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Assessment of physicochemical parameters of an *Anethum graveolens* aqueous extract and the effects on the labeling of blood constituents with technetium - 99m

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Abstract

Ethnopharmacological relevance: *Anethum graveolens* (AG) has been used both medicinally and as an aromatic herb and spice in cookery. Some pharmacological effects have been reported such as antibacterial, hypolipidemic, antihypercholesterolemic and antioxidant activities. There are no studies that evaluate the physicochemical parameters of AG and its influence with radiopharmaceutical. This study investigated the physicochemical parameters of the AG extract and the effects on the labeling of blood constituents with technetium-99m (^{99m}Tc) withdrawn from *Wistar* rats. An aqueous extract of AG was prepared and physicochemical parameters (absorption spectrum, electric conductivity, refractive index and pH was) were studied. Blood samples were incubated with AG extract or with NaCl solution 0.9%, as control. After, stannous chloride, as reducing agent, and ^{99m}Tc, as sodium pertechnetate, were added. Plasma and blood cells samples were separated. Other samples of plasma and blood cells were also precipitated with trichloroacetic acid and soluble and insoluble fractions were separated. The radioactivity in each fraction was counted and the percentage of radioactivity incorporated was calculated. Data showed an absorbance peak at 430 nm, electric conductivity and refractive index were higher and pH was more acid in highest concentration. The extract of AG did not alter the labeling of the blood constituents with ^{99m}Tc. Conclusion: The determination of these physicochemical parameters would contribute to characterize the AG aqueous extract. Moreover, probably the antioxidant properties associated with the substances of AG extract were responsible for the absence of effect of this extract on the labeling of blood constituents with ^{99m}Tc.

Key words : electric conductivity, radiolabeling, refractive index, ^{99m}Tc

INTRODUCTION

For thousands of years plants and other naturally occurring substances have been used for medicinal purposes and domestic applications [1 2]. Herbs contain a plenty of phytochemicals constituents and experimental models can be used to improve our understanding of the cellular and systemic mechanisms of action and biological effects [2-3 4]. Carmo et al. [5] and Frydman et al. [6] showed that some physical parameters like absorption spectrum, electric conductivity and refractive index are useful to aid in characterizing extract of herbs.

Anethum graveolens is an annual or biennial herb of the *Umbelliferae* family native of the Southwest Asia or Southeast Europe. The plant is used both medicinally and as an aromatic herb and spice in cookery [7 8]. The principal main compounds of *Anethum graveolens* extract are flavonoids, phenolic acids, proanthocyanidins [9], monoterpenoid, glycosides [10], saponins, tannins and alkaloids [11].

Some pharmacological effects have been reported, such as antifungal [12] antibacterial [11], hypolipidemic [13], antihyper cholesterolemic [3] and antioxidant [9] activities. It is also effective in control of food spoilage as a potential source of food preservative [14] and has significant mucosal protection and antisecretory effects of the gastric mucosa [15].

The potential side effects of natural products and interactions of drugs, mainly radiopharmaceuticals, with medicinal plants are not fully known and are of considerable concern [16]. There was no study that verifies the interactions of *Anethum graveolens* extract with radiopharmaceuticals.

Radiopharmaceuticals are used in the nuclear medicine to obtain images that have been used to measure physiological processes and identify changes related to various diseases and physiological functions [17]. Blood constituents can be labeled with technetium-99m (^{99m}Tc) using stannous chloride ($SnCl_2$) as reducing agent [18 19 20] to evaluate the vascular volume [21], recognition of gastrointestinal bleeding [22], identification of hepatic hemangioma [23] and others [17]. Sequential steps of the intracellular labeling process of blood constituents include transmembrane transport of stannous and pertechnetate ions into internal compartment of red blood cells, reduction of ^{99m}Tc as pertechnetate ($^{99m}TcO_4$) by the $SnCl_2$ and binding of the reduced ^{99m}Tc to hemoglobin [24]. *In vitro* labeling of blood constituents with this radionuclide also has been used successfully in experimental models to evaluate the influence of natural drugs [2 5 16]. Some research showed that alterations in membrane cell could modify the radiolabeling of blood constituents when treated with natural or synthetic drugs [6 25 26].

Due to the importance of the aqueous extract of *Anethum graveolens*, some authors have performed investigations to determinate biological effects and compounds such as antibacterial effects, phytochemical screening [11] and effects of blanching, freezing, and refrigerated storage on the levels of nitrates, nitrites, and oxalates [27] in different concentrations. The aim of this work is to evaluate the physicochemical parameters of the *Anethum graveolens* extract and the effects on the labeling of blood constituents with ^{99m}Tc withdrawn from *Wistar* rats.

MATERIALS AND METHODS

Preparation of the aqueous *Anethum graveolens* extract

Anethum graveolens (lot 12/10, valid 12/11), was purchased from local supermarket (Rio de Janeiro, Brazil). To prepare the extract, 0.5 g of *Anethum graveolens* was added to 50 mL of hot saline (0.9% NaCl). After 10 minutes, this preparation was filtered (paper filter), and the extract obtained was considered to be 10 mg/mL.

Spectrophotometry of *Anethum graveolens* extract

The absorbance spectrum (*Analyser Comércio e Indústria Ltda, São Paulo*, Brazil) was determined with the *Anethum graveolens* extract (10 mg/mL) prepared as described above in the range of 400700 nm. Saline solution was used as the blank. The absorbance was measured at each interval of 10 nm. The value of the absorbance of the highest concentration of the extract at 430 nm was 0.162 ± 0.01. This value was considered as the marker of the reproducibility of the conditions used to prepare the extract.

Electric conductivity of *Anethum graveolens* extract

The electric conductivity (mS/cm) of the *Anethum graveolens* extracts at different concentrations (0.62, 1.25, 2.5, 5.0 and 10 mg/mL) was performed with a conductivimeter (*Marte Balanças e Aparelhos de Precisão Ltda, São Paulo*) at room temperature. Saline solution was used as the control. The value of electric conductivity (1.821 ± 0.059) of the extract at higher concentration was used as a second marker of the reproducibility of the conditions to prepare the extract.

Refractive index of *Anethum graveolens* extract

The refractive index (%BRIX) of *Anethum graveolens* extracts at different concentrations (0.62, 1.25, 2.5, 5.0 and 10 mg/mL) was measured with a refractometer (Ningbo Utech International Co. Ltd., Ningbo, People's Republic of China) at room temperature. Saline solution was also used as the control. The value of the refractive index (1.66 ± 0.11) of the extract at higher concentration was used as a third marker of the reproducibility of the conditions to prepare the extract.

pH of *Anethum graveolens* extract

The pH of *Anethum graveolens* extracts at different concentrations (0.62, 1.25, 2.5, 5.0 and 10 mg/mL) was measured with a digital pH meter (mod. PHS-3B, PhTEK, Curitiba, Paraná) at room temperature. Saline solution was also used as the control. The value of pH (5.73 ± 0.02) of the extract at higher concentration was used as a fourth marker of the reproducibility of the conditions to prepare of the extract.

Animals

Adult male Wistar rats (3-4 months, 250-300 g) were maintained in a controlled environment: normal light/dark cycle conditions (12-h light/12-h dark; lights on at 6 am), free access to water and food and room temperature was kept at 25±2 ºC. Experimental protocols were approved by the Ethical Committee of the *Instituto de Biologia Roberto Alcantara Gomes*, *Universidade do Estado do Rio de Janeiro* (protocol number 045).

In vitro radiolabeling of blood constituents

Heparinized whole blood was withdrawn by cardiac puncture from rats under anesthesia by sodium thiopental, 60mg/kg of weight. Samples of whole blood (500 µL) were incubated with different concentrations of *Anethum graveolens* (0.6, 1.25, 2.5, 5.0 and 10 mg/mL) for 1 h (n = 7 for each *Anethum graveolens* concentration). Blood samples were incubated with saline solution (0.9% NaCl) for control. After that 500 µL of freshly prepared solution of stannous chloride (1.20 µg/mL) was added and incubated for 1 hour and, in sequence, 100 µL of ^{99m}Tc (3.7 MBq, 10 min), as sodium pertechnetate ($Na^{99m}TcO_4$), recently milked from a ^{99}Mo/^{99m}Tc generator (*Instituto de Pesquisas Energéticas e Nucleares, Comissão Nacional de Energia Nuclear, São Paulo,* Brazil) was added and the incubation was continued for 10 minutes. After that, these samples were centrifuged (1500 rpm in clinical centrifuge, 5 min, room temperature) and plasma (P) and blood cells (BC) were separated. Aliquots of P and BC were also precipitated with trichloroacetic acid (5%) and soluble (SF) and insoluble (IF) fractions were obtained. Radioactivity in P, BC, IF-P, SF-P, IF-BC and SF-BC was counted in a well gamma counter (Gamma C-12, DPC Medlab, Los Angeles, CA, USA) and the percentage of radioactivity incorporated (%ATI) on each fraction was calculated as described previously [28]. Briefly, %ATI for each fraction was obtained by ratio between the radiation counting for a fraction and the sum of the radiation counting for this fraction and the complementary fraction multiplied by 100.

Statistical analysis

Data are reported as mean ± SD values of the absorbance, electric conductivity (mS/cm), refractive index (%BRIX), pH and percentage of radioactivity (%ATI). The percentage of radioactivity (%ATI) from radiolabeling assay was also analyzed with one-way analysis of variance - ANOVA test to verify possible statistical differences ($p<0.05$) and followed by Dunnet post-test. InStat Graphpad software was used to perform statistical analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA, USA).

RESULTS

The absorbance spectrum of *Anethum graveolens* extract at higher concentration used (10 mg/mL) in the range of 400700 nm is shown in Figure 1. The data indicated an absorption peak of the extract was 0.162 ± 0.01 nm at 430 nm.

Figure 2 shows the electric conductivity at different concentrations. The electric conductivity medium (1.821 ± 0.059 mS/cm) was obtained at the higher extract concentration (10 mg/mL).

Figure 3 presents the refraction index of *Anethum graveolens* extract at different concentrations. Refraction index is dependent on the *Anethum graveolens* extract concentration, with the highest value (1.66 ± 0.11%BRIX) at the higher concentration used (10 mg/mL).

Figure 4 shows the pH of an *Anethum graveolens* extract in different concentration. The most acid concentration of an aqueous extract was observed in higher concentration (pH = 5.73 ± 0.02) and the pH increase when the concentration reduced.

The distribution of the radioactivity in plasma and cellular compartments indicates no alteration ($p>0.05$) of *Anethum graveolens* extract in distribution of ^{99m}Tc (Table 1). As in plasma and cellular compartments, *Anethum graveolens* was not capable of interfering significantly ($p>0.05$) on the fixation of the radioactivity on the insoluble and soluble fractions of plasma that are shown in Table 2. Finally, the results of fixation of radioactivity on proteins of blood cells from blood samples incubated with *Anethum graveolens* were not significant

Table 1: Effect of *Anethum graveolens* extract on the distribution of the radioactivity in plasma and cellular compartments.

Anethum graveolens	**%ATI**	
mg/mL	**P**	**BC**
0.9% NaCl	3.40 ± 2.92	96.60 ± 2.92
0.62	1.63 ± 2.06	98.37 ± 2.06
1.25	2.51 ± 2.74	97.49 ± 2.74
2.5	3.08 ± 4.27	96.92 ± 4.27
5.0	5.55 ± 9.83	94.45 ± 9.83
10.0	1.45 ± 1.28	98.55 ± 1.28

Blood samples from *Wistar* rats were incubated with *Anethum graveolens* extract for 1 hour and the labeling of blood constituents with 99mTc was carried out. Plasma (P) and blood cells (BC) were isolated, radioactivity was counted and the percentage of incorporated radioactivity (%ATI) was calculated.

Table 2: Effect of *Anethum graveolens* extract on the fixation of the radioactivity of the insoluble and soluble fractions of plasma.

Anethum graveolens	**%ATI**	
mg/mL	**IF-P**	**SF-P**
0.9% NaCl	71.09 ± 4.75	28.91 ± 4.75
0.62	69.58 ± 8.04	30.42 ± 8.04
1.25	68.65 ± 10.01	31.35 ± 10.01
2.5	70.36 ± 14.72	29.64 ± 14.72
5.0	69.63 ± 7.53	30.37 ± 7.53
10.0	61.33 ± 7.69	38.67 ± 7.69

Blood samples from *Wistar* rats were incubated with *Anethum graveolens* extract for 1 hour and labeling of blood constituents with 99mTc was carried out. Insoluble (IF) and soluble (SF) fractions from plasma (P) were isolated, radioactivity was counted and the percentage of incorporated radioactivity (%ATI) was calculated.

Table 3: Effect of Anethum graveolens extract on the fixation of the radioactivity of the insoluble and soluble fractions of blood cells.

Anethum graveolens	**%ATI**	
mg/mL	**IF-C**	**SF-C**
0.9% NaCl	92.95 ± 2.77	7.05 ± 2.77
0.62	94.93± 2.14	5.07± 2.14
1.25	94.00 ± 4.45	6.00 ± 4.45
2.5	96.17 ± 1.13	3.83 ± 1.13
5.0	96.25 ± 0.97	3.75 ± 0.97
10.0	94.29 ± 5.03	5.71 ± 5.03

Blood samples from *Wistar* rats were incubated with *Anethum graveolens* extract for 1 hour and labeling of blood constituents with 99mTc was carried out. Insoluble (IF) and soluble (SF) fractions from blood cells (BC) were isolated, radioactivity was counted and the percentage of incorporated radioactivity (%ATI) was calculated.

(p>0.05) (Table 3).

DISCUSSION

Natural products are widely used as food, food additives or as medicine to treat several diseases [29]. As the use of these natural products is increasing over the world, the development of experimental assays can contribute to verify some properties of herbs [2 3 30]. Furthermore, the developing of model that permit to evaluate pharmacologic properties of natural products is worthwhile [31].

The initial concern was to standardize the conditions of preparation of the extract used in the experiments like Carmo et al. [5] and Frydman et al. [6]. Information about the physicochemical proprieties of *Anethum graveolen* was previously investigated by Jirovetz et al. [10]. In their study the properties like refractive index, specific gravity, optical rotation, acid number and ester number was obtained by essential oil of *Anethum graveolens* for quality control. Therefore, our study proposes to evaluate the physicochemical parameters of an aqueous extract of *Anethum graveolen* to standardize the conditions of preparation for use in experimental models.

The absorbance spectrum profile with maximum absorbance at 430 nm (Figure 1) was founded to characterize the preparation of aqueous extract. Although other studies did not use the absorbance spectrum to characterize their extract, this physical parameter was used in experimental models to evaluate the radical scavenging and antioxidant activity of phenolic compounds [32], content of vitamin C [33], 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay, trolox equivalent antioxidant capacity assay, reducing power, chelating power and b-Carotene bleaching assay [9] of *Anethum graveolen* extract.

Different from Carmo et al. [5], the increase in electric conductivity of our study depends on the extract concentration where the higher value was obtained at the higher extract concentration. This result corroborates with Frydman et al. [6] where *Cordia salicifolia* (porangaba) extract is also dependent on the concentration of an aqueous extract.

In the refraction studies, we found higher refractive index at the highest concentration of *Anethum graveolens* extract (Figure 3) according to Frydman et al. [6] had described to extract of porangaba and Carmo et al. [5] had described to *Solanum melongena* extract. Even though Jirovetz et al. [10] had used essential oil of *Anethum graveolens* to verify the refractive index, the value founded was the same as founded by this study in 5.0 mg/mL concentration of our extract (1.4 %BRIX).

Our results show that the pH decreased when the concentration of an aqueous extract increased. Ameh et al. [34] verified the pH of aqueous extracts of six plants with 200mg/mL of concentration and showed that de pH of 5% solution varied between 5 and 7. Although this study did not evaluate different concentrations, their values are close to ours with the same concentration of an aqueous extract.

These physical parameters can be used in other studies to characterize the preparation conditions of an aqueous extract of *Anethum graveolens*.

Despite the fact that herbal medicine consumption is widespread throughout the world, little information is available about the possible effects of their active substances on radiolabeling process of blood constituents or interactions with radiopharmaceuticals. Mistaken interpretations of nuclear medicine exams based on radiopharmaceuticals could be obtained if patients are using herbal medicines or food [16-20].

Natural product extracts could decrease the labeling of blood constituents due to the following four factors: (i) the presence of oxidant compounds that could oxidize the $SnCl_2$, (ii) the presence of chelating agents that could form a complex with $Na^{99m}TcO_4$ and $SnCl_2$, (iii) modifications induced in the plasma membrane and (iv) the competition among the cited ions for the same binding sites [6 31]. Therefore, studies showed that aqueous extract of some herbs like *Solanum* melongena [5], *Bacopa monnieri* [35] and *Passiflora edulis flavicarpa* [16] and synthetic drugs like naproxen [36] and fenoprofen [37] did not modify the labeling of blood constituents with ^{99m}Tc.

The *Anethum graveolens* extract did not alter the labeling of the blood constituents with ^{99m}Tc. Shyu et al. [9] showed that *Anethum graveolens* extract has chelating activity against ferrous ion increased with concentration of *Anethum graveolens*. Therefore, this chelate propriety did not interact with $Na^{99m}TcO_4$ or $SnCl_2$ and did not alter the labeling of blood constituents. Components like phenols, flavonoids, proanthocyanidins, ascorbic acid, anthocyanins and tocopherols existing in *Anethum graveolens* extracts should be responsible for antioxidant abilities of this extract [8 9]. A group of biologically active polyphenolic bioflavonoids called proanthocyanidins has beneficial effects in radical scavenging and other relevant redox active properties [38]. But to confirm this, other experimental model needs to be done to clarify why the labeling of blood constituents is not modified. Moreover, considering our findings, the antioxidant property associated with the *Anethum graveolens* that could protect the stannous ions that would be responsible by the reduction of the ^{99m}Tc, as pertechnetate ion.

CONCLUSION

In conclusion, our data suggest that some physicochemical parameters could be useful to characterize the *Anethum graveolens* aqueous extract. Probably the antioxidant properties associated with the substances of the *Anethum graveolens* extract could be responsible by for the absence of effect of this extract on the labeling of blood constituents with ^{99m}Tc.

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