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Full Length Research Paper

Effects of a *Tabebuia avellanedae* extract and lapachol on the labeling of blood constituents with technetium-99m

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Tabebuia avellanedae **extract has been used in folk medicine in the treatment of some clinical disorders. Lapachol is an active compound from this medicinal plant. The procedure of labeling of blood constituents with technetium-99m (^{99m}Tc) could be used as an *in vitro* assay to evaluate some properties of natural and synthetic drugs. The aim of this work was to evaluate the effect of a *T. avellanedae* extract and lapachol solutions on the labeling of blood constituents with ^{99m}Tc. Whole blood (*Wistar* rats) was incubated with an aqueous *T. avellanedae* extract or lapachol. After, stannous chloride (reducing agent) and ^{99m}Tc (sodium pertechnetate) were added. Blood cells (BC) and plasma (P) were isolated by centrifugation. Samples of BC and P were precipitated with trichloroacetic acid to separation of soluble (FS) and insoluble (IF) fractions. The radioactivity in each fraction was counted and the percentage of incorporated radioactivity (%ATI) was determined. The data obtained showed that *T. avellanedae* extract significantly ($p<0.05$) altered the %ATI on blood constituents while no effects were observed with lapachol. As the labeling of blood constituents with ^{99m}Tc depends on the presence of a reducing, the extract of *T. avellanedae* seems to have substances with redox properties. In addition, these findings would be not associated with the lapachol.**

Key words: *Tabebuia avellanedae*, lapachol, blood, stannous ion, technetium-99m.

INTRODUCTION

Medicinal plants widely used in traditional medicine constitute an important source of new, safer and maybe biologically active compounds against many disorders in the herbal medicine in various countries. Furthermore, the scientific interest in the determination of properties associated with medicinal herbs is increasing in the world

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(Adisakwattana et al., 2011; Ma et al., 2011). In addition,some authors have studied substances isolated from medicinal herbs, as the Bisabololoxide that is isolated from the *Matricaria recutita* L. (Ogata et al., 2010).

Tabebuia avellanedae is a tree from the Bignoniaceae family and native to Central and South America. It is known as “pau d’arco” , “taheebe”, “lapacho” or “ipe roxo” and its inner bark is used as antimicrobial (Machado et al., 2001), anti-inflammatory (Lira et al., 2008), analgesic, antinociceptive (de Miranda et al., 2001), and anti-tumor drugs (Ueda et al., 1994). Phytochemical analysis of *T. avellanedae* have demonstrated the presence of quinones (Sharma et al., 1998), furanonaphthoquinones (Díaz and Medina, 1994), naphthoquinones (Manners and Jurd, 1976), benzoic acid, benzaldehyde derivatives (Wagner et al., 1989), cyclopentene dialdehyde (Koyama et al., 2000), flavonoids and iridoids (Nakano et al., 1993) and phenolic glycosides (Warashina et al., 2004).

Lapachol (2-hydroxy-3-(-3-methyl-2-butanyl)-1,4-naphthoquinone) has been isolated from *T. avellanedae* extracts. There are interest in the studies of this substance due to its anti-tumor (Balassiano et al., 2005), anti-biotic (Santos et al., 2001), anti-leishmanial (Lima et al., 2004), anti-malarial (de Andrade Neto et al., 2004), anti-ulcer (Goel et al., 2004) and anti-inflammatory activities (Lira et al., 2008). Preparation of isolated of lapachol is commercially available and it was used in this study.

Radionuclides have been in various clinical evaluations (Saha, 2010) and in experimental models (Bustami et al., 2009; Santos et al., 2013; Frederico et al., 2014,). Technetium-99m (^{99m}Tc) has been widely used in these procedures due to its optimal physical characteristics (6 h physical half-life and gamma emission) that give a negligible environmental impact (Saha, 2010). Several authors have demonstrated the effects of synthetic and natural drugs on the labeling process of blood constituents with ^{99m}Tc (Fonseca et al., 2005; Bustami et al., 2009; Carmo et al., 2011).

Blood constituents labeled with ^{99m}Tc have been used as radiobiocomplexes for a number of applications in nuclear medicine. The labeling of blood cells and cell structures is based on the transmembrane transport of a reducing agent (Sn^{+2}) and pertecnetate ($^{99m}TcO_4^-$) ions into the red blood cells, reduction of $^{99m}TcO_4^-$ by Sn^{+2}, and subsequent binding of the reduced ^{99m}Tc to internal structures. The band-3 anion transport system and calcium channels may be involved in the transportion of $^{99m}TcO_4^-$ and Sn^{+2}, respectively. The fixation of ^{99m}Tc in plasma proteins also depends on the reducing agent action occurring at different proteins sites and albumin is the principal protein involved (Saha, 2010).

The effect of drugs altering the labeling of blood constituents could be due modification of the membrane structure (Braga et al., 2013), decreasing the efficiency of transmembrane transport system of $^{99m}TcO_4^-$ and Sn^{+2} ions into cells. Redox property and/or metal chelator could be another properties associated with the drugs.

In this investigation, the effect of a *T. avellanedae* extract and of a commercial preparation of lapachol on the labeling of the blood constituents with ^{99m}Tc was evaluated.

MATERIALS AND METHODS

Animals

Adult male *Wistar* rats (3-4 months of age, body weight 250-350 g) were maintained in a controlled environment. The animals had free access to water and food and the ambient temperature was kept at 25 ± 2ºC. Experiments were conducted in accordance with the Institutional Committee of Animal Care.

Preparation of *T. avellanedae* extract

T. avellanedae was purchased from *Estrella da Terra Produtos Naturais Ltda* (Brazil). To prepare the extracts, 2 g of bark were ground in 10 ml 0.9% NaCl at 100°C for 10 min. The crude extract was filtered, centrifuged (1500 rpm, 10 min) to obtain the final extract. The supernatant was considered to be 200 mg/ml. As the quantity of lapachol is about 7% of *T. avellanedae* (American Cancer Society, 2015), it is possible to consider a concentration of 14 mg/ml of lapachol. The concentrations of *T. avellanedae* used in the experiments were 12.5, 25, 50, 100 and 200 mg/ml, and respectively the concentrations of lapachol were 0.87, 1.75, 3.5, 7 and 14 mg/ml.

Preparation of lapachol solution

Lapachol is an important chemical compound of the *T. avellanedae* extract (Balassiano et al., 2005) and it is available in the market. It was purchased from *PVP Sociedade Anônima*. (Brazil) and the solutions were prepared in 0.02 N NaOH immediately before the use.

In vitro radiolabeling of blood constituents

Heparinized blood (500 µl), was withdrawn from *Wistar* rats and incubated with 100 µl of *T. avellanedae* extract (12.5, 25, 50, 100 and 200 mg/ml) or lapachol (0.05, 0.5, 5 and 50 mg/ml) for 1 hour (room temperature). Blood samples were also incubated with saline solution (0.9% NaCl) or 0.02N NaOH as control for *T. avellanedae* or lapachol, respectively. Afterwards, 500 µl of stannous chloride (1.20 µg/ml) was added and the incubation continued for further 1 h. After this period, 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, Brazil) 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 5% trichloroacetic acid 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

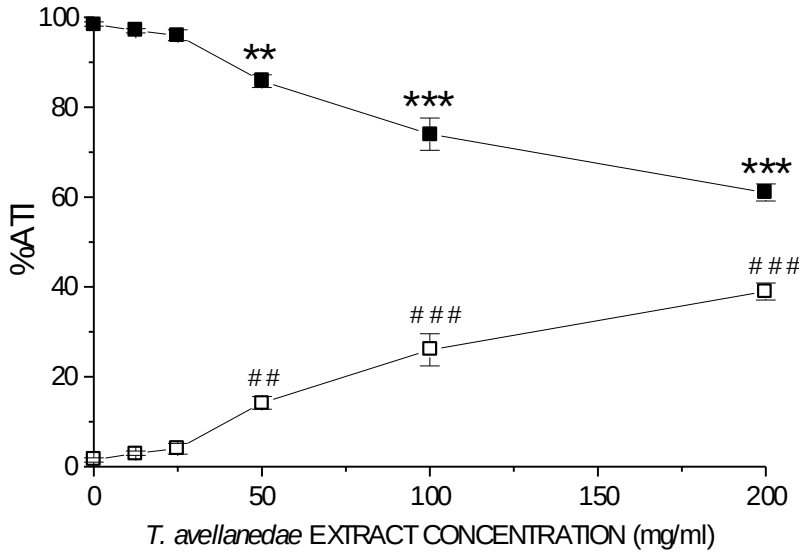

Figure 1. Effect of *T. avellanedae* extract on the distribution of the ^{99m}Tc in the plasma and blood cells (BC) compartments. Blood samples were incubated with *T. avellanedae* extract and after with $SnCl_2$ and with $Na^{99m}TcO_4$. After centrifugation, plasma (P) and blood cells (BC) were isolated. The radioactivity was counted in a gamma counter and the percentage of radioactivity incorporated (%ATI) was calculated for P and BC. ■, BC; □, P. **, $p \leq 0.01$, when compared to control group of plasma. ***, $p \leq 0.001$ when compared to control group of plasma. ##, $p \leq 0.01$, when compared to control group of blood cells. ###, $p \leq 0.001$, when compared to control group of blood cells

calculated as described elsewhere.

Histological analysis

Histological preparations were carried out with blood samples treated with various concentrations of *T. avellanedae* extract for 60 min at room temperature. Blood smears were prepared, dried, fixed and stained. After that, the morphology of the red blood cells was qualitatively evaluated under optical microscope.

Statistical analysis

Data are reported as (means ± SD) of %ATI and compared the treated (n=10 for each extract concentration) and control group (n=10) by One way analysis of variance - ANOVA, followed by Tukey post test, with a $p<0.05$ as significant level. InStat Graphpad software was used to perform statistical analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California, USA).

RESULTS

Figure 1 shows the %ATI in blood cells and plasma compartments from whole blood treated with different concentrations of *T. avellanedae* extract. The analysis of these data indicates that *T. avellanedae* extract alters significantly ($p<0.05$) the distribution of radioactivity between the two blood compartments.

Figure 2 shows the %ATI in insoluble (IF-P) and soluble (SF-P) fractions isolated from plasma separated from whole blood treated with different concentrations of *T. avellanedae* extract. The analysis of these data indicates that *T. avellanedae* extract significantly ($p<0.05$) reduced the radioactivity fixation in IF-P.

Figure 3 shows the %ATI in insoluble (IF-BC) and soluble (SF-BC) fractions isolated from blood cells separated from blood treated with different concentrations of *T. avellanedae* extract. The analysis of these data indicates that the incubation with *T. avellanedae* extract significantly alters the radioactivity fixation on insoluble blood cells fraction at the higher concentrations used (200 mg/ml).

The qualitative comparison of the shape of the RBC (non-treated and treated with natural extracts) under optical microscopy has revealed strong morphological alterations due to the treatment of blood with *T. avellanedae* extract in the concentrations of 12.5 and 200 mg/ml. The histological preparation of a sample of blood (control-non-treated) with normal shape of RBC is shown in Figure 4. Figures 5 and 6 show histological preparations of blood treated with *T. avellanedae* in which are shown qualitative and strong alterations on the shape of the RBC.

Table 1 shows the distribution of the radioactivity in BC, IF-P and IF-BC treated with different concentrations of lapachol. The analysis of the results indicates that there

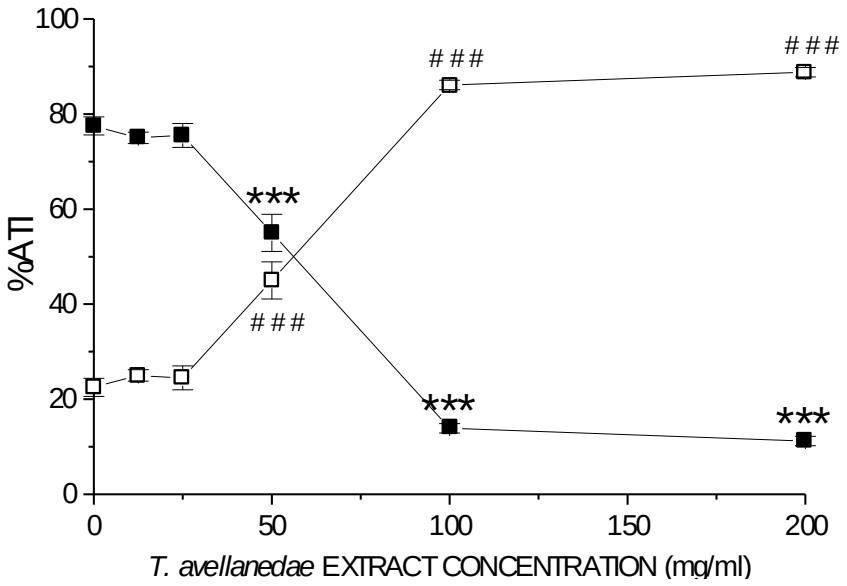

Figure 2. Effect of *T. avellanedae* extract on fixation of ^{99m}Tc by insoluble (IF-P) and soluble (SF-P) fractions of plasma (P). Blood samples were incubated with *T. avellanedae* extract (1 h) and after with $SnCl_2$ and with $Na^{99m}TcO_4$. Insoluble and soluble fractions of plasma (IF-P and SF-P) were obtained by precipitation and centrifuged. The radioactivity in these fractions were counted in a gamma counter and the percentage of radioactivity incorporated (%ATI) was calculated for each fraction. ■, IF-P; □, SF-P. ***, $p \leq 0.001$, when compared to control group of IF-P. ###, $p \leq 0.001$, when compared to control group of SF-P.

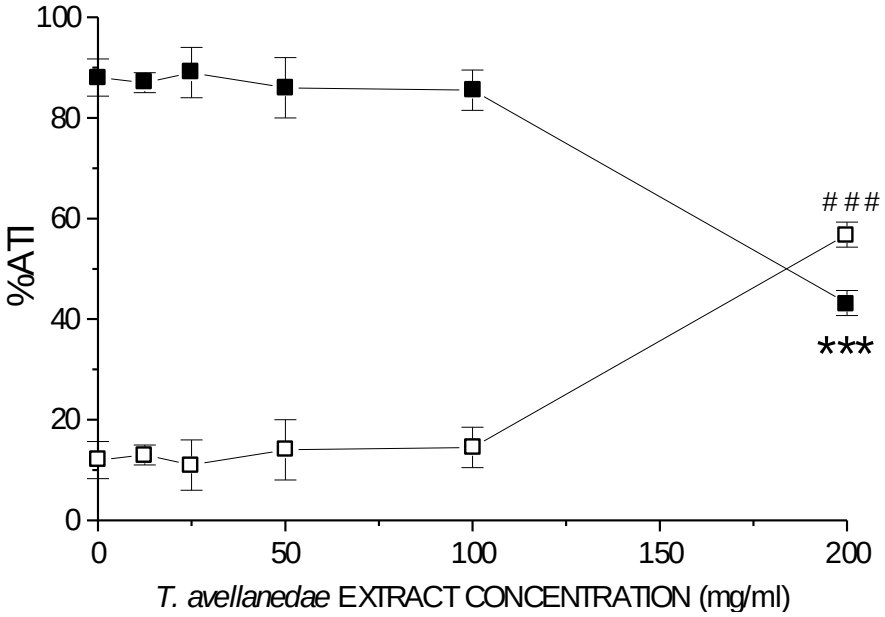

Figure 3. Effect of *T. avellanedae* extract on fixation of ^{99m}Tc by insoluble (IF-BC) and soluble (SF-BC) fractions of blood cells (BC). Blood samples were incubated with *T. avellanedae* extract, after with $SnCl_2$ and with $Na^{99m}TcO_4$. Insoluble and soluble fractions of blood cells (IF-BC and SF-BC) were obtained by precipitation and centrifuged. The radioactivity in these fractions was counted in a gamma counter and the percentage of radioactivity incorporated (%ATI) was calculated for each fraction. ■ IF-BC; □, SF-BC. ***, $p \leq 0.001$, when compared to control group of IF-BC. ###, $p \leq 0.001$, when compared to control group of SF-BC.

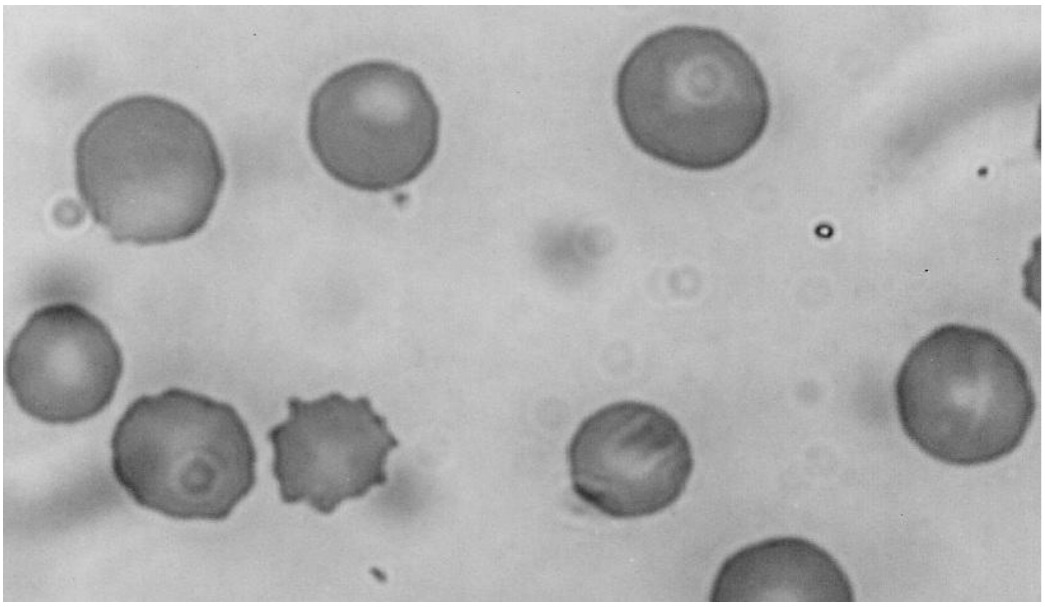

Figure 4. Samples of whole blood were incubated with 0.9% NaCl solution for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then, ^{99m}Tc, as sodium pertechnetate was added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of the red blood cells was evaluated under optical microscope (x1000).

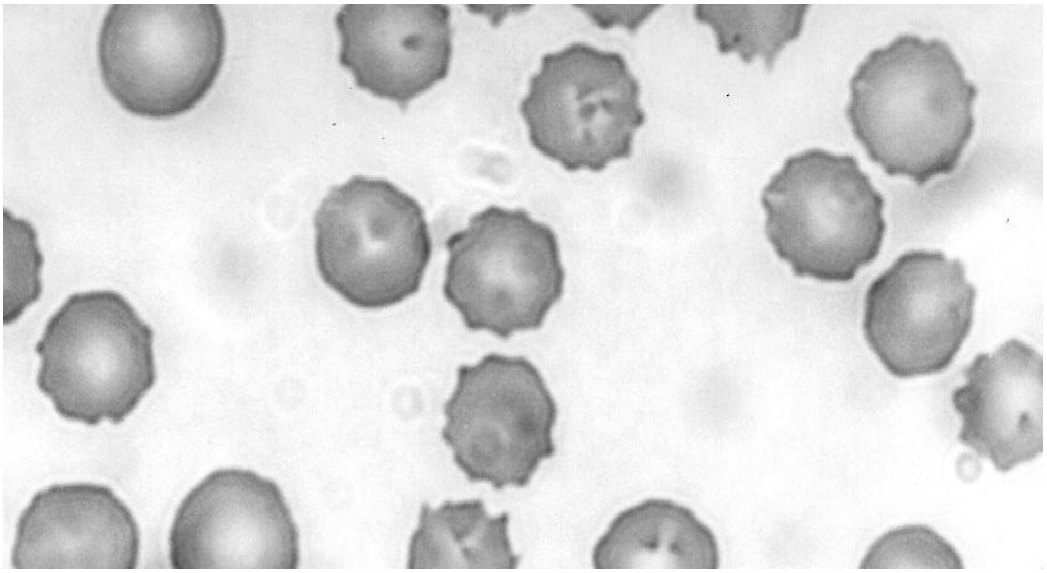

Figure 5. Samples of whole blood were incubated with 20.5 mg/ml of *T. avellanedae* extract for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then, ^{99m}Tc, as sodium pertechnetate was added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of the red blood cells was evaluated under optical microscope (x1000).

is no important alterations ($p>0.05$) of the %ATI on blood compartments, on IF-P and on IF-BC.

Heparinized blood samples of *Wistar* rats were incubated (1 h) with different concentrations of lapachol, saline solution or 0.02 N NaOH (control groups). After, stannous chloride and ^{99m}Tc were added, centrifuged and plasma (P) and blood cells (C) were separated. Another samples of P and BC were precipitated with trichloroacetic acid (5%) and insoluble fractions (IF) were separated. The radioactivity in C, IF-P and IF-BC

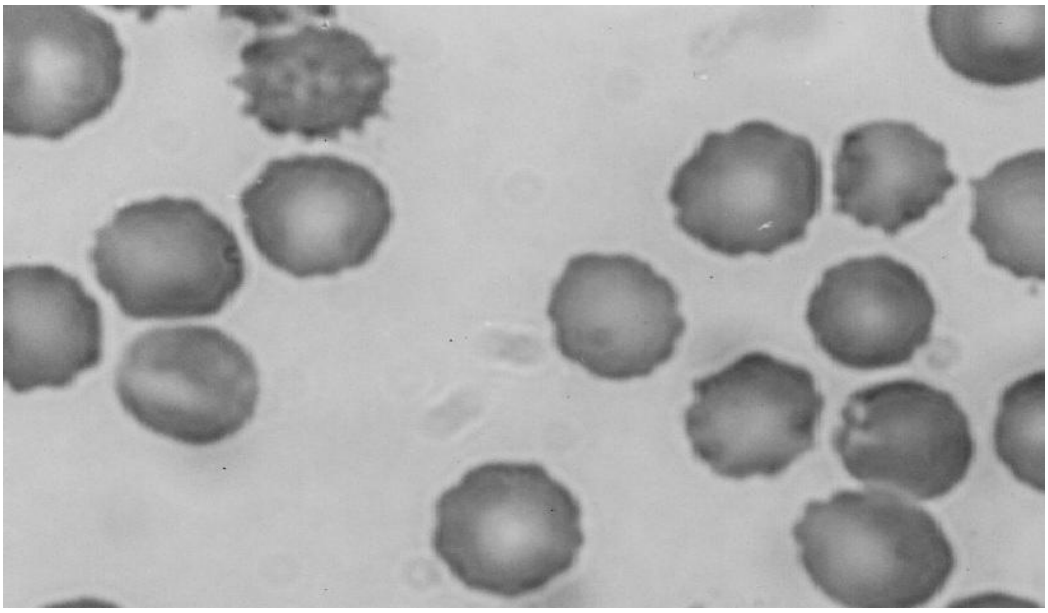

Figure 6. Samples of whole blood were incubated with 200 mg/ml of *T. avellanedae* extract for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then, ^{99m}Tc, as sodium pertechnetate was added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of the red blood cells was evaluated under optical microscope (x1000).

Table 1. Effect of different lapachol concentrations on labeling of blood constituents with ^{99m}Tc.

Lapachol (mg/ml)	Cells	IF-P	IF-BC
0.0	96.4±5.4	81.9±1.7	88.0±3.3
NaOH (0.02 N)	87.1±7.3	82.1±2.6	85.4±4.4
0.05	89.7±4.4	84.3±6.9	87.3±7.1
0.5	86.9±7.8	81.8±4.0	84.8±4.0
5.0	83.2±6.9	80.7±2.8	87.1±3.5
50	89.4±5.0	79.9±2.2	87.4±4.6

DISCUSSION

The evaluation of the influence of drugs on the labeling of blood constituents is highly relevant due to some products, as chocolate, can interfere in the quality of the examinations using red blood cells labeled with ^{99m}Tc (Bustami et al., 2009).

The analysis of data presented in Figure 1 show that the aqueous *T. avellanedae* extract can modify the distribution of ^{99m}Tc between the cellular and plasma compartments almost in all tested concentrations. However, the fixation of ^{99m}Tc in cellular proteins could be altered at high concentrations of this extract (Figure 3). The fixation of the ^{99m}Tc plasma proteins is also blocked by the presence of the *T. avellanedae* extract (Figure 2). This finding is interesting and it suggests that the entrance of the stannous and pertechnetate would be blocked on a depended matter (decreasing the radioactivity on the blood cells) (Figure 1). However, only in the highest concentration of the extract, the fixation of the ^{99m}Tc on the blood proteins would be blocked probably due to the anti-oxidant and/or scavenger activities of the substances in the *T. avellanedae* extract. These redox properties could be associated with the chemical analysis of *T. avellanedae* extracts revealed the presence of various compounds as naphthoquinones, flavonoids, quinoid compounds and phenolic glycosides (Warashina et al., 2004). The phenolic compounds presents in different herbal extracts have been described to possess antioxidant and chelating action and be able to inhibit peroxidation reaction in the living systems (Simoes-Pires et al., 2005; Soobrattee et al., 2005). On the other hand, It was described that antimicrobial effects of β-lapachol could be related to the formation of reactive oxygen species (Guiraud et al., 1994). Thus, some compounds present in *T. avellanedae* extracts could be

capable to impede or facilitate the oxidation of the stannous ions and alter the labeling of cellular proteins with ^{99m}Tc as well interfere with distribution of this radionuclide between plasma and cellular compartments.

Other hypothesis that could explain the effects of *T. avellanedae* extracts on labeling of blood cells with ^{99m}Tc is the interaction of constituents of this extract with ion channels. In fact, it was proposed that the antinociceptive effect of *T. avellanedae* may be related to an activation of the adenosine receptors (de Miranda et al., 2001). Other membrane proteins as band-3 and calcium channel may have their function altered by compounds present in *T. avellanedae* extract decreasing or impeding the transport of Sn^{+2} and $^{99m}TcO_4^-$ into blood cells and in consequence to modify de distribution of ^{99m}Tc between plasma and cellular compartments.

The data obtained in this work show that the labeling of plasma proteins with ^{99m}Tc could be decreased by the aqueous *T. avellanedae* extract used (Figure 2). Pharmacokinetics data have demonstrated that some compounds (as flavonoids) present in herbal extracts can be transported in blood attached to plasma proteins (Guiraud et al., 1994). Moreover, the already cited oxidant chelating properties of compounds present in *T. avellanedae* extract also could be related to effect obtained. Taken together, the binding in same proteins sites that the binding sites of ^{99m}Tc and oxidant/chelating properties of *T. avellanedae* extract compounds could explain the decreasing of labeling of plasma proteins with ^{99m}Tc.

In the procedure of labeling RBC with ^{99m}Tc, the stannous and pertechnetate ions pass through the plasma membrane (Gutfilen et al., 1992). Then, as reported to the tobacco extract (Oliveira et al., *2003*) and to *Maytenus ilicifolia* extract (Oliveira et al., 2000), histological alterations of the red blood cells could be responsible for modifications on the labeling of the RBC with ^{99m}Tc. Furthermore, the results obtained with the qualitative comparison of the shape of the RBC (treated and not treated with *T. avellanedae* extracts) under optical microscopy also justify the modifications in the fixation of ^{99m}Tc by the red blood cells. The achieved results have revealed strong morphological alterations due to the treatment of blood with *T. avellanedae* extract in two of the concentrations studied (Figures 5 and 6).

The analysis of Table 1 suggests that lapachol did not affect the distribution of ^{99m}Tc between cellular and plasma compartments or the binding of this radionuclide in cellular and plasma proteins. The pharmacological actions of lapachol include antitumor, antibiotic, antimalarial, antiinflammatory and antiulceric activities (Subramanian et al., 1998) besides molluscicidal, cercaricidal and trypanocidal activities (Santos et al., 2001; Lima et al., 2004). Oxidative stress and alkylation of cellular nucleophiles have been proposed to explain the lapachol effects on biological system (Bolton et al., 2000). In fact, it was described the generation of reactive oxygen species in the bioactivation of lapachol by P450 reductase (Kumagai et al., 1997) and an electrochemical study (Goulart *et al*., 2003). However, the absence of effects of lapachol labeling of blood constituents with ^{99m}Tc (Table 1) could be related to the concentrations used in this work, or this substance would be not responsible by our findings. Considering the quantity of lapachol in the *T. avellanedae*, probably the concentration of lapachol isolated used in the experiments (Table 1) would be small in comparison with the quantity of this molecule extract in the highest concentration. In consequence, the lapachol concentrations would be too low to induce any effect. In addition, the effect of a chemical compound in an extract is associated with an integrative and synergic action among several compounds (Galindo et al., 2010; Carmona and Pereira, 2013). This fact could occur with the lapachol when was used alone.

In conclusion as the labeling of blood constituents with ^{99m}Tc depends on the presence of a reducing, probably the extract of *T. avellanedae* has substances with redox properties. In addition, probably these properties are not associated with the lapachol or the concentration of lapchol used in this work was not sufficient to promote effect on the labeling process.

Conflict of Interest

The authors declare that they have no conflict of interest.

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