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An aqueous Extract of *Vitex agnus castus* Alters the Labeling of Blood Constituents with Technetium-99m

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ABSTRACT

The development of experimental assays to study properties of herbal medicine is worthwhile. Vitex agnus castus (VAC) is utilized in popular medicine and some actions have been attributed to its extract. Blood cells (BC) and plasma proteins are labeled with technetium-99m (Tc-99m) and have been used in nuclear medicine, as in basic research. This procedure uses a reducing agent and stannous ion is utilized. There are reports that drugs can alter this labeling process. The aim of this work was to evaluate the influence of an aqueous extract of VAC on the labeling of blood constituents with Tc-99m. Blood was incubated with VAC, stannous chloride and Tc-99m, as sodium pertechnetate, and centrifuged. Samples of BC and plasma were separated, aliquots of BC and plasma were also precipitated with trichloroacetic acid to obtain soluble and insoluble fractions and the percentage of radioactivity (%ATI) was determined. The results show a statistical ($p<0.05$) alteration in the %ATI on blood compartments and on the insoluble fractions of plasma and BC. Probably, this extract would have chemical compounds with oxidant properties.

Key words: Oxidant properties; Plasma proteins; Red blood cells; Technetium-99m; *Vitex agnus-castus*

INTRODUCTION

The use of natural products, as the medicinal plants, has increased in most industrialized countries of the world, as part of a resurgent belief in natural and traditional medicine. Most of the herbal therapies in use around the world have not been developed based on the findings of controlled clinical studies or modern scientific investigations. In consequence, physicians and other healthcare practitioners are feeling the pressure to inform themselves and the population about the properties of herbal medicines (Rotblatt and Ziment, 2002). Moreover, medicinal plants are used for the human being and several biological effects and the consequences for the health have not been well established yet. Many plants contain active substances that can induce biological effects and their frequent use has been correlated with a high

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incidence of diseases or undesired biological effect in the population (Fonseca *et al*, 1994).

In herbal medicine, many important properties and actions have been attributed to *Vitex agnus* castus (VAC) (Daniele et al., 2005; Atmaca et al., 2003). VAC is a deciduous shrub that is native to Mediterranean Europe and Central Asia (Daniele et al., 2005; Atmaca et al., 2003). Traditionally, VAC fruit extract has been used in the treatment of many female conditions, including menstrual disorders (amenorrhoea, dysmenorrhoea), premenstrual dysphoric disorder, corpus luteum insufficiency, hyperprolactinaemia, infertility, acne, menopause and disrupted lactation (Daniele et al., 2005; Atmaca et al., 2003; Rotblatt and Ziment, 2002). Danielle et al. (2005) have also reported the use of VAC to treat cyclic breast pain, inflammatory conditions, diarrhea and flatulence. Ohyama et al. (2003) and Ohyama et al. (2005) have evaluated the cytotoxicity and apoptotic inducibility of VAC fruit extract and have verified that the cytotoxic activity of this extract may be attributed to the effect on cell growth, that cell death occurs through apoptosis, and this apoptotic cell death may be attributed to increase intracellular oxidation by the VAC treatment. Weisskopf et al. (2005) have reported similar results of the VAC treatment using another cellular line.

In nuclear medicine, SPECT (single photon emission computed tomography) and/or PET (positron emission tomography) using radiobiocomplexes (radiopharmaceuticals) provide metabolic images that help to define the clinical diagnosis of a disease. Technetium-99m (Tc-99m) has been the most utilized radionuclide in the diagnosis in the nuclear medicine procedures (SPECT) and several chemical compounds and cellular structures used as radiobiocomplexes (Bernardo-Filho et al., 2005) are labeled with it. A reducing agent is necessary in this labeling and the stannous chloride is normally used (Hladik III et al., 1987; Early and Sodee, 1995; Saha, 2004). Tc-99m has also been used to label biological structures (Marques et al., 20004; Bernardo-Filho et al., 1993) and in several investigations in basic research (Bernardo-Filho et al., 1983; Bernardo-Filho et al., 1994; Oliveira et al., 2000; Moreno et al., 2004.

Blood constituents, as red blood cells (RBC) or plasma proteins, have been labeled with Tc-99m and used as radiobiocomplexes in many clinical applications (Hladik III et al., 1987). RBC had been labeled with this radionuclide for *in vitro*, *in vivo* or *in vivo/in vitro* techniques. This labeling depends also on a reducing agent and stannous ion (Sn^{2+}) is usually used. The fixation of the Tc-99m in the RBC is mainly in the beta-chain of the hemoglobin. However, it is also bound outside of the RBC, on the plasma proteins. This labeling process depends on optimal stannous chloride concentration, and stannous and pertechnetate ions seem to across the erythrocyte membrane. The band-3 anion transport system and calcium channels may be the ways that Tc-99m-pertechnetate and Sn^{2+} ions have, respectively, to reach the interior of the RBC (Bernardo-Filho et al., 1992; Dewanjee, 1974; Callahan and Rabito, 1990; Gutfilen et al., 1992).

Many drugs (natural and synthetic medications) have been reported to affect the labeling of blood constituents with Tc-99m (Hesslewood and Leung, 1994; Nigri et al., 2002). *Maytenus ilicifolia* (Oliveira et al., 2000), *Fucus vesiculosus* (Oliveira et al., 2003), and *Gingko biloba* (Moreno et al., 2004) decrease the fixation of the radioactivity on blood constituents and on the insoluble fractions of the blood cells and plasma. *Pneumus boldus* (Reineger et al., 1999) does not decrease this labeling process.

The labeling of blood constituents with Tc-99m has also been used to study some properties of extract of medicinal plants and we are trying to combine these results to improve an experimental model to evaluate effects of the plants used as food, additives or medicines. In this work, the influence of an aqueous VAC extract on the labeling of blood constituents with Tc-99m using an *in vitro* technique (Bernardo-Filho et al., 1983) will be evaluated.

MATERIAL AND METHODS

An *in vitro* technique used to label blood constituents (Bernardo-Filho et al.., 1983) is described elsewhere and in these experiments it was slightly modified. These experiments were performed without sacrificing the animals.

A commercial *Vitex agnus* castus (VAC) (*Herbarium, Laboratório Botânico*, Brazil, Lot 125184, April 2004 and validity August 2007) was used in the assays. As indicated by this manufacturer, lyophilized fruit of VAC was used to prepare this dried powder. In the preparation of the extract, 360 mg of the material fruit was put in

a tube with 10 ml of saline solution (0.9% NaCl) that was gently shaken in the vortex. This suspension was centrifuged in a clinical centrifuge (3000 rpm, 5 min) and the supernatant was considered to be 36 mg/ml. Stannous chloride ($SnCl_2$) was purchased from Sigma Chemical Co., St Louis, USA.

The tubes used in these experiments were previously closed with a rubber cap and a syringe was used to reduce the air atmosphere (vacuum) inside the vials. Heparinized whole blood was withdrawn from *Wistar* rats. Blood samples (0.5 ml) were incubated (100 µl) with different concentrations of VAC preparation (2.25, 4.5, 9.0, 18.0 and 36.0 mg/ml) for 1 hour at room temperature. A sample of heparinized whole blood was incubated with saline solution (0.9% NaCl) as control. Then, 0.5 ml of a stannous chloride solution (1.2 µg/ml) freshly prepared was added and the incubation continued for another 1 hour. After this period of time, Tc-99m (0.1 ml), as sodium pertechnetate, recently milked from a Molibdenium-99/Technetium-99m generator (*Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear*, Brazil), was added and the incubation continued for another 10 min. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 µl) of P and BC were also precipitated with 1ml of 5% trichloroacetic acid (TCA) 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, C5002, Packard, USA). After that, the % of radioactivity (%ATI) was calculated, as previously described (Bernardo-Filho et al., 1983).

The presented results are the averages of ten isolated assays. A statistical analysis (ANOVA test and Bonferroni post test, with significance level ($p<0.05$) was utilized to compare the results.

RESULTS

Table 1 shows the distribution of the radioactivity in the compartments plasma and blood cells from whole blood treated with different concentrations of VAC solutions. The results indicate that there is a significant ($p<0.05$) decrease in the uptake of Tc-99m by the blood cells compartment from 94.04 ± 1.80 to 66.44 ± 8.60% with the concentration of 36.0 mg/ml of the referred extract.

Table 2 shows the fixation of the radioactivity in the insoluble fraction of plasma obtained from whole blood treated with different concentrations of VAC. The results indicate that there is a significant ($p<0.05$) decrease in the fixation of Tc-99m in the plasma proteins only in the highest concentration (36.0 mg/ml) of the extract, and the radioactivity bound in the insoluble fraction of plasma decreased deeply from 69.72 ± 2.00 to 10.61 ± 2.00%.

Table 3 shows the fixation of the radioactivity in the insoluble fraction isolated from the blood cells obtained from whole blood treated with various concentrations of VAC. The results show that there is a significant decrease ($p<0.05$) in the fixation of Tc-99m in insoluble fractions of the blood cells when the concentrations of 36 mg/ml of the referred drug is used from 83.29 ± 7.10 to 73.44 ± 3.30%.

Table1 - Effect of an extract of VAC on the labeling of cells and plasma with Tc-99m

Concentrations of VAC (mg/ml)	%ATI	
	Blood Cells	**Plasma**
0.00 (control)	96.04 ± 1.8	3.96 ± 1.8
2.25	94.48 ± 0.9	5.52 ± 0.9
4.5	97.97 ± 0.7	2.03 ± 0.7
9.0	97.53 ± 1.2	2.47 ± 1.2
18.0	95.34 ± 3.8	4.66 ± 3.8
36.0*	66.44 ± 8.6	33.56± 8.6

Blood from *Wistar* rats was incubated with VAC extract for 1 hour. Then, radiolabeling procedure of blood constituents was carried out. The samples were centrifuged to plasma (P) and blood cells (BC) separation. Radioactivity in each fraction was counted and the percentage of radioactivity (%ATI) was calculated. (*) $p<0.05$ when compared with control.

Table 2 - Effect of an extract of VAC on the labeling of plasma proteins with Tc-99m.

Concentrations of VAC (mg/ml)	%ATI	
	IF-P	SF-P
0.00 (control)	69.72 ± 2.0	30.28 ± 2.0
2.25	70.66 ± 0.7	29.34 ± 0.7
4.5	66.98 ± 5.9	33.02 ± 5.9
9.0	68.44 ± 7.1	31.56 ± 7.1
18.0	69.23 ± 5.0	30.77 ± 5.0
36.0*	10.61 ± 2.0	89.39 ± 2.0

Blood from *Wistar* rats was incubated with VAC extract and labeling of blood constituents with Tc-99m was performed. Insoluble (IF) and soluble (SF) fractions from plasma (P) were isolated, radioactivity was counted and the percentage of the radioactivity (%ATI) was calculated. (*) $p<0.05$ when compared with control.

DISCUSSION

There is not yet a well-established and general model to study the interaction and/or the effect of xenobiotic drugs (natural or synthetic) in the preparation of radiobiocomplexes. Moreover, the development of experimental models could permit, in general, truth information that a particular drug, mainly the herbal medicines, is useful or useless, safe or toxic and that these conclusions are not based on suboptimal evaluations of the available data (Rotblat and Ziment, 2002). An experimental assay based on the effect of drugs on the labeling of the blood constituents with Tc-99m has permitted to obtain relevant information about the possible redox properties of various chemical compounds (synthetic and natural) (Oliveira *et al.*, 2000; Oliveira *et al.*, 2003; Nigri *et al.*, 2002; Reineger *et al.*, 1999; Fonseca *et al.*, 2005).

Table 3 - Effect of an extract of VAC on the labeling of cells proteins with Tc-99m

Concentrations of VAC (mg/ml)	%ATI	
	IF-BC	SF-BC
0.00 (control)	83.29 ± 7.1	16.71 ± 7.1
2.25	89.57 ± 0.0	10.43 ± 0.0
4.5	85.80 ± 4.0	14.20 ± 4.0
9.0	89.19 ± 1.0	10.81 ± 1.0
18.0	86.38 ± 1.8	13.62 ± 1.8
36.0*	73.44 ± 3.3	26.56 ± 3.3

Blood from *Wistar* rats was incubated with VAC extract and labeling of blood constituents with Tc-99m was performed. Insoluble (IF) and soluble (SF) fractions from blood cells (BC) were isolated, radioactivity was counted and the percentage of the radioactivity (%ATI) was calculated. (*) $p<0.05$ when compared with control.

The analysis of the results obtained with an aqueous VAC extract on the distribution of the Tc-99m in the blood cells compartment and in the fixation of this radionuclide to insoluble fractions of plasma (plasma proteins) and blood cells (blood cells proteins) seems to indicate that this effect is not dose dependent.

As the labeling of blood constituents depends on the presence of a reducing agent, it is possible to speculate that the compounds present on the VAC extract have oxidant properties could oxidize the stannous ion. This fact could reduce the fixation of the Tc-99m in specific targets on the blood constituents. Furthermore, the oxidative stress due to the chemical compounds of the VAC extract (Ohyama *et al.*, 2003; Ohyama *et al.*, 2005) could lead the generation of free radicals and in consequence, the oxidation of the stannous ions and the decrease of the labeling of the blood constituents with Tc-99m. Ohyama et al. (2003) has already suggested that a crude extract of VAC that it was prepared with ethanol from dried ripened VAC fruits growing in Israel would have cytotoxic activity that may be attributed to the effect on cell growth, that cell death occurs through apoptosis, and that this apoptotic cell death may be attributed to increased intracellular oxidation by Vitex extract treatment. Moreover,

the DNA fragmentation in Vitex extract-treated cells was inhibited by the presence of the antioxidative reagent pyrrolidine dithiocarbamate or N-acetyl-L-cysteine. These oxidation properties have already supposed to the action of other herbal medicine, as *Paullinia cupana* (Fonseca *et al.*, 1994).

It is always important have in mind that care must be taken when attempting to extrapolate experimental data to the clinical situation, once the observed effects may depend on the amount and/or nature of the drug. Our results permit to suggest that, the labeling of blood constituents with Tc-99m be altered in presence of an extract of VAC. Similarly, the fixation of radioactivity in the insoluble fraction of the blood cells and plasma proteins is modified, at least, when an in vitro technique to label RBC is used. Moreover, although the results were carried out with animals, it is suggested precaution in the interpretation of nuclear medicine procedure using radiobiocomplexes obtained with blood constituents in patients that are using extract of VAC. Morphological experiments are ongoing to try to verify the action of this extract on the shape of the red blood cells.

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RESUMO

Modelos experimentais são relevantes no estudo de propriedades de plantas medicinais. *Vitex agnus castus* (VAC) é usado na medicina popular. Células sanguíneas (CS) e proteínas plasmáticas são marcadas com tecnécio-99m (Tc-99m) com aplicações na medicina nuclear e em pesquisa. Esse procedimento utiliza um agente redutor e o íon estanoso é usado. Drogas podem alterar esse processo de marcação. O objetivo desse trabalho foi avaliar a influência de um extrato aquoso de VAC na marcação de constituintes sanguíneos com Tc-99m. Sangue foi incubado com VAC, cloreto estanoso e Tc-99m, como pertecnetato de sódio e centrifugado. Amostras de CS e plasma foram separadas, alíquotas de CS e plasma foram também precipitadas com ácido tricloroacético para obtenção de frações solúvel (FS) e insolúvel (FI) e a percentagem de radioatividade (%ATI) foi determinada. Os resultados mostraram uma alteração estatística ($p<0.05$) na %ATI dos compartimentos sanguíneos e nas FI do plasma e CS. Provavelmente, esse extrato poderia ter compostos químicos com propriedades oxidantes.

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