrifuged in a clinical centrifuge (1500 rpm, 5 min) and aliquots of 20 μl of plasma (P) and blood cells (BC) were isolated. Another aliquots of 20 μl of P and BC were separated and precipitated in 1.0 ml of trichloroacetic acid (5%) and centrifuged (1500 rpm, 5 min) to isolate soluble (SF) and insoluble fractions (IF). The radioactivity in P, BC, SF-P, IF-P, SF-BC and IF-BC were determined in a well counter (Packard, model C5002, Illinois, USA) and the percentage of incorporated radioactivity (%ATI) was calculated as described elsewhere (Oliveira et al., 2003).

Morphological evaluation of red blood cells

Histological preparations were carried out with blood samples *in vitro* treated with mate-tea extract at different concentrations during 60 min at room temperature, or with saline solution as control group. Blood smears were prepared, dried, fixed and stained by May-Grünwald-Giemsa method (Bancroft and Gamble, 2007). After that, the images of the red blood cells were acquired (Optronics, USA) from blood smears to qualitative morphology analysis under optical microscopy ($\times 1000$, Olympus, BX model, Japan).

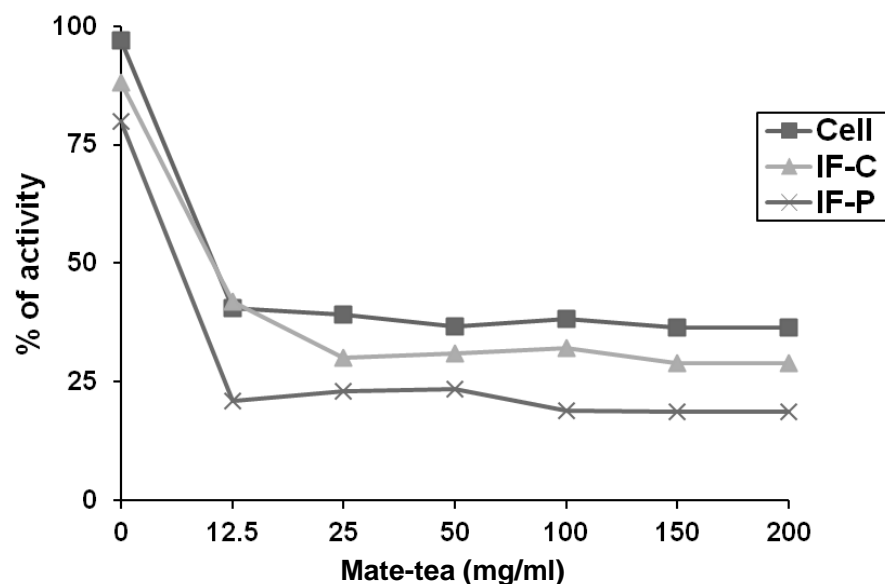


Figure 1. Effect of mate-tea extract on the uptake of the ^{99m}Tc in the blood cells (Cell), insoluble (IF-C) fractions of blood cells and insoluble (IF-P) fractions of plasma, in the radiolabeling procedure of blood constituents. Heparinized blood samples of *wistar* rats were incubated with different concentrations of mate-tea extract (1 h) and after with SnCl_2 (1.20 $\mu\text{g/ml}$, 1 h) and in sequence with $\text{Na}^{99m}\text{TcO}_4$ (3.7 MBq, 10 min). Insoluble and soluble fractions of plasma were obtained by precipitation with trichloroacetic acid (5%) and centrifugation (1500 rpm, 5 min). the radioactivity in these fractions was counted and the %ATI were calculated.

Statistical analysis

Data are reported as (means \pm SD) of %ATI. The comparison of the obtained data of the treated ($n = 10$ for each extract concentration) and control groups ($n = 10$) by one way analysis of variance – (ANOVA), followed by Tukey post test, with a $p < 0.05$ as significant level was performed. InStat Graphpad software was used in the statistical analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California, USA).

RESULTS

Figure 1 shows the distribution of the radioactivity in the cells (cellular compartment) when the blood was treated with different concentrations of the mate-tea extract (12.5, 25.5, 100 and 200 mg/ml). The results show an inhibition in the process of labeling of the cells where the efficiency of labeling was reduced significantly ($p < 0.05$) for all the concentrations of the mate-tea extract tested.

In addition, Figure 1 also shows the fixation of the radioactivity in the insoluble fractions of the plasma (IF-P) and the insoluble fractions of the cells (IF-C) when treated with different concentrations of the mate-tea extract (12.5, 25.5, 100 and 200 mg/ml). The results obtained after the treatment of the blood with the different concentrations of the mate-tea extract, show that it produced

significant reduction ($p < 0.05$) in the efficiency of labeling of the IF-C, from 88.0 ± 3.7 to 41.8 ± 3.7 , until the concentration of 12.5 mg/ml with this reduction more accentuated for the concentration of 200 mg/ml when the %ATI was reduced from 88.0 ± 3.7 to 29.0 ± 1.3 . An important consideration is that these effects are not related to a dose-response relationship. Moreover, with a strong dilution of the extract, its effect was observed.

Figures 2, 3, 4 and 5 represented photomicrographs of the blood smears from samples of whole blood treated with saline solution (control) or with an aqueous mate-tea extract with different concentrations (12.5, 50 and 200 mg/ml), respectively. The qualitative morphological analysis by the comparison between these figures suggests the treatment with mate-tea extract could induce important changes on shape of red blood cells observed under optical microscopy. Furthermore, these qualitative morphological effects seem to be independent on the dose of the extract and they appear with strong dilutions of the extract.

DISCUSSION

The knowledge about properties and effects of a natural product is worthwhile due to the consumption of these

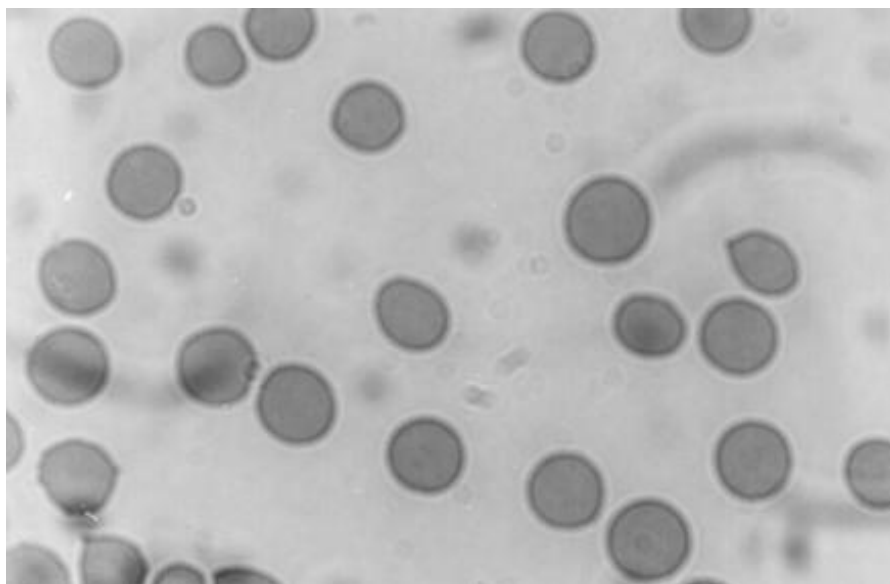


Figure 2. Photomicrography of blood smears from blood samples treated with NaCl 0.9% solution (control). Samples of whole blood from Wistar rats were treated with NaCl 0.9% solution during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy after image capture.

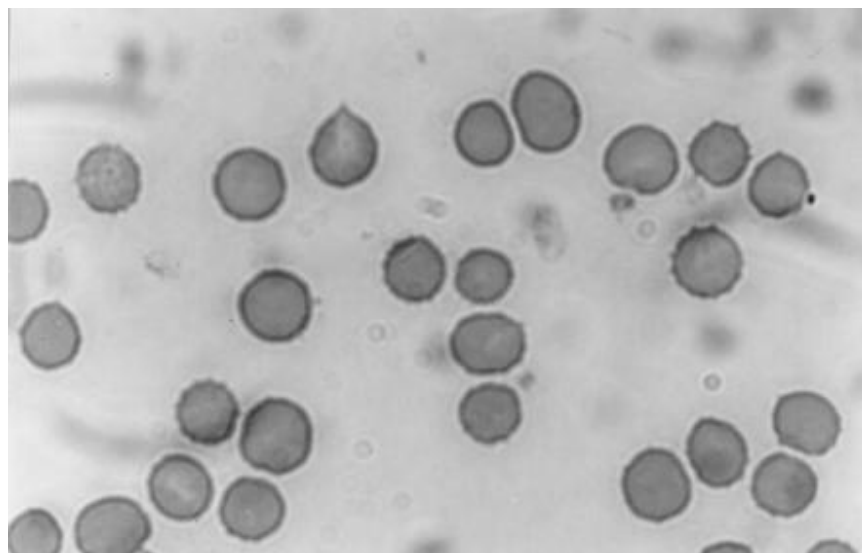


Figure 3. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (12.5 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

products which are increasing in the entire world (Rotblatt and Ziment, 2002). *I. paraguariensis*, as tea, was capable

in altering the distribution of ^{99m}Tc on plasma and cellular compartments, as well as the fixation of this radionuclide

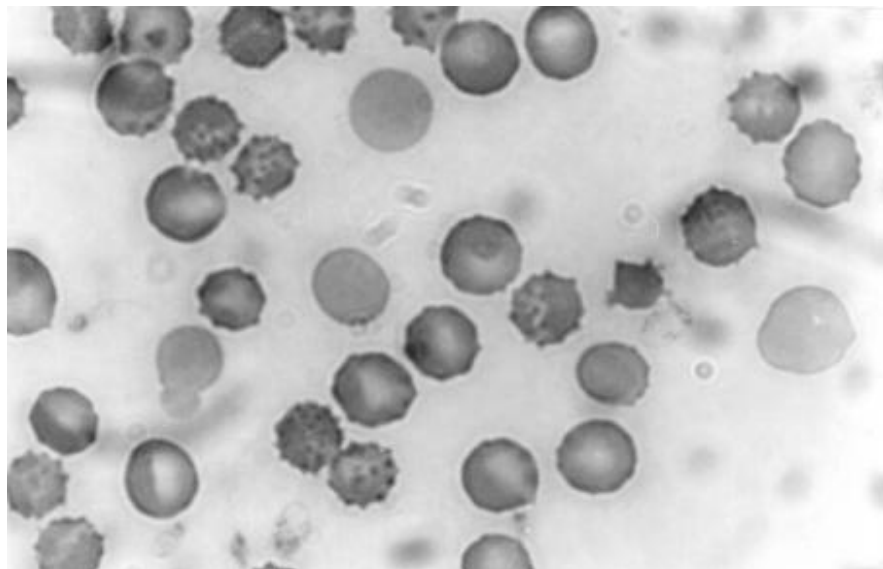


Figure 4. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (50 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

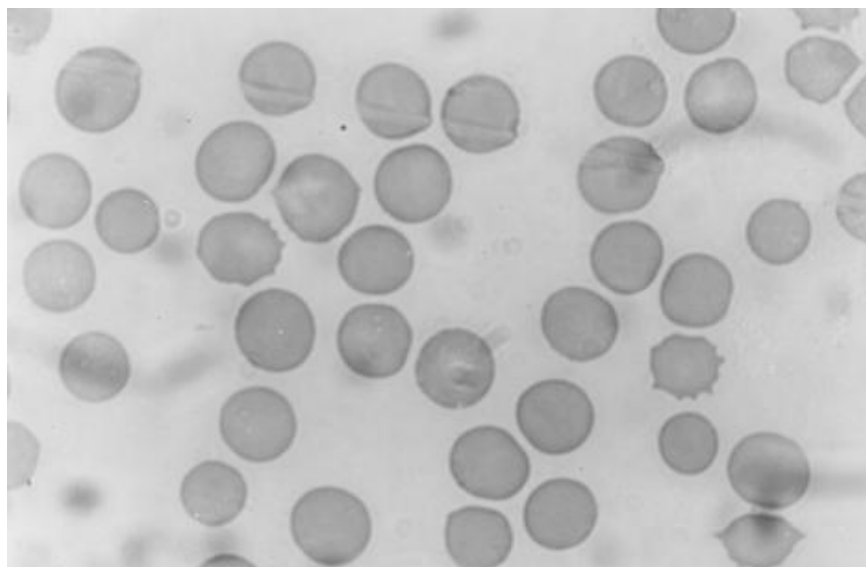


Figure 5. Photomicrography of blood smears from blood samples treated with mate-tea extract. Samples of whole blood from Wistar rats were treated with aqueous mate-tea extract (200 mg/ml) during 60 min. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy ($\times 1000$) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

in the insoluble fractions of plasma and blood cells (Figure 1). It is reported that mate-tea extracts contain

some compounds that have redoxi properties such as phenolic acids, and its properties are attributed to

methylxanthines, such as caffeine referred in folk medicine (Vieira et al., 2010). As the labeling of blood constituents depends on a reducing agent, the redox properties of the substances present in the *I. paraguariensis* could be responsible by the reduction of the labeling of blood constituents with ^{99m}Tc .

In addition, in the labeling of blood cells with ^{99m}Tc , the stannous and pertechnetate ions must reach the cell compartment by channels of erythrocyte membrane. It was observed (Figures 3, 4, 5) that independently on the dose, the extract could cause qualitative and important morphological modifications on the membrane of the red blood cells and this fact can contribute to justify the alteration on the labeling of these cells with ^{99m}Tc . Our results of morphology of RBC could be related to modifications on membrane structures involved in ions transport that could alter the transport of the stannous and pertechnetate ions into cell justifying the decrease of the labeling of blood cells with ^{99m}Tc (Figure 1).

Conclusion

Our findings could be related to the redox properties of the substances of the mate-tea extract, as well as due to the interference in the transport of ions (stannous and/pertechnetate) into the blood cells due to morphological modifications of the erythrocyte membrane. Although, our experiments were performed in controlled conditions and with blood withdrawn from rats, we suggest precaution in the interpretation of the examinations done in a Nuclear Medicine Department in patients that are undertaken mate-tea.

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