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Citotoxic effects of stannous salts and the action of *Maytenus ilicifolia*, *Baccharis genistelloides* and *Cymbopogon citratus* aqueous extracts

Efeitos citotóxicos dos sais de estanho e a ação de extratos aquosos de Maytenus ilicifolia, Baccharis genistelloides e Cymbopogon citratus

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Abstract
In nuclear medicine, stannous salts, as stannous chloride ($SnCl_2$) and stannous fluoride (SnF_2), are used as reducing agents to obtain radiopharmaceuticals labeled with technetium-99m. In the literature, the $SnCl_2$ action was studied and it seems to be mediated through free radicals (FR) production in a Fenton-like reaction. In this work it was evaluated: (i) the *in vitro* SnF_2 effects in different concentrations using pBCKS plasmid deoxyribonucleic acid (DNA); (ii) the SnF_2 effects in different *Escherichia coli* (*E. coli*) cultures, proficient or deficient in DNA repair genes, treated simultaneously with FR scavengers; and (iii) the biological effects of *Maytenus ilicifolia*, *Baccharis genistelloides* and *Cymbopogon citratus* aqueous extracts on the $SnCl_2$ action in *E. coli* culture. The SnF_2 treatment induced plasmid DNA damages (single and double DNA strand breaks), in a dose-dependent manner. Citotoxicity mediated by SnF_2 was observed and the simultaneous treatment with FR scavengers has increased the cell survival, suggesting the participation of FR on the SnF_2-deleterious effects. The vegetal extracts protected the *E. coli* cells against the $SnCl_2$ effects. The components of the extracts could be interacting with $SnCl_2$, blocking its participation in the FR formation.
Keywords: Deoxyribonucleic acid, *Escherichia coli*, Free radicals, Stannous ion.

Resumo
Em medicina nuclear, sais de estanho, principalmente sob as formas de cloreto estanoso ($SnCl_2$) e fluoreto estanoso (SnF_2), são utilizados como agentes redutores na obtenção de radiofármacos marcados com tecnécio-99m. Na literatura tem sido relatado a ação do íon estanoso, também mediada pela produção de radicais livres (RL) numa reação do tipo Fenton, em nível celular e molecular. Nesse trabalho foi avaliado (a): (i) o efeito in vitro *de diferentes concentrações de SnF_2 em plasmídio de DNA pBCKS; (ii) a ação do SnF_2 em diferentes culturas bacterianas de* Escherichia coli *(*E. coli*), proficiente e deficiente em genes de reparo de DNA, tratadas simultaneamente com aceptores de RL; e (iii) o efeito biológico de extratos aquosos de* Maytenus ilicifolia, Baccharis genistelloides *e* Cymbopogon citratus *sobre a ação do $SnCl_2$ em* E. coli*. O SnF_2 induziu danos no DNA plasmidial (quebras simples e duplas na cadeia de DNA), de maneira dose-dependente. A citotoxicidade mediada pelo SnF_2 foi observada e o tratamento simultâneo com os aceptores de RL aumentou a sobrevivência das células tratadas com o referido agente, reforçando que essa citotoxicidade seja mediada por RL. Os extratos vegetais protegeram as células contra os efeitos tóxicos do $SnCl_2$. Componentes desses extratos poderiam estar interagindo com o íon estanoso, impedindo a formação de RL.*
Palavras-chave: *Ácido desoxirribonucléico,* Escherichia coli, *Íon estanoso, Radicais livres.*

Introduction

In nuclear medicine, radiopharmaceuticals (radiobiocomplexes) labeled with technetium-99m (99mTc) are widely used as imaging agents. Red blood cells and plasma proteins labeled with 99mTc are used in cardiovascular system images, detection and localization of gastrointestinal hemorrhages (Tamm *et al*., 1995). This labeling technique is based on the reducing ability of stannous salts on 99mTc, as sodium pertechnetate, to a lower oxidation state (Tamm *et al*., 1995; Saha, 2003). The most important stannous salts used for this purpose are stannous chloride ($SnCl_2$) and stannous fluoride (SnF_2) (Saha, 2003). Furthermore, humans are widely exposed to stannous ion in food, as a result of processing and packaging (Blunden and Wallace, 2003). SnF_2 is also component of dentifrices and mouthrinses for the control of dental caries (Paraskevas *et al*., 2004). Due to their several applications, the knowledge and the understanding of the biological effects of stannous salts becomes highly relevant

Deoxyribonucleic acid (DNA) damages are related to the etiology of several diseases, like cancer (Friedberg, 2003). Metals ions can strongly bind in nucleic acid preparations (Pezzano and Podo 1980; De Mattos *et al*., 2000). In addition, some transition metals, such as iron, copper, zinc, chromium and tin are able to mediate Fenton or Fenton-like reactions that generate free radicals (FR) (Halliwell, 1994; 2003).

There is no agreement about the genotoxic and/or mutagenic activities of stannous compounds. Assays with *Bacillus subtilis* deficient in recombination repair showed an absence of those effects (Kanematsu *et al*. 1980). Triphaty *et al*. (1990) demonstrated that $SnCl_2$ is not a carcinogenic compound, using wing primordial cells from *Drosophila melanogaster*. However, McLean *et al*. (1983) reported that $SnCl_2$ produced extensive DNA damage, detected in Chinese hamster ovary cells. Genotoxic potentiality of $SnCl_2$ was demonstrated in proficient (wild-type) and deficient *Escherichia coli* (*E. coli*) strains on DNA repair genes (Bernardo-Filho *et al*., 1994). Studies have revealed that $SnCl_2$ promotes strand breaks in plasmid DNA (Dantas *et al*., 1999).

The study of the phytotherapy is increasing worldwide. Vegetal extracts have been used for medicinal purposes and nutrition. Some natural products can also be found as mainly several components of products with pharmacological activities or used in dietary supplement purposes. However, the amount of scientific information about their safe and effective use is still quite limited. Most of the information does not have scientific support and, moreover, the use as phytotherapic drug is based only on traditional folk medicine, which has been passed on from generation to generation (Ernst, 2002; Ferreira-Machado *et al*., 2004). *Cymbopogon citratus* (*C. citratus*), *Maytenus ilicifolia* (*M. ilicifolia*) and *Baccharis genistelloides* (*B. genistelloides*) are plants used in the folk medicine in various countries.

C. citratus is used to treat feverish conditions. It is a constituent of relaxants and sleeping aids. In popular medicine, it is used as antispasmodic and carminative (Almeida, 1993). Studies have revealed that this plant possess health benefits, affording protection at the vascular endothelium level (Runnie *et al*., 2004) and has inhibitory effects on the early phase hepatocarcinogenesis in rats (Puatanachokchai *et al*., 2002).

M. ilicifolia is effective in treating ulcers (regulating the production of hydrochloric acid in the stomach), restoring the intestinal flora and treating nervous disorders (Sallé, 1996). *M. ilicifolia* extract reduces acid secretion in the isolated frog gastric mucosa (Ferreira *et al*., 2004) and reduces the growth of phytopathogenical fungi (Cunico *et al*., 2002).

Some popular uses of *B. genistelloides* extract include treatment of digestive disorders, malaria, diabetes, angine, anaemia, diarrhoea, urinary inflammation, intestinal worms and leprosy (Camargo, 1985). *B. genistelloides* extract has been utilized due to its antiviral activity against herpes simplex type I and vesicular stomatitis virus (Abad *et al*., 1999). Moreover, it has been observed its anti-arthritic, hypoglycemic and hypotriglyceridemic properties in mice (Coelho *et al*., 2004).

The purposes of the present study were: (i) to study SnF_2 *in vitro* effects in pBCKS plasmid DNA treated with different concentrations of this agent; (ii) to verify the SnF_2 effects in different *E. coli* cultures, proficient or deficient in DNA repair genes, and the FR participation in the lesions produced, using FR scavengers; and (iii) to evaluate the biological effects of aqueous extracts of *M. ilicifolia*, *B. genistelloides* and *C. citratus* and the effect of these extracts on the $SnCl_2$-deleterious effects in *E. coli* culture.

Material and Methods

Reagents and extract preparation

Stannous fluoride, stannous chloride, sodium benzoate and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co., USA. Commercial dried powder of *C. citratus* and *B. genistelloides* were purchased from Refinações de Milho™ (Brazil), and *M. ilicifolia* from Herbarium Laboratório Botânico™ (Brazil). The extracts were prepared through addition of 0.9% NaCl

(1 mL) in ebullition to 0.005 g to the herbs powder and left to rest (10 min). The suspension was centrifuged and the supernatant phase was used and considered to be 5 mg/mL.

Bacterial strain and plasmid DNA

The strains used were *E. coli* AB1157, proficient in DNA repair mechanisms (Howard-Flanders and Theriot, 1966), and AB1886 deficient in the *uvr*A gene, involved in nucleotide excision repair (NER) mechanism (Boyce and Howard-Flanders, 1964). *E. coli* DH5αF,Iq (rec^-) (Hananah, 1983) strain was hosting pBCKS plasmid DNA and it was prepared according the alkaline method described earlier (Sambrook *et al*., 1989). Plasmid samples were further purified from high molecular weight RNA contaminants, performing LiCl precipitation (2.5 M), while the residual RNA contaminants, were digested by RNAse (20 mg/ml) treatment for 30 min at room temperature.

DNA treatment and electrophoresis

The DNA treatment with SnF_2 was performed and aliquots of plasmid DNA (200 ng) were incubated with increasing concentrations of SnF_2 (22, 44, 88, 110, 220 and 330 µM) in 10mM Tris-HCl buffer at pH 7.4, at 37° C for 60 min. The period of incubation were earlier established by cellular experiments (Melo *et al*., 2001). After, to stop the reaction, EDTA (metal ion chelator-25mM) was added to the samples. As a control, EDTA (25 mM) was added before incubation with $SnCl_2$.

The electrophoresis was performed using a 0.8% agarosis gel in order to separate different structural conformations of pBCKS plasmid DNA treated with SnF_2: form I (supercoiled) native conformation, form II (open circle) resulting from DNA single strand breaks, and form III (linear) resulting from DNA double strand breaks. Aliquots from each sample (100 ng) were mixed to a loading buffer (0.25% xylene cyanol FF; 0.25% bromofenol blue; 30% glycerol), applied in a horizontal gel electrophoresis chamber in Tris acetate-EDTA buffer at pH 8.0 and performed at 6 V/cm. After electrophoresis, gel was stained with ethidium bromide (0.5 mg/ml) and DNA bands were visualized by fluorescence in ultraviolet (UV) DNA transilluminator system (Germetec, Brazil). Permanent records were performed using a Polaroid MP-3 system.

Bacteria inactivation and FR scavengers

Cells from *E. coli* AB1157 and AB1886 cultures in exponential growth phase (1-2 x 10^8 cells/mL) were collected by centrifugation, washed and ressuspended in 0.9% NaCl. Samples (1mL) of these cultures were incubated on the water bath shaker with: (a) SnF_2 (25 µg/mL), (b) SnF_2 (25 µg/mL) + FR scavenger, (c) FR scavenger (100 mM), (d) 0.9% NaCl. At 60 min intervals, aliquots were withdrawn, diluted and spread onto glass Petri dishes with solid LB medium (1.5% agar). Colonies were formed after overnight incubation (37° C) and the survival fractions (SF) calculated. Experiments were carried out in triplicate with sodium benzoate and DMSO and the results presented are the average mean of three independent assays. Standard deviations did not exceed 15%.

Bacteria inactivation and medicinal plants extracts

Cells from *E. coli* AB1157 cultures in exponential growth phase (1-2 x 10^8 cells/mL) were collected by centrifugation, washed and ressuspended in 0.9% NaCl. Samples (1 mL) of these cultures were incubated in the water bath shaker with: (a) $SnCl_2$ (25 µg/mL), (b) $SnCl_2$ (25 µg/mL) + extract (25 mg/mL), (c) extract (25 mg/mL), (d) 0.9% NaCl. The procedures after 60 min incubation were exactly as described before (2.4).

Results

Results shown in Figure 1 indicate that SnF_2 modifies the plasmid DNA conformational structure (supercoiled form I changed to open circle form II and linear form III) in a dose-dependent manner. This effect was observed when the plasmid DNA was treated with SnF_2 in different concentrations. As the SnF_2 concentration grows, the SnF_2-induced single and double strand breaks also increase.

SnF_2 was able to strongly reduce the cell survival after 60 min of treatment when compared with the control (100% survival at this time). The results also show that in presence of sodium benzoate, a specific hydroxyl FR scavenger, the SnF_2 lethal effect was partly reduced (Figure 2).

Experiments examining the role of UvrA protein (NER) in the DNA lesions during stannous reduction in a Fenton-like reaction were performed using

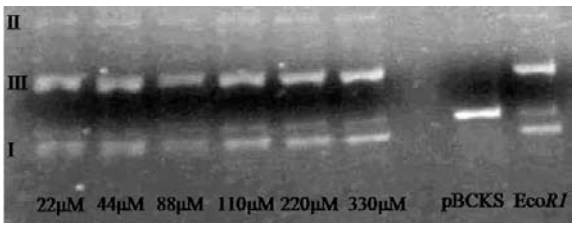

Figure 1. pBCKS plasmid DNA electrophoresis treated with increasing concentrations of SnF_2.

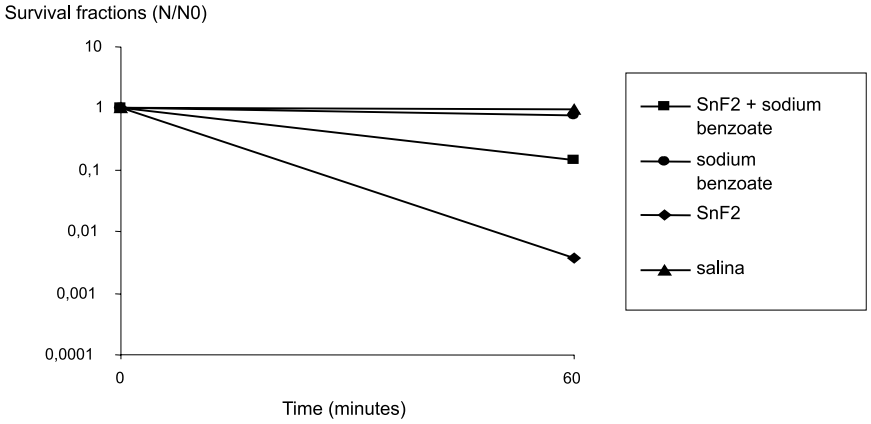

Figure 2. Survival fractions of *E. coli* (AB1157) treated with SnF_2 and FR scavenger sodium benzoate

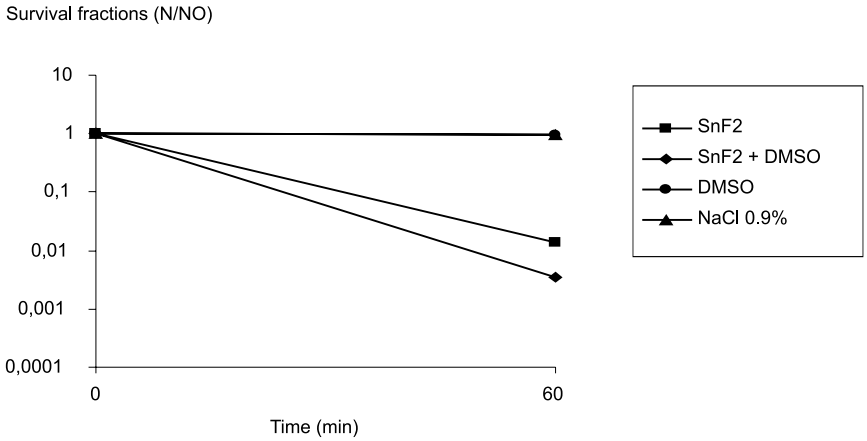

Figure 3. Survival fractions of *E. coli* (AB1886) treated with SnF_2 and FR scavenger DMSO

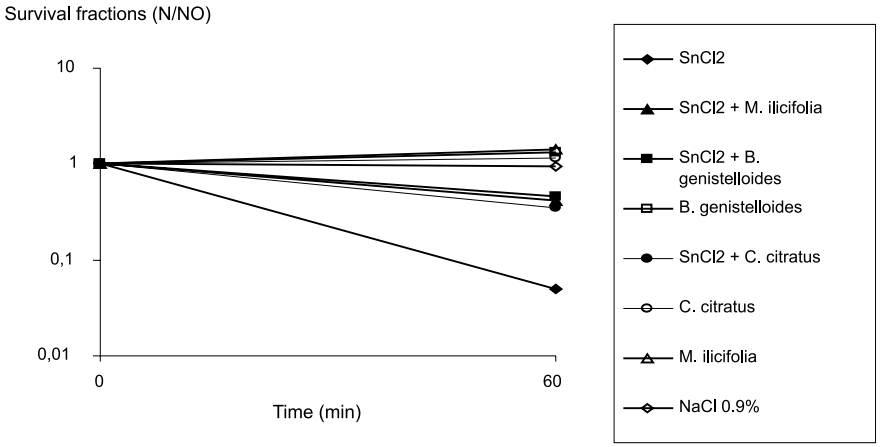

Figure 4. Survival fractions of *E. coli* (AB1157) treated with $SnCl_2$ and *Maytenus ilicifolia*, *Baccharis genistelloides* e *Cymbopogon citratus* aqueous extracts

E. coli AB1886 (*uvr*A$^-$) culture (Figure 3). It could be observed that SnF_2 was able to decrease the survival of this culture when compared with the control treatment and, moreover, this culture showed a higher rate of mortality than wild-type culture. The presence of DMSO, a non-specific FR scavenger partly abolished the SnF_2-lethal effects.

As it can be seen, in the Figure 4, in presence of the extracts studied occurs a protection of cells against the citotoxic-$SnCl_2$ effects and *M. ilicifolia* extract caused more protection than the other extracts.

Discussion

In nuclear medicine, $SnCl_2$ is the main reducing agent to the 99mTc reduction to obtain radiobiocomplexes (Tamm *et al*., 1995; Saha, 2003). In spite of the reported genotoxic effect of $SnCl_2$ (Bernardo-Filho *et al*., 1994), there is scarce information about the biological effects of the SnF_2 and the action of stannous ion when it is associated to phytochemicals (Melo *et al*., 2001).

The analysis of plasmid DNA topology has been studied in order to evaluate the *in vitro* toxicogenetics of SnF_2. This test detects genomic lesions that can lead to mutations or cell death. The analysis of pBCKS plasmid DNA treated with SnF_2 in different concentrations results (Figure 1) permits to suggest that SnF_2 can damage the DNA. As the SnF_2 concentration increases, the induced breaks (single and double DNA strand breaks) also increase. DNA treated with SnF_2 had its supercoiled form (I) changed to relaxed open circle (II) and linear form (III).

SnF_2, as $SnCl_2$, mediated the lethal effects observed on the *E. coli* cultures used (Figures 2 and 3). During the simultaneous treatment with sodium benzoate, a specific hydroxyl radical (OH^-) scavenger (Figure 1) and with DMSO (Figure 2), the cell survival increased, suggesting the participation of FR on the lethality of the cells. It could be also suggested the important role of OH^- on the oxidative stress mediated by SnF_2. The citotoxic potentiality of SnF_2 was also observed on the *E. coli* AB1886 strain, deficient in the *uvr*A gene (Figure 3). However, this culture was more sensitive to the SnF_2 lethal effects than the wild-type strain. This result could be explained due to the deficiency on NER mechanism, which consists on several enzymatic reactions to remove lesions. The absence of the UvrA protein blocks the recognizing of the lesion, stopping the repair (Grossman *et al*., 1998). This result indicates the importance of the NER on the repair of the damages mediated by SnF_2.

It has been described that plants have relevant antioxidant substances, as carotenoids, ascorbic acid, flavonoids and tannins (Mc Cune and Johns, 2002). Figure 4 shows the effect of the *M. ilicifolia*, *B. genistelloides*, *C. citratus* aqueous extracts on the inactivation induced by $SnCl_2$ in *E. coli* AB1157 strain. These extracts were capable to protect the *E. coli* cells against the lesive action of $SnCl_2$. Moreover, they were not able to interfere on the survival of this culture. *M. ilicifolia* and *B. genistelloides* aqueous extracts are capable to decrease the 99mTc radiolabeling efficiency of blood constituents (Oliveira *et al*., 2000; Braga *et al*., 2000). The extracts could be oxidizing the stannous ion, altering the radiolabeling of the blood constituents. This finding could be related to the increasing of the survival of the culture treated with these extracts and $SnCl_2$ simultaneously. These results, probably, are due to redoxi properties or chelating action on stannous ion of the chemical substances presented in these three extracts.

Conclusion

The results of agarosis gel electrophoresis presented permit to observe the increase of the lesions in DNA (single and double strand breaks) with the increase of the SnF_2 concentration. This result suggests a genotoxic potentiality of SnF_2. This salt, as $SnCl_2$, can also promote single and double DNA strand breaks and can inactivate bacterial cells. The treatment with FR scavengers protected the *E. coli* cells against the SnF_2-oxidative damages. So, it can be suggested the FR participation on the SnF_2-induced damage and the important role of OH^- in these effects, as showed by the results obtained using a specific OH^- scavenger. The biological effects of *C. citratus*, *M. ilicifolia* and *B. genistelloides* aqueous extracts against the lethal action of the $SnCl_2$ were also observed. The substances in the aqueous extract could acting as: (i) metal ion chelator on the stannous ions, avoiding the generation of FR, (ii) FR scavenger, protecting the cells against the oxidation, and/or (iii) an oxidant compounds that could act on the stannous ions, reducing the $SnCl_2$-lethal effect.

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