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Studies Involving a Commercial Extract of *Three Ballerina*: ii- Evaluation of the *in Vitro* Effect on the Labeling of Blood Constituents of rats with Technetium-99m and on the Morphology of the Red Blood Cell

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ABSTRACT

The aim of this work is to characterize (electric conductivity and refractive index) a Three Ballerina (TB) and to evaluate its in vitro effect on the labeling of blood constituents with 99mTc and on the morphology of the red blood cells (RBC). Anticoagulated whole blood (Wistar rat) was incubated with a TB extract and the labeling of the blood constituents with technetium-99m (99mTc) was performed. Plasma (P) and blood cells (BC) were isolated and aliquots were also precipitated with trichloroacetic acid to separate soluble (SF) and insoluble fractions (IF). The %ATI in these samples was calculated. The morphology of the treated RBC showed no shape's qualitative alterations. The TB extract was characterized with an electric conductivity of 1.35±0.04mSv/cm and refractive index of 2.21±0.15%BRIX. TB extract decreased significantly ($p<0.05$) the radioactivity distribution in the cellular compartment from 96.97±1.30% to 88.48±7.13%, and in IF-P from 74.29±4.12 to 14.26±5.73%. In conclusion, our data show some physical chemical parameters that could be suitable to characterize the preparation of an extract of TB. Moreover, substances present in the TB extract should probably have an effect on transport of the ions through the RBC membrane and/or should have redoxi properties and the stannous ion would decrease and could justify the effect on the fixation of the radioactivity on the plasma proteins. Moreover, although our experiments were carried out with animals, it is suggested precaution in the interpretation of the examinations that use labeled blood constituents in patients who are undergone TB extract.

INTRODUCTION

Technetium-99m (99mTc) has been the most utilized radionuclide in the single photon emission computed tomography (SPECT) (Harbert *et al.*, 1996; Prech *et al.*, 2006; Rasilla *et al.*, 2009; Schinkel *et al.*, 2010). It has also been used in basic research (Burke *et al.*, 2005; Petterson *et al.*, 2005; O'Connor *et al.*, 2009; Gropler *et al.*, 2010). Red blood cells (RBC), plasma proteins, platelets and white blood cells) have been labeled with this radionuclide and used as radiopharmaceutical (radiobiocomplex) (Rasilla *et al.*, 2009; Schinkel *et al.*, 2010; Bernardo-Filho *et al.*, 2005). 99mTc- labeled RBC scan is the nuclear study best suited for identifying slow-bleeding sources as gastrointestinal bleeding (Karacalioglu *et al.*, 2003; Manning-Dimmitt *et al.*, 2005; Kiratti *et al.*, 2009), as well as (i) to the determination of the left ventricular

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function by measuring the ejection fractions (Gropler *et al.*, 2010; Leitha et al, 2001) and (ii) to the evaluation of wall motion abnormalities in cardiovascular nuclear medicine (Prech *et al.*, 2006; Schinkel *et al.*, 2010; Sampson, 1999). RBC has been labeled with 99mTc for *in vitro*, *in vivo* or *in vivo*/*in vitro* techniques. This labeled process with 99mTc, as sodium pertechnetate, depends on a reducing agent and stannous ion (Sn^{2+}) is usually used for this purpose (Harbert *et al.*, 1999; Prech *et al.*, 2006; Oliveira *et al.*, 2003; Moreno *et al.*, 2005). When whole blood is used for the labeling of RBC with 99mTc, radioactivity is mainly found on RBC, however it is also bound on plasma proteins (Saha, 2010; Bernardo-Filho *et al.*, 1990). Besides the clinical applications of this technique involving the labeling of blood constituents with 99mTc, an experimental model has emerged with this same procedure. This model has been used for several authors to assess some effects and properties associated with natural and synthetic products (O'Neil *et al.*, 1998; Oliveira *et al.*, 2000; Lima *et al.*, 2002; Moreno *et al.*, 2002; Oliveira *et al.*, 2002; Diré *et al.*, 2004; Santos-Filho *et al.*, 2004; Santos-Filho and Bernardo-Filho, 2005; Misra *et al.*, 2007).

Qualitative morphological analysis is also a method that has been used to evaluate if the effects of drugs (synthetic and natural) on the labeling of RBC with 99mTc could be also related to changes on the shape of these blood cells (Benarroz *et al.*, 2007; Benarroz *et al.*, 2008; Oliveira *et al.*, 2003).

The use of medicinal plants has grown in world (Rotblat and Ziment, 2002; Gullett *et al.*, 2010), and the development and implementation of experimental models (Júnior and Pinto, 2005; Nakhai *et al.*, 2007) are important to permit a better comprehension of the action mechanisms of these natural products.

"3 Ballerina" Tea Dieters' Drink is blended with the premium natural herbs. Following the instructions of the manufacturer (Truong Giang Corp.), "this special formula Dieters' Drink is all natural tea, soothing and relaxing especially delightful for those desiring to adjust weight", although 'this statement has not been evaluated by the Food and Drug Administration".

An useful physical chemical property to aid to characterize and to estimate the purity or to determine the concentration of a substance or solution is the refractive index (Nautiyal and Tiwari, 2011). Electric conductivity and the absorbance spectrum profile are other physical parameters that could be measured and also used to characterize a preparation of unknown composition, such as an extract of a medicinal plant (Méhémie *et al.*, 2007).

Publications about physical properties of TB extract were not found yet in PubMed (www.pubmed.com). Moreover, since that the extract of TB can be used also humans and several effects of this natural product are not well understood; the aim of this work was to characterize some physical-chemical properties and to evaluate the effect of TB aqueous extract on the labeling of blood constituents with 99mTc using an *in vitro* experimental model, as well as to verify the consequences of this extract on the morphology of the RBC.

EXPERIMENTAL

Animals

The experiments were performed with rats maintained in a controlled environment. The animals had free access to water and food and the ambient temperature was kept at 25±2 °C. The experiments were carried out without sacrificing the animals. Heparinized whole blood was withdrawn by cardiac puncture from adult male *Wistar* rats (9 animals, 3 months of age, 250±15 g of weight). All the experimental procedures have followed the Ethical Guidelines of the *Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro* (Number CEA/024/2009).

Preparation of TB extract

As the commercial extract of *Three Ballerina* (dried powder, *Astron Comercial LTDA, São Paulo, Brasil*, Lot 1563) has only small solubility, a solution with 2.34 g of *Three Ballerina* extract was prepared with 100 ml of a hot (ebullition) 0.9% NaCl (saline). The preparation was centrifuged (clinical centrifuge, 2000 rpm, 15 min) and the supernatant was isolated. Then, the obtained solution was considered 23.4 mg/ml (100%). Saline was used in all the dilutions. All experiments were carried out during the period of validity of this product.

Spectrophotometry of TB extract

Absorbance spectrum (Spectrophotometer, 800M, *Analyser Comércio e Indústria Ltda., São Paulo, Brazil*) was determined with the TB extract (23.4 mg/ml) prepared as described above in the range of 400–700 nm. Saline solution was used as the blank. The absorbance was measured at each interval of 10 nm. This value was considered as the marker of the reproducibility of the conditions used to prepare all the extracts utilized in the assays.

Electric conductivity of TB extract

Electric conductivity (mS/cm) of the TB extract was performed with a conductivimeter (*Marte Balanças e Aparelhos de Precisão Ltda, São Paulo*). Saline solution was used as the control. This method was considered as the second marker of the reproducibility of the conditions used to prepare all the extracts utilized in the assays.

Refractive index of TB extract

The refractive index (%BRIX) of TB extract was measured with a refractometer (Ningbo Utech International Co. Ltd., Ningbo, People's Republic of China) at room temperature. Saline solution was also used as the control. This method was considered as the third marker of the reproducibility of the conditions used to prepare all the extracts utilized in the assays.

In vitro radiolabeling of blood constituents

Samples of 0.5 ml of whole blood were incubated with 100 µl of different concentrations of diluted (0.9% NaCl) TB extract (6.25, 12.5, 25, 50 and 100%) for 1 hour at room

temperature. A sample of heparinized whole blood was incubated with saline solution (NaCl 0.9%) as control. All the tubes used in this experiment were previously closed with a rubber cap and a syringe was used to reduce the air atmosphere (vacuum) inside the vials. Then, 0.5 ml of a freshly prepared stannous chloride solution (1.2 µg/ml), as $SnCl_2$ (Sigma, USA) was added and the incubation continued for another 1 hour. After this period of time, 99mTc (0.1 ml with 370kBq), as sodium pertechnetate, recently milked from a 99Mo/99mTc generator (*Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, Brasil*), was added and the incubation continued for another 10 min. These samples were centrifuged and plasma (P) and BC cells were separated. Samples (20 µl) of P and BC were also precipitated with 1 ml of trichloroacetic acid 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in P, BC, IF-P, SF-P, IF-BC and SF-BC were determined in a well counter (Automatic Gamma Counter, Packard Instrument Co, USA). After that, the % of radioactivity (%ATI) was calculated as described elsewhere (Oliveira *et al.*, 2003; Oliveira *et al.*, 2002).

Morphological evaluation of RBC

Preparations for morphological (microscopic) analyses were carried out with blood samples treated *in vitro* with TB extract at different concentrations for 60 minutes at room temperature or with saline solution as the control group. Blood smears were prepared, dried, fixed, and staining by the May-Grünwald-Giemsa method. After that, images of the RBCs were acquired (CANON, model Power Shot SX200 IS) from blood smears to analyze the qualitative morphology by optical microscopy (NIKON, model E 200, x 1000). Three independent researches with expertise in analysis of blood smears have done a qualitative analysis of the RBC.

Analysis of the results

The data were analyzed using the GraphPad InStat (version 3.01 for Windows 95/NT, GraphPad Software, San Diego Ca, USA). Data from the analyses were tested for any differences between treatments using one-way analysis of variance (ANOVA). The means and standard errors of the means (mean ± SE) are also reported. Test with the significance level being $p<0.05$.

RESULTS

Physical chemical determinations, as electric conductivity and refractive index of the extract of TB were performed. The value of electric conductivity (1.35±0.04mS/cm) of the extract at higher concentration was used as a marker of the reproducibility of the conditions used to prepare the extract. The value of the refractive index (2.21±0.15%BRIX) of the extract at higher concentration was used as another marker of the reproducibility of the conditions used to prepare the extract.

Table 1 shows the distribution of the radioactivity in the cellular and plasma compartments isolated from whole blood treated with different concentrations of the TB extract. The results indicate that there is a significant decrease ($p<0.05$) on the distribution of the 99mTc in the cellular compartment from 96.97±1.30% to 88.48±7.13% in presence of TB extract.

Table. 1: Effect of TB extract on the distribution of the radioactivity in the cellular and plasmatic compartments.

TB concentrations (%)	Cellular compartment	Plasma compartment
0.00 (control)	96.97±1.30	3.03±1.30
6.25	96.56±0.39	3.43±0.39
12.50	98.73±0.22	1.26±0.22
25.00	97.14±0.67	2.85±0.67
50.00	98.24±1.28	1.75±1.28
100.00	88.48±7.13	11.51±7.13

Samples of heparinized blood were incubated with different concentrations of TB extract. A sample of heparinized whole blood was incubated with saline solution (NaCl 0.9%) as control. Then, stannous chloride (1.2µg/ml) and 99mTc, as sodium pertechnetate were added. The % ATI in plasma and cellular compartments were determined in a well counter and the percent of radioactivity was calculated.

Table 2 shows the distribution of the radioactivity on the soluble and insoluble fractions of the cellular compartment isolated from whole blood treated with different concentrations of the TB extract. There is no alteration in the 99mTc fixation by the cellular proteins (IF-BC) in presence of TB extract.

Table 3 shows the distribution of the radioactivity on the soluble and insoluble fractions of the plasma compartment isolated from whole blood treated with different concentrations of the TB extract. There is a significant and strong ($P<0.05$) decrease on the radioactivity fixation in the plasma proteins (IF-P) in presence of TB extract from 74.29±4.12 to 14.26±5.73%.

Table. 2: Effect of TB extract in the labeling of soluble (SF) and insoluble (IF) fractions of the BC with 99mTc .

TB concentrations (%)	IF-BC	SF-BC
0.00 (control)	93.45±7.64	6.54±7.64
6.25	96.49±1.69	3.50±1.69
12.50	98.26±0.72	1.73±0.72
25.00	95.45±4.67	4.54±4.67
50.00	97.66±0.99	2.33±0.99
100.00	96.43±1.95	3.56±1.95

Samples of heparinized blood were incubated with different concentrations of TB extract. A sample of heparinized whole blood was incubated with saline solution (NaCl, 0.9%) as control. Then, stannous chloride (1.2µg/ml) and 99mTc, as sodium pertechnetate were added. ATI% in IF-BC and SF-BC were determined in a well counter and the % of radioactivity was calculated.

Table. 3: Effect of TB extract in the labeling of soluble (SF) and insoluble (IF) fractions of the plasma (P) with 99mTc

TB concentrations (%)	IF-P	SF-P
0.00 (control)	74.29±4.12	25.70±4.12
6.25	72.11±0.84	27.88±0.84
12.50	71.38±0.23	28.61±0.23
25.00	8.64±7.59	91.35±7.59
50.00	9.58±8.95	90.41±8.95
100.00	14.26±5.73	85.73±5.73

Samples of heparinized blood were incubated with different concentrations of TB extract. Sample of heparinized whole blood was incubated with saline solution (NaCl, 0.9%) as control. Then, stannous chloride (1.2µg/ml) and 99mTc, as sodium pertechnetate were added. The %ATI in IF-P and SF-P were determined in a well counter and the percent of radioactivity was calculated.

Figures 1 and 2 show photomicrographs of smears from blood treated with saline solution (control) and with TB extract at the higher concentration used (23.4mg/ml), respectively. The

qualitative morphological analysis suggests that the treatment with TB extract does not induce important changes in the shape of RBC observed under optical microscopy.

DISCUSSION

To our knowledge, there is only a description about physical chemical properties of TB extracts. Pinto et al has reported that a TB extract has an absorbance spectrum with a peak in 490 nm (Pinto *et al.,* 2011). This fact has also stimulated this investigation about another physical chemical property of a commercial TB extract. This study shows that TB extract could be characterized by an electric conductivity of 1.35±0.04mSv/ml and a refraction index of 2.21±0.15%BRIX. These physical parameters could be used in other studies to characterize the preparation conditions of an aqueous TB extract.

An experimental model has emerged from the studies involving the effects of drugs (natural and synthetic) on the labeling of blood constituents with 99mTc and important findings have been reported by several authors (Sampsom, 1999; Oliveira *et al.,* 2003; Moreno *et al.,* 2002; Oliveira *et al.,* 2002; Diré *et al.,* 2004; Caprilles *et al.,* 2002; Fonseca *et al.,* 2005; Frydman *et al.,* 2004; Fernandes *et al.,* 2005). This procedure has been proposed as an *in vitro* assay to study some properties, as chelating/redoxi activities or interactions on cellular membrane, of products used daily by humans (Benarroz *et al.,* 2007; Fonseca *et al.,* 2007.

The analysis of the data presented in Tables 1 indicates that the TB extract could alter the distribution of 99mTc between P and cellular compartments. In addition, the alteration of the fixation of this radionuclide on plasma proteins was also found (Table 3). In general, the labeling of blood constituents could decrease because of action of drugs by (a) alteration of the plasma membrane structure or modifying the transport systems of stannous and pertechnetate ions into cells, (b) direct oxidation or generation of free radicals that could oxidize the stannous ion, (c) direct inhibition (chelating action) of the stannous and pertechnetate ions, or (d) binding at same sites on the blood constituents.

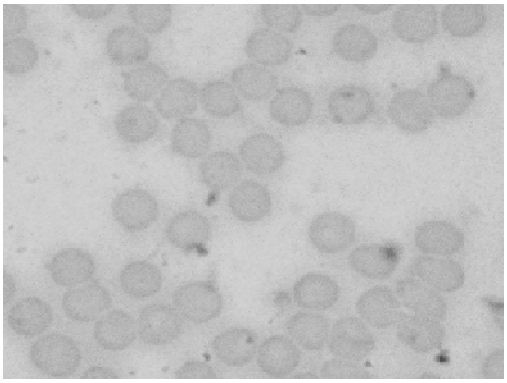

Fig 1: Photomicrograph of blood smear from blood samples treated with saline (control). Blood samples from Wistar rats were incubated with saline (0.9% NaCl) for 1 hour. After that, blood smears were prepared, dried, and stained by the May-Grünwald-Giemsa method. The slides were analyzed by optical microscopy (X 1,000).

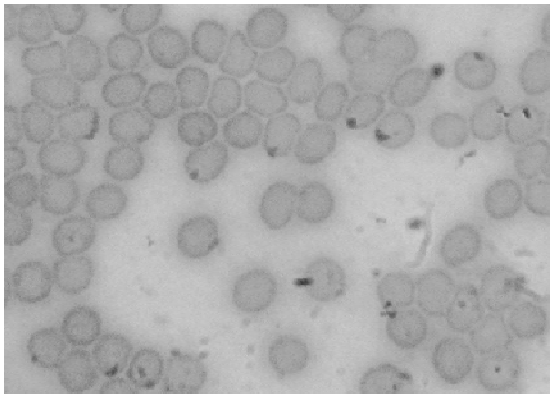

Fig. 2: Photomicrograph of blood smear from blood samples treated with TB extract. Blood samples from Wistar rats were incubated with TB extract (23.4mg/ml) for 1 hour. After that, blood smears were prepared, dried, and stained by the May-Grünwald-Giemsa method. The slides were analyzed by optical microscopy (X 1,000).

Interactions involving the ion transport systems could alter the transport of stannous and pertechnetate ions, decreasing the labeling of RBC with 99mTc and explain, in part, the data obtained (Table 1). In fact, other researchers have demonstrated that drugs that interact with calcium channels (Gutfilen *et al.,* 1992) and band-3 protein (Callahan and Rabito, 1990) to alter the labeling of RBC with 99mTc. Moreover, no modifications, at least under optical microscopy, of the shape of RBC were not found when blood samples were incubated with TB extract (Figures 2 and 3). This fact suggests that other mechanisms could be involved in the decrease of the labeling efficiency of RBC with 99mTc (Table 1), as alteration of the transport of the ions related with this labeled process or direct or indirect oxidation of the stannous ion or chelating of the stannous and pertechnetate ions.

The redoxi properties of phenolic compounds present in medicinal plants have been related to various mechanisms: free radical scavenging activity, transition metal chelating, and singlet oxygen-quenching capacity (Shan *et al.,* 2005). These substances could act as chelators on stannous ions, decreasing the distribution of radioactivity in blood cell compartment (Table 1) and the fixation of 99mTc on plasma proteins (Table 3). In fact, experimental data have suggested antioxidant and pro-oxidant actions of caffeine and its metabolites (Azam *et al.,* 2003). Other data have suggested that caffeine could alter the labeling of blood constituent at higher concentrations (Frydman *et al.,* 2004). These findings and our morphological data from RBC could indicate that TB extract could alter the labeling of blood constituents with 99mTc due to chelating/redox properties of chemical compounds at the highest concentration of TB.

Although scientific information about the pharmaco-kinetics and pharmacodynamics of the products present in TB is scarce, in general, the actions of drugs has been shown to be depend on the plasma protein binding (Musteata *et al.,* 2006). Despite previous data indicating that caffeine at low concentrations does not alter the radiolabeling of plasma and cellular proteins (Frydman *et al.,* 2004), other chemical compounds in TB extract could decrease the fixation of 99mTc on

these proteins (Table 3). An unexpected finding is the no alteration of the labeling of the blood cell proteins in presence of extract of TB (Table 2). Probably, the quantity of the TB that is inside the RBC is not capable to interfere also on the fixation of the 99mTc on the blood cell proteins.

CONCLUSION

Our data show some physical chemical parameters that could be suitable to characterize the preparation of an extract of TB. Moreover, substances present in the TB extract should probably have an effect on transport of the ions through the RBC membrane and/or should have redoxi properties and the stannous ion would decrease and could justify the effect on the fixation of the radioactivity on the plasma proteins. Moreover, although our experiments were carried out with animals, it is suggested precaution in the interpretation of the examinations that use labeled blood constituents in patients who are undergone TB extract.

DECLARATION OF INTEREST

All the authors declare that there are no conflicts of interest in this article.

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