

## Experimental Models Using Radionuclides for Assessment of the Effects of Natural Drugs

Silvana Ramos Farias Moreno<sup>1,2\*</sup>, Jorge José de Carvalho<sup>3,5</sup>, Bernardo Rebelo<sup>1,2,5</sup>, Emely Kazan Rocha<sup>4,5</sup>, Margareth de Oliveira Timóteo<sup>1</sup>, Beni Olej<sup>1</sup>, Mário Bernardo Filho<sup>2,5</sup> and Luiz Querino de Araújo Caldas<sup>1</sup>

<sup>1</sup>Universidade Federal Fluminense; Marquês de Paraná, 303; srfmoreno@hotmail.com; 24030210; Niterói - RJ - Brasil. <sup>2</sup>Departamento de Biofísica e Biometria; Rio de Janeiro - RJ - Brasil. <sup>3</sup>Departamento de Histologia e Embriologia; Rio de Janeiro - RJ - Brasil. <sup>4</sup>Departamento de Biologia Celular e Genética; Rio de Janeiro - RJ - Brasil. <sup>5</sup>Instituto de Biologia Roberto de Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Avenida 28 de Setembro, 87, Rio de Janeiro, 20551030, Brasil

### ABSTRACT

The aim of this study was to evaluate the effect of the extracts of *Nectandra membranacea* (*N. membranacea*), *Ginkgo biloba* (*EGb*) and *Passiflora* (*PEF*) on the morphology of red blood cells (RBC), on the biodistribution of sodium pertechnetate (<sup>99m</sup>TcO<sub>4</sub>Na), on the morphology of duodenum and on the labeling of blood constituents (BC, IF-P, IF-BC) with technetium-99m (Tc-99m). Morphometry studies also were performed. The results show that *EGb* promotes alteration of the labeling of BC, IF-P and IF-BC ( $p < 0.05$ ). The *N. membranacea* extract does not promote significant alteration of the radiolabeling, and *PEF* extract alters the IF-P labeling. *N. membranacea*, *EGb* and *PEF* extracts were able to alter the RBC morphology ( $P < 0.05$ ). *N. membranacea* extract and *EGb* modifies the biodistribution of the <sup>99m</sup>TcO<sub>4</sub>Na, and *EGb* influences the morphometry of duodenum isolated from rats ( $P < 0.05$ ).

**Keywords:** *Nectandra membranacea* (*N. membranacea*), *Ginkgo biloba* (*EGb*), *Passiflora edulis flavicarpa* (*PEF*), blood constituents (BC) labeling, technetium-99m, biodistribution

### INTRODUCTION

The use of radionuclides, as radiobiocomplexes, in nuclear medicine has been an important resource for the diagnose and treatment of many diseases (Bernardo-Filho et al., 2005; Zolle, 2007). Blood constituents (BC) are usually labeled with technetium-99m (Tc-99m) and used as radiocomplexes (Bernardo-Filho et al., 2005; Moreno et al., 2005; Zolle, 2007). Blood samples are incubated with the stannous ion and then

exposed to Tc-99m, as sodium pertechnetate (Zolle, 2007). Various factors that can influence the labeling of blood constituents have been described, such as inadequate preparation of reducing agent (stannous ion), the interference of disease and/or the presence of drugs in the patient's plasma (Saha, 2004; Zolle, 2007).

The bioavailability of radiobiocomplexes can be notably altered by a wide variety of conditions, such as drug therapy, radiation therapy, diseases and medicinal plant therapy (Hesslewood and

\* Author for correspondence

Leung, 1994; Gomes et al., 2002; Oliveira et al., 2003; Bernardo-Filho et al., 2005; Moreno et al., 2007a; Moreno et al., 2007b).

*Nectandra membranacea* (*N. membranacea*), from South America, is a plant frequently found in Brazil and has been used by populations to treat several health problems, such as inflammation, hypertension and various other diseases (Simões et al., 2007). *N. membranacea* extracts contain tannins, alkaloids and flavonoid catequins (Simões et al., 2007). *Ginkgo Biloba* extract (EGb) is a medicinal herb that increases the blood flow, acts as platelet activating factor antagonist and prevents the membrane against the damage caused by free radicals (Rotblatt et al., 2002; Simões et al., 2007). Passion fruit, *Passiflora edulis f. flavicarpa* (PEF), is a fruit used to treat increased levels of cholesterol and to evaluate glucose tolerance. It contains pectin, tryptophan, grax acid and sulphured aminoacids (Talcoot et al., 2003).

The possible interference of a *N. membranacea* extract, *Ginkgo biloba* extract and *Passiflora edulis f. Flavicarpa* on the labeling of blood constituents with Tc-99m and on the morphological architecture of red blood cells (RBCs) has not been well documented, and the effect of these plants on the biodistribution and on the morphology of organs has not been evaluated. The aim of this work is to study the *in vitro* effect of the *N. membranacea*, *Ginkgo biloba*, *Passiflora edulis flavicarpa* extracts on: (i) the labeling of RBCs, cellular and plasma proteins with Tc-99m, (ii) the morphology of RBC; as also to evaluate (iii) the effect of the extracts of *N. membranacea* and EGb on the bioavailability of the sodium pertechnetate ( $^{99m}\text{TcO}_4\text{Na}$ ) and (iv) the effect of EGb on the morphometry of duodenum isolated from rats.

## MATERIAL AND METHODS

*N. membranacea* leaves were ("Maciço da Pedra Branca", Rio de Janeiro, RJ, Brazil) identified by a certified biologist and treated by percolation using ethanol 95% (cold extraction). The solvent was completely evaporated under a rotating evaporator under reduced pressure (*Química Orgânica, Universidade Federal Rural do Rio de Janeiro, UFRuRJ, Seropédica, RJ, Brazil*) to obtain an ethanol-free residual extract, as previously reported by Moreno et al. (2007a). The dry extract product was re-dissolved in an aqueous saline

solution (NaCl 0.9 %), and 300 mg of *Nectandra membranacea* was placed in a container with 10 mL of NaCl 0.9 %, to obtain an aqueous preparation of 30 mg/mL (Moreno et al., 2007a). A commercial solution of *Ginkgo biloba* extract (EGb 761, *China Jiangsu Medicines and Health Products* Lot GB 001128, w/w, Galena, RJ, Brazil) containing 24% of flavonoids was prepared in NaCl 0.9%. Saline preparations containing 40 mg/mL were obtained for labeling of blood constituents, as also 400 mg/mL of the commercial extract were prepared for use in biodistribution and morphometry studies. A commercial preparation of *Passiflora edulis flavicarpa*, Peel passion fruit flour (PFE), was obtained from A.S.S. *Neto's Alimentos LTDA.*, RJ, Brazil, (Lot 0001415). In the preparation of the extract, 500 mg of the flour was diluted to 10 mL in NaCl 0.9% to obtain a solution of 50 mg/mL.

Female *Wistar* rats (2 month-old, 180-210 g) were obtained from the *Laboratório de Radiofarmácia Experimental (Universidade do Estado do Rio de Janeiro, RJ, Brazil)*. Experiments were conducted in accordance with the Committee of Animal Care and in compliance with national laws and Guidelines for the Use of Animals in Biomedicals Research (Giles, 1987). Blood samples (0.5 mL) obtained from the rats (n=6) were incubated (60 minutes), and gently mixed, with 100  $\mu\text{L}$  of solution of the extracts (*N. membranacea*, 30 mg/mL; EGb, 40 mg/mL; PEF, 50 mg/mL) or with NaCl 0.9% as controls. Then, 0.5 mL of freshly prepared stannous chloride solution (1.2  $\mu\text{g/mL}$ , *Sigma Chemical Co. St Louis, USA*, Lot 65H26736), was added under vacuum conditions and the incubation was continued for at least 60 minutes. Then, 100 $\mu\text{L}$  of Tc-99m (3.7MBq/mL, *Instituto de Pesquisas Energéticas e Nucleares, CNEN, São Paulo, Brazil*, from a  $^{99}\text{Molybdenium}/^{99m}\text{Technetium}$  generator) as sodium pertechnetate was added (10 minutes). These samples were centrifuged for 5 minutes, and plasma (P) and blood cells (BCs) were separated. Samples (20  $\mu\text{L}$ ) of P and BCs were also precipitated with 1 mL of trichloroacetic acid (TCA, 5 %) and soluble (SF) and insoluble fractions (IF) were separated. The radioactivity in BC, IF-P and IF-BC were determined in a sodium iodide well counter (Automatic Gamma Counter, C5002, Packard, USA). The results are presented as mean $\pm$ SD, with a statistical analysis performed (ANOVA test, Tukey-Kramer test). The

morphometry study of RBC was performed with blood samples incubated with *N. membranacea*, EGb and Peel passion fruit flour (PEF) extracts or with NaCl 0.9% (control) and Tc-99m. One drop of each sample was smeared onto glass slides (5 slides for each sample) and the May-Grünwald-Giemsa (MGG) method was performed. The morphometry of the RBC images was evaluated using Software Image Pro plus (media Cybernetics), for evaluating of the ratio of area and perimeter of the RBCs. A statistical analysis (Kruskal-Wallis with post-test Dunns,  $p < 0.05$ ) was used to compare the experimental data.

For evaluation of the effects on the biodistribution, saline preparations containing 400 mg/mL of EGb commercial extract were administrated to female *Wistar* rats ( $n=5$ ) during 6 days (intra-gastric via). The control group has received 1 mL of a solution of NaCl 0.9%. After that, 0.3 mL of a Tc-99m solution (sodium pertechnetate) was injected into the ocular plexus and the animals were rapidly sacrificed (after 10 minutes). The organs were isolated (brain, liver, duodenum, heart, kidney, lung, stomach, pancreas, blood, bone and thyroid) and the radioactivity was determined. The percentages of radioactivity per gram of each organ (%ATI/gram) were calculated. This procedure was also performed for study of *N. membranacea* extract (30 mg/mL). A statistical analysis of the results (ANOVA test, with Dunnet test,  $p < 0.05$ ) was performed.

Histological preparations were carried out with pieces of duodenum isolated from the animals that had received EGb (400 mg/mL) per intra-gastric via. These pieces (treated and control) were fixed in 2.5% glutaraldehyde, in 0.1 M cacodylate buffer

(pH 7.2) and 0.25% tannic acid (Merck). The tissues were dehydrated in acetone and embedded in Epon. Thin sections (2  $\mu\text{m}$ ) were stained with toluidine blue (*Vetec*, Brazil) and observed under light microscopy (*Olympus BH2-RFCA*, USA). Images of these tissues were recorded on a computer employing the *Nero Pro plus Image* Program. It was used the morphometry analysis with the Mann Whitney test ( $P < 0.05$ ). The Frequency cells/area was the parameter considered to compare the findings of the controls and of the treated animals with EGb.

## RESULTS AND DISCUSSION

It has been reported that phytoterapics may alter the labeling efficiency of blood constituents with Tc-99m, the morphology of RBCs (Diré et al., 2003; Neves et al., 2007) and the bioavailability of radiobiocomplexes (Bernardo-Filho et al., 2005; Moreno et al., 2007a; Moreno et al., 2007b).

Table 1 shows the distribution of the radioactivity in the blood cells (BCs) and in the insoluble fractions of plasma (IF-P) and cells (IF-BCs) from whole blood treated with extracts of *N. membranacea*, EGb and PEF extracts. The results indicate that there is no significant alteration in the distribution of radioactivity for all studied fractions isolated from the whole blood treated with *N. membranacea* extract (Table 1). Probably the *Nectandra membranacea* extract has compounds with anti-oxidant properties that could protect the stannous ion from the oxidation process.

**Table 1**-Effect of *N. membranacea*, EGb and PEF on the radiolabeling of blood constituents.\* $p < 0.05$

	<i>N. membranacea</i>	BC	IF-P	IF-BC	EGb	BC	IF-P	IF-BC	PEF	BC	IF-P	IF-BC
Control	97.8 $\pm$ 0.7	70.4 $\pm$ 8.8	87.33 $\pm$ 3.4	97.2 $\pm$ 1.9	80.7 $\pm$ 2.9	86.6 $\pm$ 5.0	95.5 $\pm$ 1.7	72.7 $\pm$ 3.4	92.8 $\pm$ 1.9			
Treated	97.3 $\pm$ 1.0	61.0 $\pm$ 3.9	89.12 $\pm$ 2.3	*64.9 $\pm$ 6.5	*20.2 $\pm$ 2.2	*75.5 $\pm$ 9.0	95.8 $\pm$ 3.2	*53.9 $\pm$ 6.7	90.6 $\pm$ 2.8			

Figure 1 shows a photomicrograph of blood smears, both control (non incubated, Figure 1A) and treated (incubated with the *N. membranacea*, 30 mg/mL). In the sample incubated with the extract, some blood cells show spikes on their membranes (Figure 1B) differing from normal blood cells, which have a naturally round shape (Figure 1A, control). The results were confirmed by image analysis and the statistical test, that

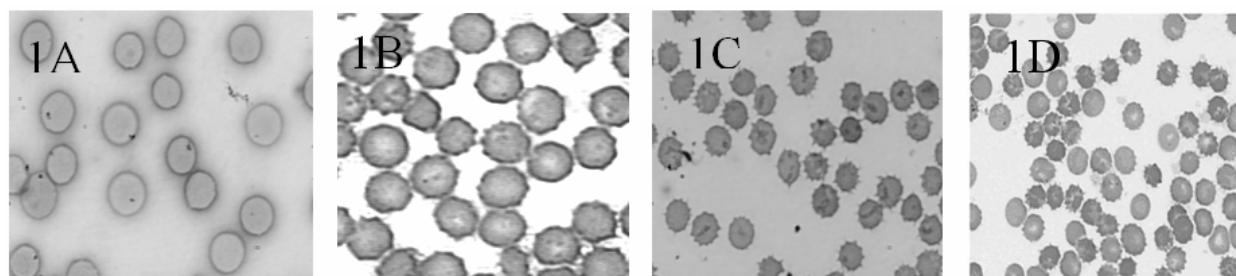
indicated a significant difference ( $p < 0.05$ ) on the morphometry of the RBC treated with *N. membranacea* extract (Figure 1B) when compared with control RBC (Figure 1A). Quantitative analysis of the results showed that the cellular shape perimeter/area ratio was altered ( $P=0.021$ ) in the treated samples, when compared with control samples in figure 1A. The alterations on the morphology of the RBCs induced by the *N.*

*membranacea* extract probably do not interfere with the transportation of the stannous and pertechnetate ion through the RBC membrane, and thus the labeling of RBCs is not modified.

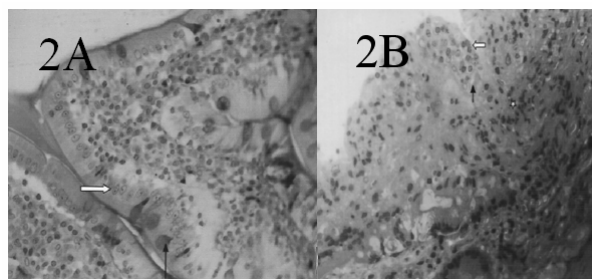
The interference of the *Ginkgo biloba* extract on the uptake of Tc-99m by the BC, IF-P and IF-BC was observed ( $p < 0.05$ ) in Table 1. Figures 1A and 1C show the light microscopy of RBC and important morphological alterations were found (Figure 1C). Diameter/area ratios in the treated RBC were reduced ( $p < 0.05$ ) due to the treatment of the samples with EGb when compared with control samples (Figure 1A). We suggest that the chemical agents present in the EGb could create active metabolites, could directly inhibit the labeling of the stannous/pertechnetate ions, could damage plasma membrane or compete with the cited ions for same bindings sites or generate reactive oxygen species that could oxidize the stannous ion. The active metabolites or the direct action of compounds present in the EGb could also to promote alterations in the morphology of RBCs. Table 1 also shows the effect of the Peel passion fruit flour on the distribution of the radioactivity in cellular compartments, plasma proteins and cellular proteins. The results indicate no significant ( $p > 0.05$ ) alteration of the distribution of Tc-99m between cellular compartments and IF-BC in the samples treated. The results indicate that there is a significant decrease ( $p < 0.05$ ) of the fixation of Tc-99m on the plasma proteins (IF-P), from  $72.7 \pm 3.4$  to  $53.9 \pm 6.7\%$ . Figure 1D shows morphological alterations in samples treated with PEF. Some blood cells present spikes on the membrane, when compared with samples incubated with NaCl 0.9% solution (non

incubated, figure 1A). A perimeter/area ratio of the RBCs was also significantly altered ( $p < 0.05$ ). It is possible to suggest, that the morphological alterations produced on the membrane of RBC by chemical compounds of this extract did not interfere with the ions transport mechanism into the cell and in consequence the labeling of the blood cells was not altered.

The results of the effect of the EGb on the biodistribution of the  $^{99m}\text{TcO}_4\text{Na}$  show a significant ( $p < 0.05$ ) decrease of the uptake of the radiobiocomplex in the duodenum after treatment with EGb from  $1.0 \pm 0.3$  to  $0.5 \pm 0.3$  (Table 2, % ATI). The results of the effect of the *N. membranacea* on the biodistribution of the  $^{99m}\text{TcO}_4\text{Na}$  show a significant ( $p < 0.05$ ) increase of the uptake of the studied radiobiocomplex in the heart, kidney and thyroid after the treatment with this extract from  $0.4 \pm 0.0$  to  $0.8 \pm 0.2$ , from  $0.3 \pm 0.2$  to  $1.0 \pm 0.2$  and from  $3.4 \pm 0.8$  to  $11.8 \pm 2.8$ , respectively (Table 2, % ATI). These data suggest that the metabolization in rats of the extracts of EGb and *N. membranacea* could generate active metabolites to influence the bioavailability of  $^{99m}\text{TcO}_4\text{Na}$  radiobiocomplex. The results about the effect of the EGb on the morphology of the duodenum are indicated in the Figures 2A and 2B. In the control animals, the caliciform and absorptive cells present normal aspect (Figure 2A). In the treated animals these caliciform cells are poorly visible, absorptive cells are irregular and inflammatory cells are present (Figure 2B). The frequency of cells by area (morphometry) also revealed what caliciform cells were altered in animals treated ( $P < 0.05$ ).



**Figure 1** - Photomicrograph of blood smears prepared with samples of whole blood (control, 1A; treated with *N. membranacea*, EGb and PEF extracts, respectively, figures 1B, 1C, 1D). X 100.



**Figure 2** - Photomicrograph of the duodenum obtained of animals treated with EGb: 2A, Control animals, calciform cells. 2B, Treated animals, calciform cells poorly visible. X 400.

**Table 2** - % of ATI per gram of tissue after the treatment with EGb and *N. membranacea*. \*p<0.05.

Organ	EGb 400 mg/mL		<i>N. membranacea</i> 30 mg/mL	
	NaCl (Control)	EGb 400 mg/mL	NaCl (Control)	<i>N. membranacea</i> 30 mg/mL
1. Brain	0.04±0.02	0.06±0.02	0.21±0.03	0.11±0.09
2. Liver	0.64±0.32	0.73±0.36	6.41±1.44	6.98±1.83
3. Duodenum	1.01±0.37	0.51±0.36*	0.23±0.15	0.34±0.25
4. Heart	0.42±0.17	0.35±0.08	0.44±0.07	0.82±0.24*
5. Kidney	0.56±0.18	0.56±0.27	0.35±0.23	1.08±0.27*
6. Lung	0.61±0.35	0.55±0.19	1.12±0.35	1.58±0.24
7. Stomach	5.55±3.30	3.38±1.01	9.95±3.57	13.34±3.88
8. Pancreas	0.21±0.15	0.09±0.04	0.21±0.16	0.40±0.18
9. Blood	3.43±1.50	3.34±0.54	1.10±0.49	1.21±0.58
10. Bone	0.28±0.14	0.21±0.03	0.22±0.05	0.35±0.11
11. Thyroid	2.72±1.18	1.10±1.14	3.47±0.87	11.81±2.86*

The presence of inflammatory cells (Figure 2B) in the duodenum of animals that received EGb, may indicate the existence of an oxidative stress agent in the such biological system due to the action of the metabolites. Similar findings were described in substances (flavonoids, diterpenes and terpenes) present in the EGb and other natural products (Veiga-Júnior et al., 2005; Simões et al., 2007). In conclusion, the *in vivo* treatment with EGb in these experiments generated active metabolites that may modify the bioavailability of the studied radiobiocomplex in the treated animals and may promote the morphometry alterations observed in the cells of duodenum.

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Sciences, Vanderbilt University, Nashville, for revisions to the English grammar in the paper.

## RESUMO

O objetivo deste estudo foi avaliar o efeito de um extrato de *Nectandra* (*N. membranacea*), de *Ginkgo* (EGb) e de *Passiflora e. flavicarpa* (PEF) na marcação de constituintes sanguíneos (BC, IF-P, IF-BC) com Tc-99m, na morfologia de hemácias (RBC), na biodistribuição do  $^{99m}\text{TcO}_4\text{Na}$  na morfologia do duodeno. Amostras de sangue foram incubadas com os extratos. Tc-99m foi adicionado e as frações do plasma (IF-P) e da célula (IF-BC) foram isoladas. Estudos morfométricos foram realizados. Os resultados mostram que EGb promove alteração na marcação de BC, IF-P e IF-BC. *N. membranacea* não altera a radiomarcação e PEF altera a marcação de IF-P. O extrato de *N. membranacea*, EGb e PEF

alteraram a morfologia de RBC ( $p < 0.05$ ). Os extratos de *N. membranacea* e EGb modificam a biodistribuição do  $^{99m}\text{TcO}_4\text{Na}$ , e o EGb influencia a morfometria ( $p < 0.05$ ) do duodeno de ratos.

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