Assessment *In Vitro* Of The Biological Effect Of A Herbal Product Extract: Morphological And Radiolabeling Analysis

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Abstract: An increasing number of people in the world are using natural products as medicine which led many scientists to contribute to the research in this field. Also a few pharmacologists, after an initial phase of correct criticisms, today recognize the possibility of investigating the scientific value of medicinal products composed essentially of vegetable extracts. The constituents of. herbal products can cause adverse effects. We evaluated the influence of a chayotte (*Sechium edule*) extract on the morphology of red blood cells and on the radiolabeling of blood elements with technetium-99m (99mTc). In our study, blood was withdraw from *Wistar* rats. Samples of blood were treated with chayotte extract (decoct) in different concentrations (100; 50; 25; 12.5 and 6.25%v/v,) during 1 hour. After that blood was incubated with stannous chloride for more 1 hour. Elapsed this time 99mTc as Sodium pertechnetate was added and the incubation continued for more 10 minutes. Plasma (P) and blood cells (BC) were isolated, also precipitated with trichloroacetic acid and soluble (SF) and insoluble fractions (IF) separated. For the morphology analysis, samples of the blood were collected and smears were prepared. The blood smears were dried, fixed and stained. The analysis was done by video optical microscope using image pro-plus program. In our results it was observed that the referred extract was not capable of altering the radiolabeling of blood elements although it was capable to alter the morphology of red blood cells in the highest concentration. The effect of chayotte extract probably, could be explained by an effect which might alter the stabilizing activity of the red blood cell membrane. [The Journal of American Science. 2008;4(2):68-77]. (ISSN 1545-1003).

Key words: chayotte, red blood cells, morphology, technetium-99m, *in vitro*

Introduction

Natural medicines are increasingly used throughout the world, as they are considered to be effective and to have few side-effects (1). Traditional herbal medicines have been reported to cause serious hematological adverse effects. It is well-known, that lipid antioxidants can retard the oxidative rancidity of foods caused by atmospheric oxidation, and thus protect oils, fats, and fat-soluble components from their quality degradation. In the last few years, much emphasis has been put on the promotion and use of natural antioxidants, commonly occurring in many fruits and vegetables and thereby produced from various natural extracts (2). Many drugs and vegetable extracts have been reported to affect the radiolabeling of blood elements with 99mTc as well as the bioavailability of sodium pertechnetate (3,4). *Sechium edule* (chayotte) a subtropical vegetable with potent diuretic action, is a cucurbitaceus species which is used as food or as medication in popular medicine. It was reported a case of severe hypokalemia pregnancy and that a chayotte preparation was implicated, as the potassium level returned to normal, without recurrence of hypokalemia, once the ingestion of this vegetable was stopped (5, 6). Gordon (2000) described the antihypertensive effect of chayotte. Diré et al, 2001 have noticed that chayotte extract (macerated) was capable of altering the biodistribution of sodium pertechnetate. In a *in vivo* study, Diré et al, 2002 observed the extracts (decoct and macerated) of chayotte were capable to alter the radiolabeling of blood elements with 99mTc. The effects of natural products on the radiolabeling of blood elements have been studied by different researchers (9, 10, 11, 12, 13, 14, 15, 16). Mongelli et al, 1997 have showed that *Bolax gummifera* extract was used as a treatment of wounds probably due its properties related to the stabilizing activity of the red blood cell membrane. In a *in vitro* study developed by Oliveira et al, 2003 was remarked through a qualitative analysis that the *Fucus vesiculosus* extract altered the morphology of red blood cells. *F.*

vesiculosus extract was capable to alter the radiolabeling of blood elements with 99mTc. In other study, Oliveira et al (2002), have shown that the *Paullinia cupana* extract have promoted alterations in the shape of red blood cells and on the radiolabeling of blood elements with 99mTc. Similar results were obtained by Braga et al, 2000 in a comparative study with *Thuya occidentalis* and *Nicotiana tabacum*. In a qualitative *in vitro* study, was noticed a lightly morphological alterations on the red blood cells due to treatment of blood with *Maytenus ilicifolia*. In this same study was verified that the studied extract has altered the labeling of blood constituents with 99mTc (13). Tetechnetium-99m (99mTc) has been the most utilized radionuclide in nuclear medicine procedures (18) and it has also been used in basic research (19). The wide utilized in nuclear medicine is due to its optimal physical characteristics as half-life of 6h, gamma rays energy of 140 keV and minimal dose to the patients, convenient availability from 99Mo/99mTc generator and negligible environmental impact. There are many applications of 99mTc-labeled red blood cells (99mTc-RBC), as in cardiovascular evaluations, in the detection of gastrointestinal bleeding and in the determination of the RBC mass in patients. RBC have been labeled with 99mTc through of *in vitro*, *in vivo* or *in vivo*/ *in vitro* techniques (20, 21). The 3 foundations of nuclear medicine are radiation conscious personnel, specific radiopharmaceuticals and equipment. The trend in molecular radiopharmacy is to develop new radiopharmaceuticals targeting peptides and receptors. 99mTc-radiopharmaceuticals give important clinical and molecular information especially in endocrinology, oncology and cardiology. Nevertheless, there is not a well established model to study the influence of drugs (synthetic or natural) on the labeling of blood elements as well as molecules as peptides and receptors. Here, we have evaluated the influence of a chayotte extract on the labeling of blood elements with 99mTc using an *in vitro* technique and on morphology of the red blood cells (22).

Material and Methods

Characterization of the chayotte sample:

Chayotte was purchased from a local market in Rio de Janeiro city, RJ, Brazil. To prepare the extract, 50 g of skin of chayotte were mixtured with 500 mL of water in an electric extractor. This preparation was filtered and this extract was considered 100%.

The presence of toxic compounds was evaluated and we did not find them in the extracts of chayotte used in our experiments. The method to verify the presence of these toxic products is based on inhibition of acetylcholinesterase in the presence of the pesticides. In this method, brain acethylcholinestarase is utilized as an *in vitro* detector of organophosphorus and carbamate insectides. Briefly, a preparation of acetylcholinesterase was obtained after extraction of a rat brain microsomal fraction with Triton X-100 and was incubated with the extract of chayotte. Enzyme assay was performed by a potentiometric method based on the formation of acetic acid in the incubation mixture (preparation of acetylcholinesterase and extract of chayotte)

Preparing of the extract:

Heparinized whole blood was withdrawn from *Wistar* rats. Samples of 0.5 ml of blood were incubated with 100 µl of different concentrations (100; 50; 25; 12.5 and 6.25% v/v) of a preparation (decoct) of chayotte. To prepare the decoct of cahyotte, this vegetable (50 g) was put in an Erlenmeyer with 500 mL of saline solution (0.9% NaCl) and it was boiled on slow heat for ten minutes. After that, the solution was filtered and the watery extract was obtained.

Radiolabeling process:

Sechium edule preparation was incubated with samples of blood (0.5 mL) for 1 hour at room temperature. Samples of heparinized blood (0.5mL) which were incubated with saline solution (NaCl 0.9%) were utilized as control. Then, 0.5 mL of stannous chloride (1.2 µg/mL), as $SnCl_2.2H_2O$ (Reagen, Quimibrás Indústrias Químicas SA, Brazil) was added and the incubation continued for another 1 hour. After this period of time, 99mTc (0.1 mL), as sodium pertechnetate, recently milked from a 99Mo/99mTc 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 (clinical centrifuge) and plasma (P) and blood These samples were centrifuged 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 well counter. After that, the % of radioactivity (%ATI) was calculated. Statistical analysis (Kruskal Wallis test) was utilized to compare the experimental data.

Preparing of the blood smears

The blood homogenized was distended in the surface of a smear (26x76mm), after adequate cleaning, forming an angle of 45º. The distention was dried, agitating the smear in the air. The rapid desiccating is indispensable for a good conservation of the morphology of the cells and other elements.

Staining: May-Grünwald-Giensa method

The May-Grünwald-Giensa is a technique of staining which was utilized in this study. It has permitted us to visualize the cells through their distinct characteristics of staining, namely, according to the affinity of various cellular compounds towards the used dyes (23).

Technique of staining

The smears were putted upon a appropriated support and the staining technique followed: (i) distention with the dye of May-Grünwald-Giensa (Merck, Germany), for a period of 5 minutes, homogenized with a pipe; (ii) after that, the smear was covered with the dye of GIENSA (Merck, Germany), and again the surface of the smear was homogenized and left the dye acting for 10 minutes; (iii) the dye was despised and the smear was washed in the fluent water, dried in the room temperature in the vertical position.

The smears were evaluated under optical microscope of clear field (Eclipse E 400 ^{TM}), in the immersion objective (100x), with photographer ocular.

Morphometric analysis

The aim of this study was to evaluate the effect of the different concentrations of. a chayotte extract on the morphology of. red blood cells. It was analyzed the following parameters: the area (μm^2); maximum diameter (μm); minimum diameter (μm); perimeter (μm); spherecity (no dimensional-[/]) e perceptual of area (no dimensional - [/]), which correspond to the percentage of the number of cells counted per area. The quantification of the data was realized by the following equipment: Software image pro plus (media Cybernetics), according to the procedures: (1) capture of images in the gray schedule of 256 tons; (2) transformation of the image from gray schedule to binary schedule according to the calibrating threshold to detach red blood cells; (3) counting of cells with the use of. the function *count/size* of the program which has permitted us: (i) title the objectives by the size; (ii) seep the objectives by the relation major and minor axis (iii) measure automatically the area, the perimeter and the spherecity; (4) the gauges were impressed in the archives ASCII, being exported to electronics tables and formatted to the programs STATISTICA® e SPSS® where the statistical tests were performed.

In the morphometric analysis were employed: (i) optical microscope of. clear field (Nikon); (ii) photographic ocular; (iii) video camera CCD Sony DXC-151 A; (iv) microcomputer Pentium MMX 166 MHz, with 64 Mb of RAM memory, 256 de memory *Cache* and equipped with *frame grabber Matrix Vision* to the capture and to process the image; (vi) auxiliary monitor SONY KX-14CP1, (vii) printer HP 692C.

Results

Table 1 has shown the effect of a chayotte extract on the distribution of the radioactivity on the red blood cells and in the plasma. The analysis of the results indicates that there is not an alteration ($p>0.05$) in the uptake of 99mTc by the RBC in the plasma.

Table 1- Effect of a chayotte extract on the labeling of blood cells and plasma with 99mTc.

Sechium edule	Blood Cells	Plasma
	Percentage radioactivity	
control	94.81 ± 2.57	5.19 ± 2.57
6.25%	90.94 ± 6.18	9.06 ± 6.18
12.5%	93.93 ± 2.93	6.07 ± 2.93
25%	92.65 ± 3.45	7.35 ± 3.45
50%	90.68 ± 5.66	9.32 ± 5.66
100%	93.04 ± 4.97	6.96 ± 4.97

Sample of blood were incubated with different concentrations of chayotte extract. In the control blood was treated with saline solution (NaCl 0.9%). After that, stannous chloride and 99mTc were added. The ATI% was calculated. A statistical analysis (Kruskal Wallis test, n=5) was used to compare the results.

Table 2 has shown the effect of a chayotte extract on the distribution of the radioactivity in the plasma proteins. The analysis of the results indicates that there is not an alteration ($p>0.05$) in the liaison of 99mTc in the plasma proteins.

Table 2- Effect of a chayotte extract on the labeling of plasma proteins with 99mTc.

Sechium edule	Insoluble fraction	Soluble fraction
	Percentage radioactivity	
control	77.67 ± 7.44	22.33 ± 7.44
6.25%	81.53 ± 4.45	18.47 ± 4.45
12.5%	76.96 ± 8.63	20.01 ± 8.63
25%	72.22 ± 10.40	27.78 ± 10.40
50%	74.03 ± 9.76	25.97 ± 9.76
100%	72.69 ± 9.55	27.31 ± 9.95

Sample of blood were incubated with different concentrations of chayotte extract. In the control blood was treated with saline solution (NaCl 0.9%). After that, stannous chloride and 99mTc were added. The ATI% was calculated. A statistical analysis (Kruskal Wallis test, n=5) was used to compare the results.

Table 3 has shown the effect of a chayotte extract on the distribution of the radioactivity in the blood cells proteins. The analysis of the results indicates that there is not an alteration ($p>0.05$) in the fixation of 99mTc in the blood proteins.

Table 3- Effect of a chayotte extract on the labeling of cell proteins with 99mTc.

Sechium edule	Insoluble fraction	Soluble fraction
	Percentage radioactivity	
control	91.26 ± 3.57	8.74 ± 3.57
6.25%	90.03 ± 2.63	9.97 ± 2.63
12.5%	89.15 ± 3.34	10.85 ± 3.34
25%	91.37 ± 3.44	8.63 ± 3.44
50%	91.94 ± 1.77	8.06 ± 1.77
100%	91.36 ± 2.29	8.64 ± 2.29

Sample of blood were incubated with different concentrations of chayotte extract. In the control blood was treated with saline solution (NaCl 0.9%). After that, stannous chloride and 99mTc were added. The ATI% was calculated. A statistical analysis (Kruskal Wallis test, n=5) was used to compare the results.

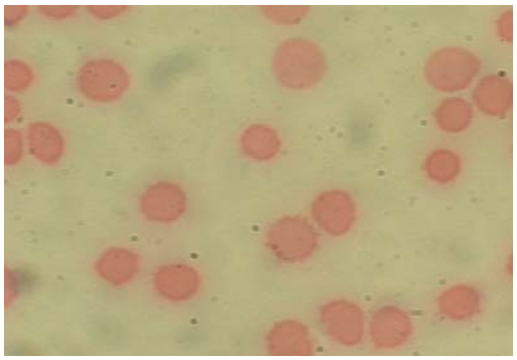

Figure 1. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of whole blood were incubated with NaCl 0.9% solution (control) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

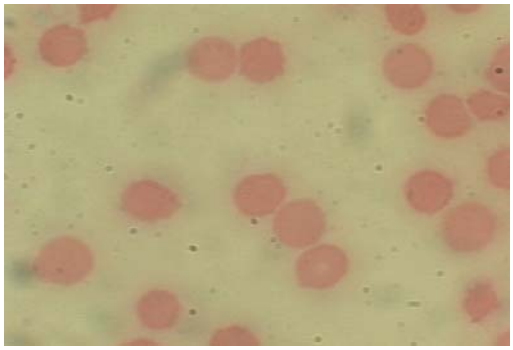

Figure 2. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of whole blood were incubated with chayotte extract (6.25%) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

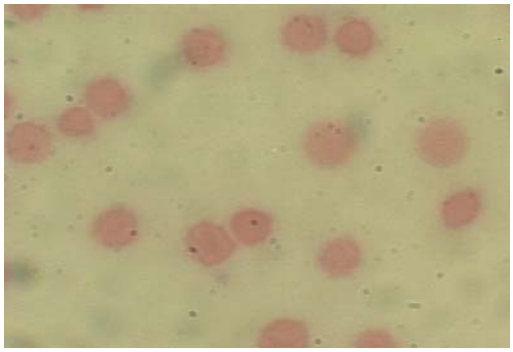

Figure 3. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of. whole blood were incubated with chayotte extract (12.5%) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

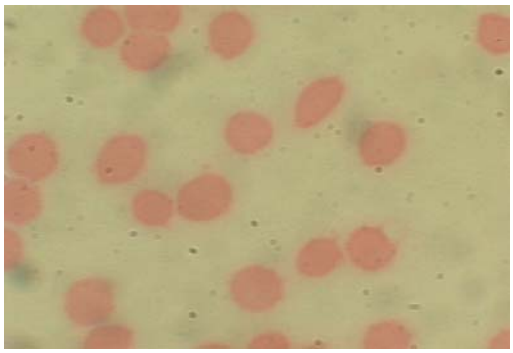

Figure 4. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of. whole blood were incubated with chayotte extract (25%) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

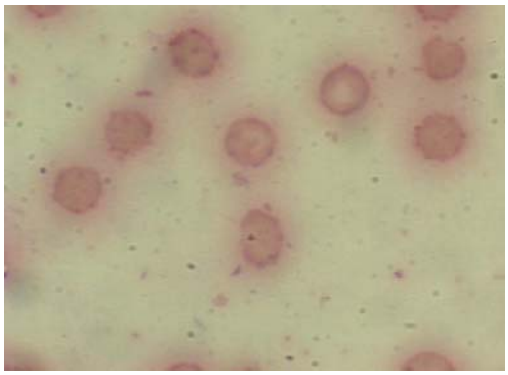

Figure 5. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of whole blood were incubated with chayotte extract (50%) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

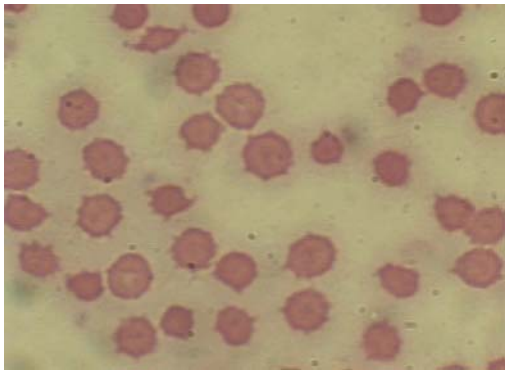

Figure 6. Photomicrography of blood smears prepared with samples of whole blood used to label blood elements with 99Tc. Samples of whole blood were incubated with chayotte extract (100%) for 60min. After that, stannous chloride was added and the incubation continued for 60min. Then, 99mTc, as sodium pertechnetate was added. Blood smears were carried out, dried, fixed and staining. Afterwards, the morphology of. red blood cells was evaluated under an optical microscope (x1000).

Table 4 has shown the effect of a chayotte extract on the morphology of red blood cells. The analysis of the results indicates that there is an alteration ($p<0.05$) on the shape of the cells.

Table 4- Effect of a chayotte extract on the morphometry of red blood cells.

Concentration %	Perimeter/ Area (μm/ μm^2)
Control	0.72 ± 0.07
6.25	0.71 ± 0.03
12.5	0.74 ± 0.01
25	0.76 ± 0.04
50	0.81 ± 0.09
100	0.91 ± 0.08

The blood smears were observed under optical microscope. In the treated group blood was incubated with chayotte extract (different concentrations 6.25; 12.5; 25; 50 and 100%) during 1 hour. In the control group blood was incubated with saline solution (NaCl 0.9%). The morphometric results were compared employing the ANOVA and Dunnet tests.

Discussion

The red blood cells may have alterations in their morphology which can indicate states of abnormality of the organism. It is of the fundamental importance the acquaintance of these alterations so as to the clinical full measure can have more efficacy due to the diagnostic of the patient. (24). Extracts obtained from various medical plants can alter the labeling of blood elements with 99mTc as well as the morphology of red blood cells (3, 4, 9, 10, 11, 12, 13, 14, 15, 16). The developing of models that permit evaluation of biologic properties of natural products is worthwhile. The evidence that drugs can affect either the radiolabeling as the biodistribution of red blood cells or the morphology of them in the context of nuclear medicine clinic has come to light only comparatively recently and it is an important factor in the interpretation of scintigraphic images. A great number of workers have turned their attention to *in vitro* and *in vivo* evaluation of drugs in the process to label blood cells and in the biodistribution of radiopharceutical (25, 26, 27, 28).

We have studied the effect of *Sechium edule* extract (decoct): (i) on the labeling of blood elements and (ii) on the morphology of red blood cells. In the labeling process of blood elements with 99mTc needs a reducing agent, and probably the stannous ion would be oxidized. In *in vitro* studies was verified that extracts of *Thuya ocidentallis* (9), *Nicotiana tabacum* (10) and *Maytenus ilicifolia* (13), possibly, would have oxidants compounds, and the labeling of blood elements decrease in the presence of these extracts. By of a qualitative analysis it was remarked that *N. tabacum* and *T. occidentalis* have altered the shape of red blood cells and the radiolabeling of blood elements (12). In qualitative study, Oliveira et al, 2000 have shown that the extract of *M. illicifolia* was only induced a lightly morphological alterations of red blood cells. In a research was verified that *Paullinia cupana* extract was capable to alter the radiolabeling of blood elements as well as to alter quantitatively the shape of red blood cells (15). In other *in vitro* study with *Fucus vesiculosus* extract was noticed that the referred extract has induced a qualitative alterations on the morphology of red blood cells together with alterations on the labeling of blood elements with 99mTc (16). In a *in vivo* studies Diré et al, 2002, have demonstrated that the chayotte extracts were capable to alter the radiolabeling of blood elements. Sastre et al, 1998, described that *Ginkgo biloba*, used for treating cognitive disturbances, has been reported to produce an anticoagulant effect by inhibition of platelet activating factor. In other study, Moreno et al, 2002, eyed that in a *in vitro* study the extract of *Ginkgo biloba* altered the morphology of red blood cells, the opposite, was observed in a *in vivo* study which this fact may be explained by the generate of metabolites *in vivo* without direct action on the morphology of red blood cells. It was reported by Santos-Filho & Ribeiro et al, 2002, that the extracts of *Mentha crispa L*. (mint) and *Piper methysticum* (Kava Kava) were capable to alter the morphology of red blood cells notwithstanding mint extract has also altered the radiolabeling process. Braga et al, 2000, in a *in vitro* study demonstrated that *Peumus boldus* did not alter the labeling of blood elements with 99mTc. Lima et al, 2002 in a *in vivo* study have shown that an extract of cauliflower (leaf) was not capable of altering the labeling of blood elements with technetium-99m. Diré et al, 2002, in a *in vitro* study eyed that the chayotte extracts were not capable to alter the radiolabeling of blood constituents. In the procedure of labeling RBC with 99mTc, the stannous and pertechnetate ions pass through the plasma membrane (19, 32). Then, as reported to the tobacco extract (10) and *Maytenus ilicifolia* extract (13), histological alterations of red blood cells could be responsible for the modifications on the labeling of RBC with 99mTc. Furthermore, we can speculate that if the chemical compounds present in the extracts could complex with these ions as a chelating agent, this fact could explain the decrease in the fixation of radioactivity on the blood elements. Diré et al, 2001, in a qualitative analysis *in vivo*, have eyed that a chayotte extract (macerated) has induced alteration on the shape of red blood cells. In this study the chayotte extract did not alter the radiolabeling although it was capable to induce qualitative and quantitative alterations on the shape of red blood cells, in

question to this fact, we can suggest like observed by Mongelli et al, 1997, in a study with *Bolax gummifera* extract, that the chayotte extract is able to stabilizer the active of red blood cell membrane even though the morphology of the cell has been altered.

Conclusion

In general, we can conclude that *Sechium edule* extract is not capable of altering the labeling of blood elements with 99mTc although it has altered the morphology of red blood cells. This fact could be due to the presence of compounds which were not strong enough to complex with pertechnetate and stannous ions but the sufficient to modify the architecture of plasma membrane without interfere in the transport of ions by the cell.

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