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Artigo

Assessment of the effect of *Bacopa monnieri* (L.) Wettst. extract on the labeling of blood elements with technetium-99m and on the morphology of red blood cells

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RESUMO: "Avaliação do efeito do extrato de *Bacopa monnieri* (L.) Wettst. na marcação de elementos sanguíneos com tecnécio-99m e na morfologia de células vermelhas do sangue." ***Bacopa monnieri*** (L.) Wettst. (BM), uma planta tradicional da medicina ayurvédica, é usada por séculos para problemas de memória, antiinflamatória, antitérmica, sedativa e como agente anti-epiléptico. O extrato BM têm sido extensivamente investigada por diversos autores por seus efeitos neurofarmacológicas. Na medicina nuclear, os glóbulos vermelhos (RBC) marcados com tecnécio-99m (99mTc) tem várias aplicações clínicas. Entretanto, os dados demonstraram que drogas sintéticas ou naturais podem modificar a marcação de hemácias com 99mTc. Como *Bacopa monnieri* é amplamente utilizado em medicina, foi avaliada a sua influência na marcação de hemácias e proteínas plasmáticas com tecnécio-99m (99mTc). Este procedimento de marcação depende de um agente redutor e normalmente o cloreto estanoso é usado. Sangue foi incubado com os extratos BM. Solução de cloreto estanoso e 99mTc foram adicionados. O sangue foi centrifugado e o plasma (P) e células sangüíneas (CS) foram isolados. Amostras de P ou BC também foram precipitadas, centrifugadas e fração insolúvel (FI) e fração solúvel (FS) foram separadas. A porcentagem de radioatividade (%ATI) em BC, IF-BC e SE-P foram calculados. A As% ATI diminuiu significativamente em BC de 95,53±0,45-35,41±0,44, no IF-P de 80,20±1,16 para 7,40±0,69 e no IF-BC em 73,31±1,76-21,26±1,40. O estudo da morfologia de hemácias revelou alterações morfológicas importantes devido a tratamentos com extratos BM. Sugere-se que a ação do extrato BM poderia ser explicada por uma inibição dos íons estanoso e pertecnetato ou oxidação do íon estanoso ou por danos na membrana plasmática.

Unitermos: *Bacopa monnieri,* Scrophulariaceae, tecnécio-99m, células sanguíneas vermelhas, plasma, proteinas, morphologia.

ABSTRACT: *Bacopa monnieri* (L.) Wettst. (BM), a traditional Ayurvedic medicine, used for centuries as a memory enhancing, anti-inflammatory, antipyretic, sedative and antiepileptic agent. BM extract have been extensively investigated by several authors for their neuropharmacological effects. In nuclear medicine, red blood cells (RBC) labeled with technetium-99m (99mTc) have several clinical applications. However, data have demonstrated that synthetic or natural drugs could modify the labeling of RBC with 99mTc. As *Bacopa monnieri* is extensively used in medicine, we evaluated its influence on the labeling of RBC and plasma proteins using technetium-99m (99mTc). This labeling procedure depends on a reducing agent and usually stannous chloride is used. Blood was incubated with BM extracts. Stannous chloride solution and 99mTc were added. Blood was centrifuged and plasma (P) and blood cells (BC) were isolated. Samples of P or BC were also precipitated, centrifuged and insoluble fraction (IF) and soluble fraction (SF) were separated. The percentage of radioactivity (%ATI) in BC, IF-BC and IF-P were calculated. The %ATI significantly decreased on BC from 95.53±0.45 to 35.41±0.44, on IF-P from 80.20±1.16 to 7.40±0.69 and on IF-BC from 73.31±1.76 to 21.26±1.40. The morphology study of RBC revealed important morphological alterations due to treatment with BM extracts. We suggest that the BM extract effect could be explained by an inhibition of the stannous and pertechnetate ions or oxidation of the stannous ion or by damages induced in the plasma membrane.

Keywords: *Bacopa monnieri,* Scrophulariaceae, technetium-99m, red blood cells, plasma, proteins, morphology.

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INTRODUCTION

The use of natural products, as medicinal plants, has increased in the last decades all over the world (Oliveira et al., 2003a, 2003b; Gobindarajan et al., 2005; Barbosa-Filho et al., 2007; Biavatti et al., 2007; Cardoso-Lopes et al., 2008; Quintans-Júnior et al., 2008; Rangel et al., 2008; Rodríguez et al., 2008; Sousa et al., 2008; Mariath et al., 2009). *Bacopa monnieri* (L.) Wettst. (Brahmi) is a creeping annual plant found throughout the Indian subcontinent in wet, damp and marshy areas. *Bacopa monnieri* (BM) is an important constituent of the Ayurvedic material medica and is used to improve memory, intellect (medhya) and epilepsy. Extensive investigations (Russo et al., 2005; Kishore & Singh, 2005; Bhattacharya et al., 2000) indicate that the extract of BM facilitated learning acquisition, improved retention of learning (memory) and to increase the activity of antioxidative enzymes (e.g. superoxide dismutase (SOD), glutathione peroxidase and catalase) (Jyoti et al., 2007). BM extract contains a mixture of triterpenoid, saponins designated as bacoside A and B and number of alkaloids (Singh & Dhawan, 1992; Chowdhuri et al., 2002; Paulose et al., 2007).

In Nuclear medicine technetium-99m (99mTc) has been the most utilized radionuclide both in diagnosis nuclear medicine procedures and in basic scientific research (Srivastava, 1987; Early & Sodee, 1999; Saha, 2003; Hladik et al., 1987; Gutfilen et al., 1993; Bernardo-Filho et al., 1994a, 1994b; Aquino et al., 2007; Paoli et al., 2008; Mousinho et al., 2009). This wide use in nuclear medicine is due to its optimal physical characteristics, convenient availability from Mo-99/Tc-99m generator and negligible environmental impact (Hladik et al., 1987; Early et al., 1999; Saha et al., 2003).

There are several important applications of 99mTc labeled red blood cells (99mTc-RBC), the most important being cardiovascular nuclear medicine. Some other applications include the blood pool of other organs, detection of gastrointestinal bleeding sites, and determination of RBC mass in patients (Hladik et al., 1987; Srivastava, 1987; Callahan & Rabito, 1990; Gutfilen et al., 1993; Porter et al., 1993; Early & Sodee, 1999; Saha., 2003; Bernardo-Filho et al., 1994a). RBCs have been labeled with 99mTc by *in vitro* (Bernardo-Filho et al., 1983; Hladik et al., 1987; Early & Sodee, 1999; Saha, 2003; Bernardo-Filho et al., 1994a, 1994b), *in vivo* (Hladik et al., 1987; Early & Sodee, 1999; Saha, 2003) or by a combination of these two, called *in vivo/in vitro* labeling (Atkins et al. 1980; Srivastava et al., 1984; Hladik et al, 1987; Srivastava, 1987; Bernardo-Filho et al., 1994a; Harbert et al., 1996) techniques. Plasma proteins are also labeled with 99mTc and used for evaluation of lung perfusion and location of placenta (Harbert et al., 1996; Moreno et al., 2002). These labeling techniques involve the pre-tinning of the blood constituents with stannous ions, followed by exposure to 99mTc, as sodium pertechnetate, which is reduced within the cell and remains trapped intra cellularly by the binding in the beta chain of hemoglobin (Harbert et al. 1996; Moreno et al., 2002; Bernardo-Filho et al., 1994a). Sequential steps of the intracellularly labeling process of blood constituents include: (1) transmembrane transport of reducing agent (Sn^{2+}) and $99mTcO4^-$ ions into the internal compartment of the RBC, (2) reduction of 99mTc($99mTcO4^-$) by Sn^{2+}, and (3) subsequent binding of the reduced 99mTc to hemoglobin (Dewanjee et al., 1982; Abreu et al., 2006). The band-3 anion transport system and calcium channels may be involved in transport of these ions (Callahan & Rabito, 1990; Sampson, 1996).

Unexpected patterns of radiopharmaceutical biodistribution can be associated with a disease and dietary conditions (Oliveria et al., 2002). Any chemical, physical or biological agent which alters the chemical identity of the tracer or modifies the physiological status of the organ of interest or modifies its binding capability to plasma proteins or other blood element could be expected to alter the radiopharmacokinetics and the disposition of the radiopharmaceutical in the specific target (Hladik et al., 1987; Srivastava & Straub, 1992; Santos et al., 1995; Oliveira et al., 2002; Moreno et al., 2004). The labeling of red blood cells with 99mTc has been also influenced by patient medications (Hladik et al., 1987; Oliveira et al., 2002).

Authors have reported that many natural or synthetic drugs can alter the labeling of blood elements with 99mTc (Haldik et al., 1982, 1987; Srivastava & Straub, 1992; Gutfilen et al., 1993; Santosh et al., 1995; Oliveira et al., 1997; Oliveira et al., 2003a; 2003b; Frydman et al., 2004; Moreno et al., 2004; Fonseca et al., 2005). There are some studies about the effect of the medicinal plants (*Thuya occidentalis, Nicotiana tabacum, Peumus boldus, Maytenus ilicifolia, Paullinia cupana,* cauliflower) on the labeling of RBC with 99mTc (Hladik et al., 1987; Hesslewood & Leung, 1994; Sampson, 1996; Oliveria et al.,1997, 2002, 2003a; Vidal et al., 1998; Dire et al., 2003). The drugs (natural/synthetic) could alter the labeling of blood constituents acting as antioxidant agent, modify the membrane structure or decrease the efficiency of transmembrane transport system of stannous and pertechnetate ions into cells (Abreu et al., 2006). In this context, we have evaluated the influence of aqueous extract of *Bacopa monnieri* on the labeling of RBC and plasma proteins with 99mTc using an *in vitro* (Bernardo-Filho et al., 1994a, 1994b) technique and we have also studied qualitatively the morphology of the RBC under an optical microscope.

MATERIAL AND METHODS

Preparation of the plant extract

The whole *Bacopa monnieri* plant was dried in shade and then crushed. The crushed materials were

extracted with water (Bhattacharya et al., 2000; Jyoti et al., 2007; Paulose et al., 2007). This extract was dried in vacuum. From this dried product, different concentrations (25.0, 50.0, 100.0 and 200.0 mg/mL) of BM extract were prepared in NaCl 0.9% solution by established method (Oliveira et al., 2000; Moreno et al., 2004).

Female Sprague Dawley rats (250-300 g) were used. The animals were received a standard pelleted rat diet and water, and were maintained under constant environmental conditions (22±5 ^{0}C, 12 h of light/dark cycle). Experiments were conducted in accordance with the Departmental Committee of Animal Ethics and with the Institutional Guidelines of Indian Institute of Chemical Biology, Kolkata, India.

In vitro radiolabeling of blood elements

An *in vitro* technique employed to label RBC and plasma proteins (Bernardo-Filho et al., 1983, 1994a; Oliveira et al., 2000) described elsewhere was used with minor modification. Heparinized whole blood was withdrawn from Sprague Dawley rats. Blood samples of 0.5 mL were gently mixed and incubated with 100 µL of BM extract at different concentrations (25.0, 50.0, 100.0 and 200.0 mg/mL) for 1 h at room temperature. A sample of heparinized whole blood was incubated with 100 µL of NaCl 0.9% as a control. Then, 0.5 mL of stannous chloride (1.2 µg/mL) was added and the incubation continued for another 1 h. After this period of time, 99mTc (0.1 mL), as pertechnetate, ($99mTcO4^-$ used in the labeling procedure was obtained by 2-butanone extraction from a 5N NaOH solution of 99Mo (Misra et al., 1994)) was added and the incubation continued for another 10 min. 99-molybdenum/99m-technetium (kits from Board of Radiation and Isotope Technology (BRIT), Mumbai, India) generator was used for radiopharmaceutical preparation. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 µL) of P and BC were precipitated with 1.0 mL of trichloroacetic acid (TCA) 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-type counter (Gamma ray spectrometer Model: GRS 23C, ECIL). After that, the percentage of radioactivity (% ATI) was calculated, as previously described method (Bernardo-Filho et al., 1983; Bernardo-Filho et al., 1994a, 1994b; Oliveira et al., 2000).

The experiments were repeated ten times and the means and S.D.s were determined. A statistical analysis (Variance analysis, with significance level $P<0.05$, $n = 10$) was utilized to compare the values found.

Histological preparations were carried out with blood samples treated with the BM extract for 60 min at room temperature. Blood smears were prepared, dried, fixed and staining (Junqueira & Carneiro, 1992; Oliveira et al., 1997). After that, the morphology of the red blood cells was evaluated under an optical microscope (X1000).

RESULTS

Table 1 shows the distribution of the radioactivity in plasma and blood cells from blood treated with different concentrations of BM extracts. The analysis of the results indicates that there is a significant decrease in the uptake of 99mTc by the red blood cells (from 95.53 ± 0.45 to 35.41 ± 0.44) with the concentration of 200.0 mg/mL of the referred extracts.

Table 2 shows the distribution of the radioactivity in the insoluble fraction of plasma (IF-P) and Soluble fraction of Plasma (SF-P) obtained from whole blood treated with different concentrations of BM extracts. The analysis of the results indicates that there is a significant decrease in the radioactivity fixation of 99mTc in the plasma proteins (IF-P) with the BM concentration of 25, 50, 100 and 200 mg/mL, and the radioactivity bound in the insoluble fraction of plasma decreased deeply from 80.20±1.16 to 7.40±0.69.

Table 3 shows the fixation of the radioactivity in the insoluble fraction of blood cells (IF-BC) and soluble fraction of the blood cells (SF-BC) obtained from whole blood treated with various concentrations of BM extracts. The analysis of the results indicates that there is a significant decrease in the fixation of 99mTc in insoluble fractions of the blood cells when all concentrations of the BM extracts are used (from 73.31 ± 1.76 to 21.26 ± 1.40).

The qualitative comparison of the shape of the RBC (not treated and treated with the BM extracts) under optical microscopy has revealed important morphological alterations due to the treatment of blood with BM extract in the different concentrations (25, 50, 100 and 200 mg/mL). Figure 1 shows the histological preparation of a sample of blood (control–not treated), and Figure 2 and Figure 3 shown the histological preparations of blood treated with BM extract 25 and 200 mg/mL.

Table 1. Effect of *Bacopa monnieri* extract on the labeling of blood cells (BC and plasma (P) with 99mTc.

Bacopa monnieri extract concentrations (mg/mL)	Percentage radioactivity (%ATI)	
	Blood cells (BC)	Plasma (P)
00.0 (control)	95.53±0.45	04.47±0.45
25.00	75.20±0.37	24.80±0.37
50.00	61.44±0.53	38.56±0.53
100.00	47.71±0.72	52.29±0.72
200.00	35.41±0.44	64.59±0.44

Heparinized blood samples were incubated with different concentrations of BM extracts (25, 50, 100 and 200 mg/mL). Then, stannous chloride (1.2 µg/mL) and 99mTc were added. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. The radioactivity in P and BC were determined in a well counter and % ATI in P and BC were calculated. The results were averages (S.D.s of ten isolated experiments, $p<0.05$).

Table 2. Effect of *Bacopa monnieri* extract on the labeling of insoluble fraction of plasma (IF-P) and soluble fraction of plasma (SF-P) with 99mTc.

Bacopa monnieri extract Concentrations (mg/mL)	Percentage radioactivity (%ATI)	
	Insoluble fraction (IF-P)	Soluble fraction (SF-P)
00.0 (control)	80.20 ± 1.16	19.80±1.16
25.00	66.28 ± 0.51	33.72±0.51
50.00	48.85 ± 0.71	51.15±0.71
100.00	17.11 ± 0.55	82.89±0.55
200.00	07.40 ± 0.69	92.60±0.69

Heparinized blood samples were incubated with B extracts (25, 50, 100 and 200 mg/mL). Then, stannous chloride (1.2 µg/mL) and 99mTc were added. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 µL) of P were precipitated with trichloroacetic acid (TCA) 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in SF-P and IF-P were determined in a well counter and % ATI in SF-P and IF-P were calculated. The results were averages (S.D.s of ten isolated experiments, $p<0.05$).

Table 3. Effect of *Bacopa monnieri* extract on the labeling of insoluble fraction of blood cells (IF-BC) and soluble fraction of blood cells (SF-BC) with 99mTc.

Bacopa monnieri extract Concentrations (mg/mL)	Percentage radioactivity (%ATI)	
	Insoluble fraction (IF-BC)	Soluble fraction (SF-BC)
00.0 (control)	73.31 ± 1.76	26.69±1.76
25.00	54.30 ± 1.24	45.70±1.24
50.00	43.94 ± 1.74	56.06±1.74
100.00	29.45 ± 1.23	70.55±1.23
200.00	21.26 ± 1.40	78.74±1.40

Heparinized blood samples were incubated with B extracts (25, 50, 100 and 200 mg/mL). Then, stannous chloride (1.2 µg/mL) and 99mTc were added. These samples were centrifuged and plasma (P) and blood cells (BC) were separated. Samples (20 µL) of BC were precipitated with trichloroacetic acid (TCA) 5% and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in SF-BC and IF-BC were determined in a well counter and % ATI in SF-BC and IF-BC were calculated. The results were averages (S.D.s of ten isolated experiments, $p<0.05$).

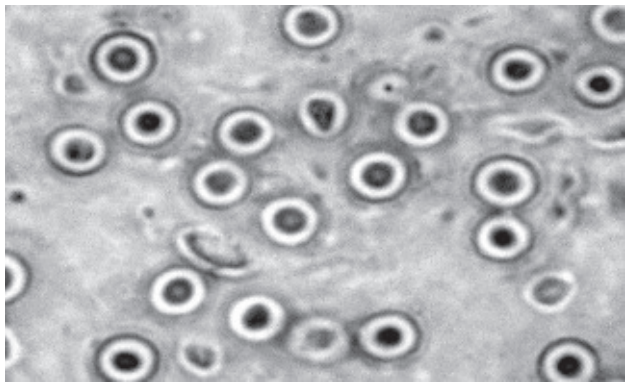

Figure 1. Photomicrography of blood smears prepared with samples of whole blood used to label RBC with 99mTc (control). Samples of whole blood were incubated with NaCl 0.9% solution for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then 99mTc, as sodium pertechnetate was added. Blood smears were prepared, dried, fixed and staining. After that, the morphology of the red blood cells was evaluated under an optical microscope (X1000).

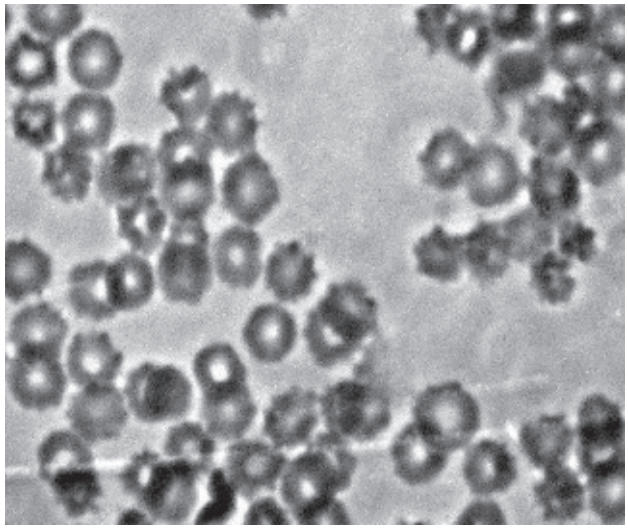

Figure 3. Photomicrography of blood smears prepared with samples of whole blood used to label RBC with 99mTc (blood samples were previously treated BM extract 200 mg/mL). Samples of whole blood were incubated with BM extract (200 mg/mL) for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then, 99mTc, 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 an optical microscope (X1000).

DISCUSSION

There are evidences that various medicinal plants can affect either radiolabeling or biodistribution of red cells in the context of the nuclear medicine clinic, and a number of workers have turned their attention to in vitro testing of the drug (synthetic/natural) with 99mTC labeled blood cells (Hladik et al., 1982, 1987; Sampson, 1996; Frydman et al., 2004; Fonseca et al., 2005). Furthermore, the developing of model that permits to evaluate pharmacologic properties of natural products is worthwhile.

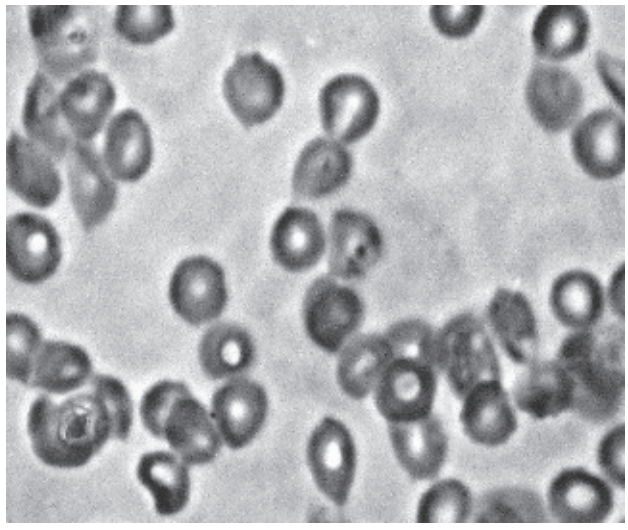

Figure 2. Photomicrography of blood smears prepared with samples of whole blood used to label RBC with 99mTc (blood samples were previously treated BM extract 25 mg/mL). Samples of whole blood were incubated with BM extract (25 mg/mL) for 60 min. After that, stannous chloride solution was added and the incubation continued for 60 min. Then, 99mTc, 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 an optical microscope (X1000).

The use of natural products is increasing over the world. BM extracts are used in popular medicine due to their reported important biological activities (Bhattacharya et al., 2000; Russo & Borrelli, 2005; Kishore & Singh, 2005; Gobindarajan et al., 2005; Jyoti et al., 2007). We have studied the BM extract effect on the labeling of RBC with 99mTc and the fixation of this radionuclide to insoluble fraction of plasma (plasma proteins) and blood cells (blood cells proteins). The results of this study showed that BM extracts reduced the fixation of radioactivity in plasma proteins and blood proteins.

In this labeling process, labeling of RBC with 99mTc depends on the entry of stannous and pertechnetate ions into these cells through ionic channels (Callahan & Rabito, 1990). Then, as reported to the tobacco extracts (Vidal et al., 1998), *Maytenus ilicifolia* (Oliveria et al., 2000), *Sechium edule* (Dire et al., 2003), *Mentha crispa* (Santos-Filho et al., 2002), *Paullina cupana* (Oliveira et al., 2002), *Gingko biloba* (Moreno et al., 2002, 2004), *Fucus vesiculosus* (Oliveira et al., 2003a) and *Psidium guajava* (Abreu et al., 2006) extracts, histological alterations of red blood cells could be responsible for the modifications on the labeling of RBC with 99mTc. However, the results obtained with the qualitative comparison of the shape of the RBC (not treated and treated with natural extracts) under optical microscopy did not justify the modifications in the uptake of 99mTc by the red blood cells in presence of BM extracts. The achieved results have revealed only lightly morphological alterations due to the treatment of blood with BM extracts in the concentrations of 25 and 200 mg/mL.

It is described that natural and synthetic products can alter the labeling of blood constituents with 99mTc (Frydman et al., 2004; Valenca et al., 2005; Fonseca et al., 2005). The labeling of blood constituents could decrease due to the action of drugs (natural and synthetic) in (1) binding at the same sites on the blood constituents, (2) direct inhibition (chelating action) of the stannous (Sn^{+2}) and pertechnetate ions ($99mTcO^{-}$), (3) direct oxidation or generation of free radicals that could oxidize the stannous ion, (4) antioxidant action impeding or decreasing the stannous ion oxidation, and (5) alteration of the plasma membrane structure or modifying the transport systems of stannous and pertechnetate ions into cells.

As in this radiolabelling process of red blood cells with 99mTc depends on the entry of stannous and pertechnetate ions into these cells through plasma membrane (Callahan & Rabito, 1990; Gutfilen et al., 1993), we suggest that BM extracts effect (decrease in the fixation of the radioactivity on RBC, IF-P and IF-BC) might be explained by an inhibition of the transport of these ions, or oxidation of the stannous to stannic ion or by damages induced in plasma membrane or by generating of ROS, as already reported by other medicinal plant extracts (Vidal et al.,1998; Oliveria et al., 2003a, 2003b; Santos-Filho et al., 2004; Moreno et al., 2004; Abreu et al., 2006).

CONCLUSION

In conclusion, our experimental results indicates that 99mTc-RBC, 99mTc-IF-P and 99mTc-IF-BC can be decreased in presence of BM extract and we can suggest that this effect may be due to the active compounds presents in this *Bacopa monnieri* extracts that may (i) complex with these ions (stannous and pertechnetate), or (ii) have direct or an indirect effect on intracellular stannous ion concentration. Experiments with different extracts of medicinal plants are in progress to evaluate the possibility of the generation of these free radicals. Furthermore, this study suggest that the aqueous extracts of *Bacopa monnieri* could present antioxidant action and/or effects on the membrane structures involved in ions transport altering the radiolabeling of blood constituents with 99mTc and that precaution should be taken in examinations of nuclear medicine based on this procedure in patients using *Bacopa monnieri* extracts.

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