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Full Length Research Paper

Alteration of the labeling of blood constituents with technetium-99m and the morphology of red blood cells by *Baccharis trimera* extract

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Baccharis trimera **(carqueja) has been used in folk medicine to treat rheumatism, diabetes and digestive disorders, and suggested as anti-inflammatory, analgesic, anti-ulcerative and antihepatotoxic. Blood constituents have been labeled with technetium-99m (^{99m}Tc) and used in nuclear medicine. The influence of natural and synthetic drugs in this procedure has been reported. In this work, the influence of an aqueous carqueja extract on the labeling of blood constituents with ^{99m}Tc and morphology of red blood cells was evaluated. Blood samples of Wistar rats were incubated with different concentrations of carqueja extract. The labeled process with ^{99m}Tc was the performed. Blood cells (BC) and plasma (P) were isolated. Aliquots of BC and P were precipitated and soluble (SF) and insoluble (IF) fractions separated. The radioactivity in each fraction 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 and IF-P as well as morphology of red blood cells from blood incubated with carqueja extract. The results suggest that aqueous carqueja extract could presents an antioxidant action and/or alters the membrane structures involved in ions transport into cells decreasing the radiolabeling of blood constituents with ^{99m}Tc.**

Key words: *Baccharis trimera*, blood constituents, radiolabeling, technetium-99m, stannous ion.

INTRODUCTION

Baccharis trimera is a green herb that presents a nearly vertical aspect with a height of 1 up to 2 ms and yellow-white flowers at the top (Lonni et al., 2005). The species of *Baccharis* genus are common in Southern Brazil, Northern Argentina, Paraguay, Uruguay and Bolivia. In Brazil, this genus is represented by several species thatare popularly known as carqueja (Lonni et al., 2003).

Various properties of this plant in the form of tea have already been described and it has been popularly used to treat rheumatism (Souza et al., 1991), diabetes (Oliveira et al., 2005) and digestive disorders (Torres et al., 2000). It has also been related to anti-inflammatory, analgesic and anti-ulcerative (Gené et al., 1996) and antihepato-toxic (Soicke, 1987) properties for extracts of carqueja. In addition, data have demonstrated that this phytotherapic presents anti-proteolytic and anti-hemorrhagic properties (Januario et al., 2004). The phytochemical analysis of carqueja extracts has revealed the presence of diterpenes (Torres et al., 2000), saponins and flavonoids

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(Gené et al., 1996; Soicke, 1987).

Blood constituents have been labeled with technetium-99m (^{99m}Tc) and used in several procedures in nuclear medicine. These radiobiocomplexes can be used for important applications, including imaging of cardiovascular system (Niemeyer et al., 1995), peripheral arterial blood flow (Blond and Madsen, 2000; Harel et al., 2005), evaluation of gastrointestinal bleeding (Wong et al., 2004; Zaman et al., 2004; Olds et al., 2005), measurement of red cell volume (Hladik III et al., 1987), hepatic hemangiomas (Artiko et al., 2004; Verdu et al., 2005), renal carcinoma (Cortes et al., 2003), splenic reticulo-endothelial system (Jin et al., 2004; Slart et al., 2004) and imaging infection (Stoeckli et al., 1996). The labeling procedure of blood constituents with ^{99m}Tc has been also considered as an experimental model to evaluate some properties and effects of natural and synthetic products (Oliveira et al, 2003a; Oliveira et al, 2003b; Frydman et al., 2004; Moreno et al., 2004; Fonseca et al., 2005; Bustani et al., 2009).

The labeling process of red blood cells (RBC) is based on the transmembrane transport of reducing agent (Sn^{+2}) and pertechnetate ($^{99m}TcO_4^-$) ions into the RBC, reduction of $^{99m}TcO_4^-$ by Sn^{+2}, and subsequent binding of the reduced ^{99m}Tc to internal structures (Dewanjee et al., 1982). The band-3 anion transport system and calcium channels may be involved in transport of $^{99m}TcO_4^-$ 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 (Early and Soddee, 1995). Data have demonstrated the effects of synthetic and natural drugs on this radiolabeling process (Oliveira et al., 2003a, b; Frydman et al., 2004; Moreno et al., 2004; Fonseca et al., 2005; Bustani et al., 2009). In consequence, the labeling of blood constituents with ^{99m}Tc has been used as an *in vitro* assay for the screening of synthetic or natural products that could affect related to the band-3 and calcium channels or antioxidant/oxidant properties. Moreover, qualitative and quantitative morphological analysis 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 (Oliveira et al., 2003a).

The aim of this study was evaluate the effect of an aqueous extract of carqueja 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 ± 2°C. Experiments were conducted in accordance with the Institutional Committee of Animal Care.

Preparation of carqueja extract

To prepare the extracts, 8 g of dry leaves 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 160 mg/ml.

In vitro labeling of blood constituents assay

Heparinized blood (500 µl), was withdrawn from Wistar rats and incubated with 100 µl of carqueja extract at different concentrations (20, 40, 80, 120 and 160 mg/ml) or with a saline solution alone, as control, for 1 h (room temperature). Afterwards, 500 µl of stannous chloride (1.20 µg/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 ($Na^{99m}TcO_4$), recently milked from a $^{99}Mo/^{99m}$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 was 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 (Bernardo-Filho et al., 1983).

Morphological evaluation of red blood cells

Histological preparations were carried out with blood samples *in vitro* treated with carqueja 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-Giensa method (Junqueira and Carneiro, 2002). After that, the images of the red blood cells were acquired (Optronics, USA) from blood smears to qualitative morphology analysis under optical microscopy (×1000, Olympus, BX model, Japan).

Statistical analysis

Data are reported as (means ± standard deviation (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 %ATI in the compartments related to the blood cells (BC) and plasma (P) isolated from whole

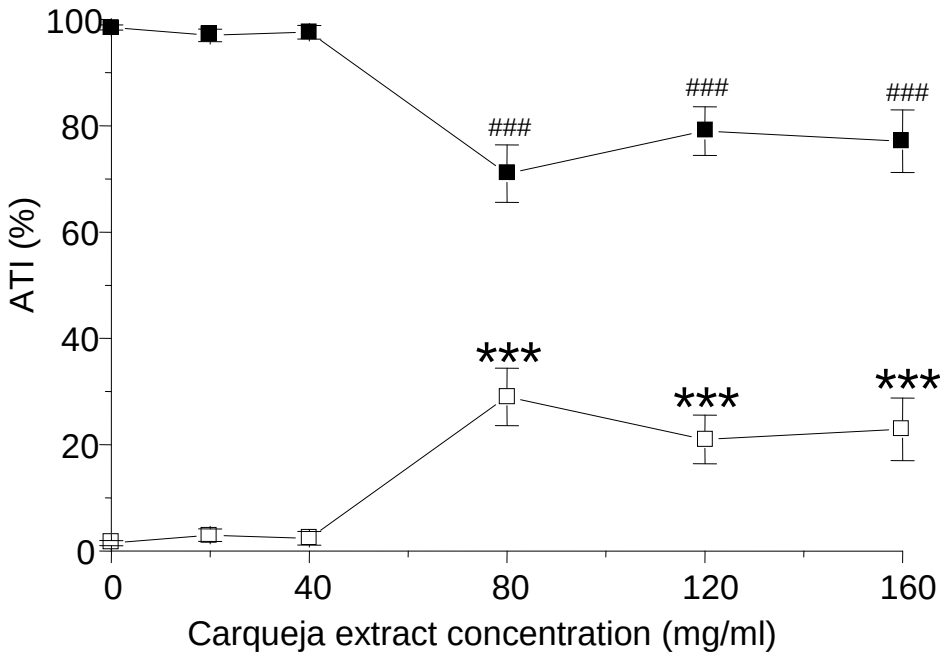

Figure 1. Effect of carqueja extract on the distribution of the ^{99m}tc in the plasma and blood cells (BC) compartments in the radiolabeling procedure of blood constituents. Heparinized blood samples of Wistar rats were incubated with different concentrations of carqueja extract (1 h) and then with $SnCl_2$ (1.20 µg/ml, 1 h) and in sequence with $Na^{99m}TcO_4$ (3.7 MBq, 10 min). After centrifugation, plasma (P) and blood cells (BC) were isolated. The radioactivity was counted and the %ATI calculated. (■) BC and (□) P. ***P ≤ 0.01, when compared to control group of plasma and $^{###}$P ≤ 0.01, when compared to control group of blood cells.

blood treated with different concentrations of carqueja extract. The analysis of these data indicates that carqueja extract significantly (P < 0.05) alters the distribution of radioactivity in these two compartments (BC and P) at the highest carqueja extract concentrations studied (80, 120 and 160 mg/ml).

Figure 2 shows the %ATI in insoluble (IF-BC) and soluble (SF-BC) fractions isolated from samples of blood cells separated from whole blood treated with different concentrations of carqueja extract. The analysis of these data indicates that the incubation with carqueja extract not significantly (P > 0.05) alters the radioactivity uptake in insoluble blood cells fraction at all concentrations used. Figure 3 shows the %ATI in insoluble (IF-P) and soluble (SF-P) fractions isolated from plasma separated from whole blood treated with different concentrations of carqueja extract. The analysis of these data indicates that carqueja extract significantly (P > 0.05) alters the radioactivity uptake in IF-P at highest concentrations studied (40, 80, 120 and 160 mg/ml).

Figures 4 and 5 show the photomicrographs of the blood smears from samples of whole blood treated with saline solution (control) or with an aqueous carqueja extract at the highest concentration used (160 mg/ml), respectively. The qualitative morphological analysis by the comparison between these figures suggests that treatment with carqueja extract could induce important changes on shape of red blood cells observed under optical microscopy.

DISCUSSION

The knowledge about properties and effects about a natural product is worthwhile due to the fact that the consumption of these products are increasing in the entire world. *B. trimera*, as tea, has several properties that has stimulated its use in the treatment of various clinical disorders, and the analysis of our results indicates that the aqueous carqueja extract could alters the distribution of ^{99m}Tc between cellular and plasma compartments (Figure 1).

In the labeling of blood cells with ^{99m}Tc, the stannous and pertechnetate ions must reach the cell compartment by calcium and band-3 ions channels, respectively (Dewanjee et al., 1982; Callahan and Rabito, 1990; Gutfilen et al., 1992). It is possible to suppose that compounds of the carqueja extract could complex with these ions outside of the cells or decrease the efficiency of the ions transport system altering the distribution of radioactivity between plasma and cellular compartments. Moreover, these effects would not alter the fixation of

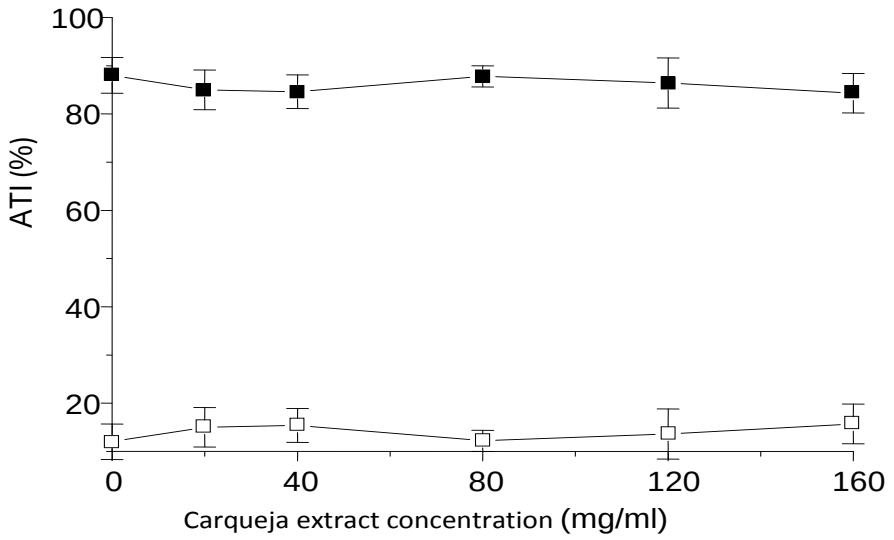

Figure 2. Effect of carqueja extract on uptake of ^{99m}Tc by insoluble (IF-BC) and soluble (SF-BC) fractions of blood cells (BC), in the radiolabeling procedure of blood elements. Heparinized blood samples of Wistar rats were incubated with different concentrations of carqueja extract (1 h), and then with $SnCl_2$ (1.20 µg/ml, 1 h) and in sequence with $Na^{99m}TcO_4$ (3.7 MBq, 10 min). Insoluble and soluble fractions of blood cells (IF-BC and SF-BC) 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. (■) IF-BC and (□) SF-BC.

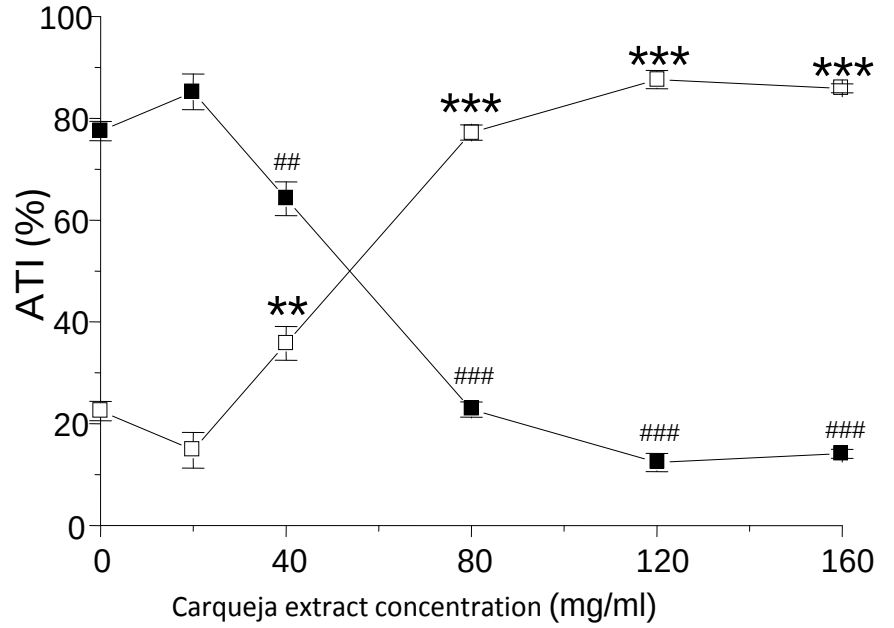

Figure 3. Effect of carqueja extract on uptake of ^{99m}Tc by insoluble (IF-P) and soluble (SF-P) fractions of plasma (P), in the radiolabeling procedure of blood elements. Heparinized blood samples of Wistar rats were incubated with different concentrations of carqueja extract (1 h) and then with $SnCl_2$ (1.20 µg/ml, 1 h) and in sequence with $Na^{99m}TcO_4$ (3.7 MBq, 10 min). Insoluble and soluble fractions of plasma (IF-P and SF-P) 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. (■) IF-P and (□) SF-P. $^{\#\#}P \leq 0.01$; $^{\#\#\#}P \leq 0.001$, when compared to control group of IF-P and $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, when compared to control group of SF-P.

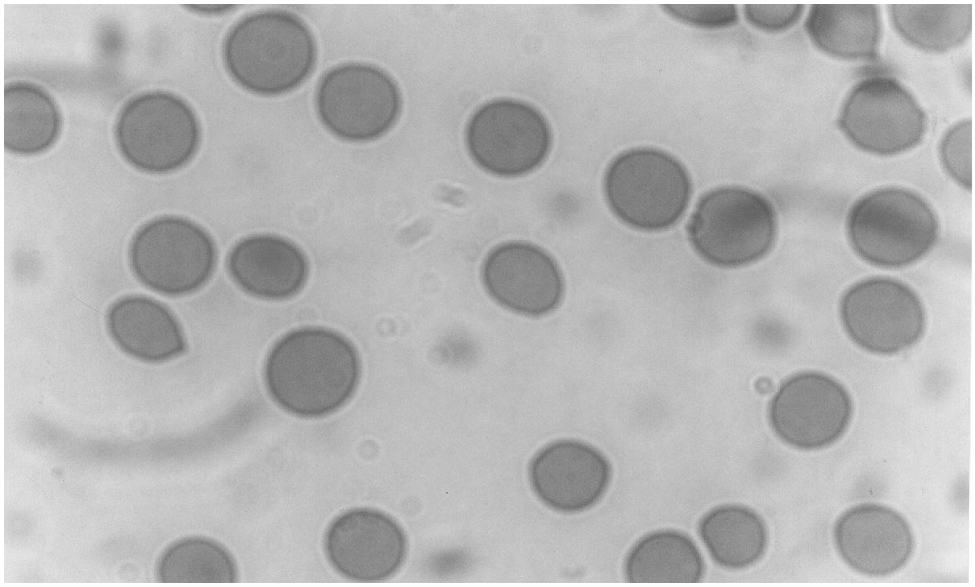

Figure 4. Photomicrography of blood smears from blood samples treated with NaCl 0.9% solution (control). Samples of whole blood from Wistar rats were treated with NaCl 0.9% solution for 60 min. Blood smears were prepared, dried, fixed and stained by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy after image capture.

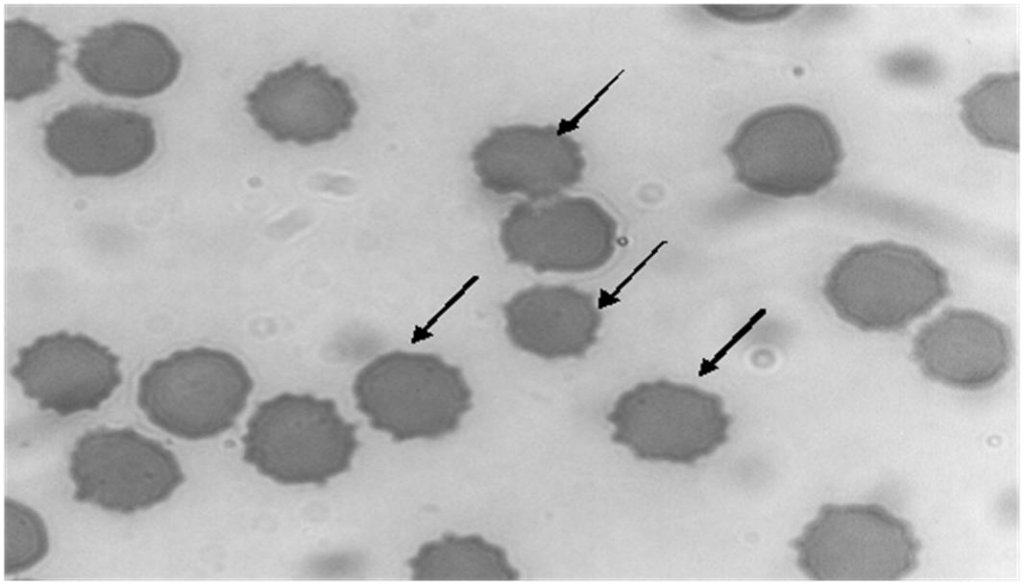

Figure 5. Photomicrography of blood smears from blood samples treated with carqueja extract. Samples of whole blood from Wistar rats were treated with aqueous cinnamon extract (160 mg/ml) for 60 min. Blood smears were prepared, dried, fixed and stained by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy (×1000) after image capture. The arrows indicate the alterations on the erythrocyte membrane.

^{99m}Tc at cellular proteins explaining the data obtained in this work (Figure 2). In fact, it was verified that carqueja extracts contain some compounds that could block ionic currents through calcium channels promoting smooth muscle relaxation and improvement of blood circulation referred in folk medicine (Torres et al., 2000).

Nevertheless, these compounds could also interfere with the fixation of the ^{99m}Tc on the plasma proteins (Figure 3) and a strong effect is observed.

To verify if the aqueous carqueja extract could cause morphological modifications at membrane of red blood cells and to alter the labeling of these cells with ^{99m}Tc, qualitative (Figures 4 and 5), morphological analysis was carried out. The analysis suggested that the carqueja extract used could induce modifications on the shape of RBC. Our results of the morphology of RBC could be related to modifications on membrane structures involved in ions transport that could alter the internal cellular conditions and/or stannous and pertechnetate ions transport into cell explaining the decrease in the radio-labeling of blood cells with ^{99m}Tc (Figure 1).

Another possibility is related to antioxidant property of some compounds in carqueja extract (Simoes-Pires et al., 2005). These compounds acting out cellular compartment could interfere on oxidization state of stannous ions decreasing the pertechnetate ion reduction and alter the radiolabeling of blood cells.

By *in vivo* and *in vitro* assays, it was verified that a component of carqueja extract could bind to metalloprotease snake venom inhibiting its proteolytic and hemorrhagic properties (Januario et al., 2004). Moreover, other compounds (as flavonoids) present in several medicinal plants are transported in blood attached to plasma proteins (Podhajcer et al., 1980). The binding of carqueja components in plasma proteins at same binding sites of ^{99m}Tc could decrease labeling with ^{99m}Tc of these blood constituents and to justify the data obtained in this work.

In conclusion, our experimental data, with the suggested assays, indicate that the labeling of blood constituents with ^{99m}Tc and morphology of red blood cells can be altered in the presence of aqueous carqueja extracts. Accordingly, additional experiments are going in our laboratory to elucidate the specific mechanisms involved in the effects of carqueja extracts on labeling of blood constituents with ^{99m}Tc.

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