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Effects of a *Nectandra Membranacea* Extract on Labeling of Blood Constituents with Technetium-99m and on the Morphology of Red Blood Cells

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***Abstract*—**The aim of this *in vitro* study was to evaluate the possible interference of a *Nectandra membranacea* extract (i) on the labeling of blood cells (BC), (ii) on the labeling process of BC and plasma (P) proteins with technetium-99m (Tc-99m) and (iii) on the morphology of red blood cells (RBC). Blood samples were incubated with a *Nectandra membranacea* crude extract, stannous chloride, Tc-99m (sodium pertechnetate) was added, and soluble (SF) and insoluble (IF) fractions were isolated. Morphometry studies were performed with blood samples incubated with *Nectandra membranacea* extract. The results show that the *Nectandra membranacea* extract does not promote significant alteration of the labeling of BC, IF-P and IF-BC. The *Nectandra membranacea* extract was able to alter the erythrocyte membrane morphology, but these morphological changes were not capable to interfere on the labeling of blood constituents with Tc-99m.

***Keywords*—**in vitro study, Nectandra membranacea, red blood cell, technetium-99m

I. Introduction

THE ingestion of herbs as food or as medicinal plants has increased worldwide [1], [2]. Unexpected interactions between the use of natural products and orthodox therapeutic treatments are reported, and the understanding of these effects may be difficult [1], [2]. These findings have stimulated the development of assays to know better about the biological effects of the natural products [2]. There are numerous species of the Lauraceous plant family; as the *Nectandra membranacea* (*N. Membranacea*, White cinnamon). This species, from South America, was firstly described and classified by Moura, 1997 [3]. Plants from this genus have a well known geographic distribution [4].

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They are frequently found in Brazil and have been used by populations to treat several health problems, such as inflammation, hypertension and various other diseases [5], [6]. Some strains of *Nectandra* also have been investigated as possible anti-tumor agents, and some studies have showed that the chemotherapeutic potential of this plant could be associated with the presence of neolignan constituents [5], [6]. Phytochemistry analysis carried out by Moura in 1997 showed that *N. Membranacea* contain tannins, alkaloids and the flavonoid catequins [3] and that these constituents may carry calcium ions into cells [1], [2]. It has also been suggested that tannins and flavonoids may play important roles in the scavenging activities of free radicals [7].

Red blood cells (RBC) and plasma proteins are labeled with technetium-99m (Tc-99m) and used as radiocomplexes [8]- [12]. Blood samples are incubated with the stannous ion and then exposed to Tc-99m, as sodium pertechnetate [10]. Various factors that can influence the labeling of blood constituents have been described, such as inadequate preparation of reducing agent (stannous ion), the influence of disease and/or the presence of drugs in the patient's plasma [8], [11], [13]. The mechanisms of this interaction may also be related to the competition of the drugs with stannous/pertechnetate ion and blockage of calcium channels promoted by exogenous substances [14]. Zolle et al. (2007) noted that under normal conditions, there is no evidence of morphological alterations of RBCs after *in vitro* and *in vivo* Tc-99m-labeling [9]. In addition, some extracts of natural products migth alter the labeling efficiency of blood constituents with Tc-99m, as well as the morphology of RBC [15]- [19]. Moreover, these investigations have permitted to develop an experimental model to assess biological effects of extracts of natural products [9], [12], [13], [15]- [19]. To our understanding only a reference is found in the PubMed. Furthermore a small number of references about *Nectandra* is found in this databank. This fact has stimulated to study the *in vitro* effect of a *Nectandra membranacea* extract: (i) on the labeling of blood cells, cellular and plasma proteins with Tc-99m and (ii) on the morphology of RBC.

II. Material And Methods

A. PubMed strategy

Searches were performed in the PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) using the keywords *Nectandra* or *Nectandra membranacea* at January 12, 2011.

B. Preparation of the Nectandra membranacea extract

N. membranacea leaves were taken from the "Maciço da Pedra Branca" (Rio de Janeiro, RJ, Brazil) and identified by a certified biologist. *Nectandra membranacea* leaves were 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 [20].The dry extract product was re-dissolved in an aqueous saline solution (NaCl 0.9 %), and 300mg of *Nectandra membranacea* was placed in a container with 10 ml of saline solution, to create an aqueous preparation of 30mg/mL [20]. This preparation of the natural product was then diluted with saline to obtain other solutions (50%, 25%, 12.5%, 6.2%) of the extract.

C. Animals

Female *Wistar* rats (2 month-old, 180-210 g) were obtained from the *Laboratório de Radiofarmácia Experimental* (*Departamento de Biofísica e Biometria, Universidade do Estado do Rio de Janeiro*, RJ, Brazil). Experiments were conducted in accordance with the Committee of Animal Care, within institutional guidelines, and in compliance with national laws and Guidelines for the Use of Animals in Biomedicals Research [21].

D. Radionuclide and in vitro assay

Tc-99m was obtained from the *Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear*, São Paulo, Brazil, from a ^{99}Molybdenium/^{99m}Technetium generator. Blood samples (0.5mL) obtained from the *Wistar* rats (n=6) were incubated (60 minutes), and gently mixed with 100μL of different dilutions of the *Nectandra membranacea* extract (100%, 50%, 25%, 12,5%, 6,2%) or with 0.9% NaCl as control. Then, 0.5ml of freshly prepared stannous chloride solution (1.2 μg/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, 100μl of Tc-99m (3.7 MBq/ml) as sodium pertechnetate was added and the incubation was continued for another 10 minutes. These samples were centrifuged for 5 minutes, and plasma (P) and blood cells (BC) were separated. Samples (20 μL) of P and BC were also precipitated with 1 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 sodium iodide well counter (Automatic Gamma Counter, C5002, Packard, USA). After that, the percent of administered radioactivity (% ATI) was calculated, as previously described [22]. The results are presented as mean and standard deviation (SD), with a statistical analysis performed (ANOVA test, Tukey-Kramer test and Dunnet test).

E. Morphological studies

The morphometry study was performed with blood samples incubated with *Nectandra membranacea* extract (100%, 50%, 25%, 12.5%, 6.2%) or with 0.9% NaCl (control), stannous chloride 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. After setting the stain (10 minutes), the smear was washed in water and dried at room temperature in a vertical position [23]. The images obtained from smears were evaluated under a clear field light microscope (Eclipse E 400^{TM}). The morphometry of the RBC images was evaluated using Software Image Pro plus (media Cybernetics), for image capture in a 256 shade gray schedule; the image was transformed to a binary format for study of the red blood cells, evaluating of the ratio of area and the perimeter of the RBCs. A statistical analysis (Kruskal-Wallis with post-test Dunns, $p<0.05$) was used to compare the experimental data.

III. RESULTS

The search with the keyword *Nectandra membranacea* in the PubMed has shown only one publication and with *Nectandra* was found 27 references. Table I shows the distribution of the radioactivity in blood cells and plasma compartments from whole blood treated with different concentrations of *Nectandra membranacea* extract. The results indicate no significant alteration in the distribution of Tc-99m in these blood compartments for any extract concentration.

TABLE I
%ATI OF SAMPLES OF PLASMA AND CELLS: EFFECT OF A *NECTANDRA MEMBRANACEA* EXTRACT (MG/ML) ON THE LABELING OF BLOOD CELLS (BC) WITH TC-99M

Nectandra	P	BC
Control	2.19 ±0.70	97.80 ± 0.70
100%	2.60 ± 1.08	97.30 ± 1.08
50%	2.90± 1.05	97.08 ± 1.05
25%	3.09± 0.30	96.9 ± 0.30
12.5%	2.20±0.60	97.10± 0.60
6.25%	2.01±0.98	97.20± 0.98

Note that the difference between the %ATI of treated samples with the extract (100%, 50%, 25%, 12.5%, 6.25%) and controls is not significant ($p>0.05$).

Table II shows the uptake of the radioactivity in the insoluble and soluble fractions of plasma obtained from whole blood treated with different concentrations of *Nectandra membranacea* extract. No significant alteration in the fixation of Tc-99m in plasma proteins (insoluble fraction of plasma) is seen for any concentration of the medicinal plant. The same result was found with samples of cells proteins (insoluble fraction of BC) treated with this extract (Table III).

TABLE II
%ATI OF SAMPLES OF SOLUBLE FRACTION OF PLASMA (SF-P) AND INSOLUBLE FRACTION OF PLASMA (IF-P): EFFECT OF A *NECTANDRA MEMBRANACEA* EXTRACT (MG/ML) ON THE LABELING OF PLASMA PROTEINS WITH TC-99M

Nectandra	SF- P	IF-P
Control	29.57 ±8.88	70.40 ± 8.88
100%	38.95 ± 3.90	61.04± 3.92
50%	39.40± 4.90	60.50 ± 4.90
25%	35.01± 0.60	64.90 ± 0.60
12.5%	38.20± 0.80	65.10± 0.80
6.25%	39.43± 3.81	60.57± 3.81

Note that the difference between the %ATI of treated samples with the extract (100%, 50%, 25%, 12.5%, 6.25%) and controls is not significant ($p>0.05$).

TABLE III
%ATI OF SAMPLES OF SOLUBLE FRACTION OF CELL (SF-BC) AND INSOLUBLE FRACTION OF CELL (IF-BC): EFFECT OF A *NECTANDRA MEMBRANACEA* EXTRACT (MG/ML) ON THE LABELING OF CELLULAR PROTEINS WITH TC-99M

Nectandra	P	BC
Control	12.67 ± 3.41	87.33 ± 3.41
100%	10.86 ± 2.39	89.12 ± 2.30
50%	14.70 ± 4.70	85.20 ± 4.70
25%	11.01 ± 1.04	88.90 ± 1.04
12.5%	12.22 ± 1.11	87.78 ± 1.11
6.25%	12.11 ± 0.81	87.89 ± 0.81

Note that the difference between the %ATI of treated samples with the extract (100%, 50%, 25%, 12.5%, 6.25%) and controls is not significant ($p>0.05$)

The qualitative analysis of the shape of RBC (incubated sample with *Nectandra membranacea*) under light microscopy has shown important qualitative morphological alterations, mainly, in samples treated with the highest concentrations, due to the incubation of whole blood with the extract (figure 1E, 1F). Figure 1 shows a photomicrography of blood smears, both control (non incubated, figure 1A) and treated samples (incubated with the extract in the 6.2%, 12.5%, 25%, 50%, 100% concentrations). In the samples incubated with the extract, some blood cells show spikes on their membranes (Figures 1E, 1F) differing from normal RBC, which have a naturally round shape (Figure 1A**,** control). The results were confirmed by image analysis and the Dunns post-test from the Kruskal-Wallis test, that indicated a significant difference ($p<0.05$) on the morphometry of the RBC treated with *N. membranacea* extract (Figure 1E, 1F) 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, in the 50% concentration from 0.22±0.10% to 0.77±0.12% and in the 100% concentration from 0.22±0.10% to 0.73±0.06%) when compared with control samples in figure 1A. The samples treated with lowest concentrations (6.25%, 12.5% and 25%) did not demonstrate a significant alteration in the perimeter/area ratio ($p>0.05$).

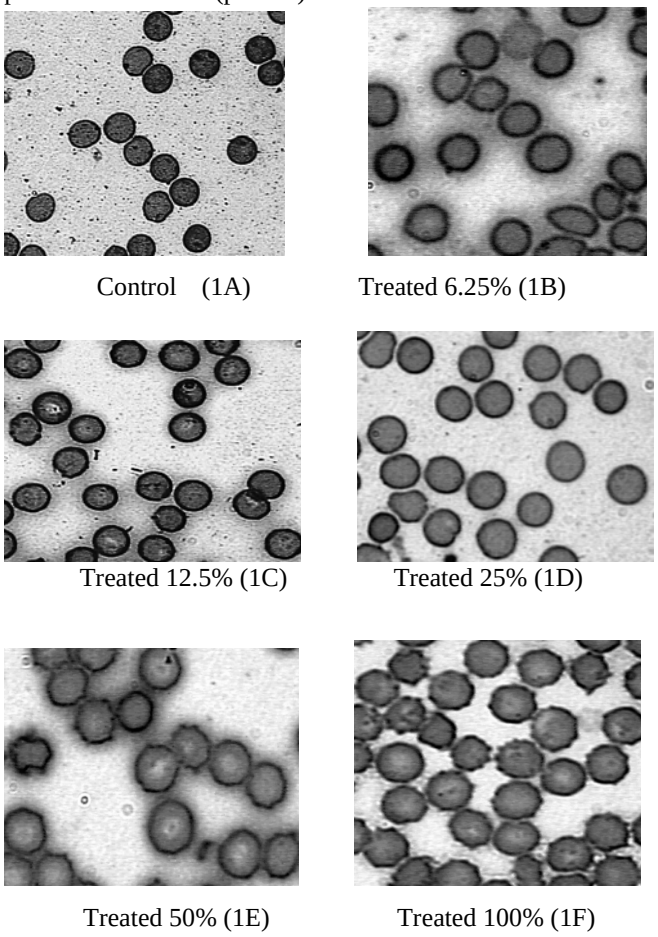

Fig. I Photomicrography of blood smears prepared with samples of whole blood (control and treated with *Nectandra membranacea* extract) used to label RBC with Tc-99m

Samples of whole blood were incubated with NaCl solution (Control Figure 1A) or different concentrations (Figures 1B, 1C, 1D, 1E, 1F) of *Nectandra membranacea* extract for 60 minutes. The morphology of the red blood cells was evaluated under a light microscope (x100). Observe that some RBC have presented spikes on the membrane (figures 1E, 1F) differing from normal blood cells with a round shape (figure 1A**,** control), like also from blood cells treated with decreased concentrations of the extract (Figures 1B, 1C, 1D).

IV. DISCUSSION

Unexpected interactions of pharmaceuticals and phytopharmaceuticals, as well as undesirable effects have been reported of these natural products. This fact could be associated with a small scientific information about the medicinal plant [1], [2]. These conditions may justify more careful and responsible use of extracts medicinal plants. The development of assays that permits evaluation of possible extract interactions with biological systems is very important. Concerning to the studies with *Nectandra membranacea* only one paper is found in the PubMed. Moreover, the search with *Nectandra* has also shown a reduced number of publications.

The utilization of methods using cell radiolabeling for study of the interaction of natural drugs has contributed to increase the understanding about the some properties of these products [1],[9].

The model using the radiolabeling of blood constituents is useful to try explain the interaction between natural drugs and cells or other drugs, and may reveal a possible effect on the biodistribution of radiobiocomplexes [12], [20], [24] used in nuclear medicine examinations [8], [25]. Several studies have reported the effect of natural products on the labeling of blood constituents [17], [26]-[30]. It has been described that *Thuya occidentalis* [26], *Paullinia cupana* [27], *Fucus vesiculosus* [28], *Ginkgo biloba* [18] and *Vellozia pusilla* [30] extracts may alter the labeling of blood constituents with Tc-99m. There also are extracts such as *Pfaffia sp*. that apparently do not alter the labeling of blood constituents with Tc-99m [29]. The effects of synthetic drugs [14], [31], [32], as well as other conditions (e.g. those cited by Sampson 1996 and Kawabe et al. 2003) have been evaluated using the labeling of blood cells with Tc-99m. However it is important to note that the studies of Sampson (1996) and Kawabe et al. (2003) used an *in vivo* model, and we have used an *in vitro* model in our work [8], [33].

The results obtained on this study indicate that there is no alteration in the uptake of radioactivity for all studied fractions isolated from the whole blood treated with *Nectandra membranacea*, independent on the concentrations of the extract of this medicinal plant. Once this labeling process begins, depending on the action of the reducing agent, probably the *Nectandra membranacea* extract has compounds with anti-oxidant properties that could protect the stannous ions from the oxidation process, as suggested by other authors to another natural products [7], [34].

The morphological analysis showed a change of the red blood cells perimeter/area ratio in samples treated *in vitro* with *Nectandra membranacea*. It is possible that some substances present in the extract promote important morphological alterations in the RBC membrane. Alterations on the erythrocyte membrane have also been reported to other extracts as *Cinnamomum zeylanicum* [15], *Coffea arabica* [35], *Mentha crispa* [36], *Fucus vesilosus* [28], and *Ginkgo biloba* [19], but in these studies, when a morphological alteration occurred, the RBC labeling with Tc-99m was also altered by the extracts. Extracts of *Pfaffia sp*. apparently do not alter the morphology of the RBC [29]. Probably the alterations on the membrane of the RBC (Figures 1E, 1F) induced by the *N. membranacea* would be not sufficent to interfere in the transportation of the stannous and pertechnetate ion through the RBC membrane. In consequence, the labeling of the RBC is not modified (Table 1). Concerning to the alkaloids, flavonoids and tannins (identified in constituents of the extract), some authors have reported that these substances do not interfere in the transportantion of the calcium ion [1], [2]. Our results are in accordance with these findings due to the similarity between calcium and stannous ions [37].

As reported in previous publications, RBCs may suffer alterations in the presence of ethanol [38]-[40]. In our research, similar findings were observed (Figure 1E, 1F). In this case, as the extract studied is free from ethanol, the alterations could be due to its constituents, possibly the alkaloids [1], [2], [41]. In the *Nectandra* extract are present organic compounds that have chemical structures like alkaloids, tannins and flavonoids, probably with the presence of hydroxyl radicals (OH) [1], [2], [41]. The alkaloids may have one hydroxyl of alcohol function in their structures that could have similar characteristics to that of the ethanol hydroxyl (alcohol function) [41]. This could justify the morphological alterations on RBC morphology (Figures 1E, 1F, confirmed by statistical analysis).

V. Conclusion

In conclusion, the *Nectandra membranacea* extract may alter the RBC membrane morphology, but the morphological alterations produced by the extract did not alter the labeling of blood constituents with Tc-99m. Moreover, although our assays were performed with animals, we suggest precaution with the use of extract of *Nectandra membranacea.*

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