Modulatory activity of brazilian red propolis on chemically induced dermal carcinogenesis[1]

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ABSTRACT

PURPOSE: To evaluate modulatory effects of a hydroalcoholic extract of Brazilian red propolis (HERP) on dermal carcinogenesis using a murine model.

METHODS: The HERP was used at concentrations of 10, 50 and 100 mg/kg (PROP10, PROP50 and PROP100, respectively) to modulate dermal carcinogenesis induced by the application of 9,10-dimetil-1,2-benzatraceno (DMBA) on the backs of animals.

RESULTS: The chemical compounds identified in HERP included propyl gallate, catechin, epicatechin and formononetin. PROP100 treatment resulted in significantly decreased tumor multiplicity throughout the five weeks of tumor promotion ($p<0.05$), and this concentration also resulted in the highest frequency of verrucous tumors ($p<0.05$). All of the tumors that developed in DMBA-treated animals were regarded as squamous cell carcinomas and were either diagnosed as non-invasive verrucous carcinomas or invasive squamous cell carcinomas (SCCs). The average score for malignancy was significantly lower in the PROP100-treated group than the non-treated group ($p<0.05$), but there was no difference between the other groups ($p>0.05$).

CONCLUSION: The oral administration of hydroalcoholic extract of Brazilian red propolis at a dose of 100 mg/kg had a significant modulatory effect on the formation, differentiation and progression of chemically induced squamous cell carcinoma in a murine experimental model.

Key words: DMBA. Squamous Cell Cancer. Skin Cancer. Rodents. Propolis

Introduction

Cancer chemoprevention represents the prevention or delay of carcinogenesis due to the ingestion of dietary or pharmaceutical agents[1]. Due to the side effects associated with the long-term use of synthetic compounds for the prevention of skin cancer, research has been directed towards identifying chemopreventive compounds from natural products[2].

Propolis is a natural resinous hive product that honeybees manufacture by mixing their own waxes and salivated secretions with resins collected from the cracks of the tree bark and leaf buds. Moreover, propolis collected from different regions of Brazil has been shown to display distinct colors and chemical compositions depending on the local flora at the site of collection[3]. A new type of Brazilian propolis, which is popularly known as "red propolis", has been described and characterized. The major chemical constituents of this variety, as identified by high-performance liquid chromatography from ethanolic extracts, include the flavonoids pinocembrin, formononetin and isoliquiritigenin[4]. Studies have demonstrated that Brazilian red propolis displays cytotoxic effects on cell lines derived from human malignant tumors[5].

The chemopreventive effects of chemical compounds on skin cancer have been assessed using experimental models of chemically induced dermal carcinogenesis[6]. DMBA (7,12-dimethylbenz(a)anthracene) is a site- and organ-specific carcinogen commonly employed to experimentally induce skin cancer, and the topical application of DMBA has been shown to induce dermal carcinogenesis in mice[7]. DMBA is a polycyclic aromatic hydrocarbon that is metabolized into dihydrodiol epoxide, a compound able to bind to and damage DNA. Therefore, DMBA-induced skin carcinogenesis is regarded as an ideal tool to evaluate the chemopreventive efficacy of different anti-cancer agents in rodent models[8].

The purpose of this study was to assess the antitumor activity of different doses of a hydroalcoholic extract of Brazilian red propolis (HERP) on dermal carcinogenesis induced by DMBA in a murine model.

Methods

Ethical principles for experiments in animals were applied in this study. The experimental protocols and procedures were previously approved by the University Tiradentes Animal Care and Use Committee (CEUA nº 020913).

Animals management and groups formation

Ninety adult male mice (*Mus musculus*) weighing 30-35g were randomly assigned into six experimental groups (n=15), as described in Table 1. Animals were kept in plastic cages with wood shavings bedding, replaced daily, under controlled temperature (22±2˚C) and 12 h light/darkness scale, with water and food *ad libitum* (diet Labina®, Purina, Sao Paulo, Brazil).

TABLE 1 - Distribution of animals in experimental groups according to treatment.

Groups	Topical application	Oral administration
CTR1	Distilled water	Diluent (tween 2%)
CTR2	Distilled water	100 mg/Kg HERP
TUM	DMBA a 0.5 %	Diluent (tween 2%)
PROP10	DMBA a 0.5 %	10 mg/Kg HERP
PROP50	DMBA a 0.5 %	50 mg/Kg HERP
PROP100	DMBA a 0.5 %	100 mg/Kg HERP

Gathering propolis

Brazilian red propolis samples were gathered at an apiary in Brejo Grande, Southeast Brazil (10° 25′ 28″ S, 36° 27′ 44″ W) from Langstroth-type boxes. The material was labeled, placed in sterile, refrigerated containers and sent to the laboratory.

Preparation of Hydroalcoholic Extracts of Red Propolis (HERP)

Propolis samples (1g) were extracted with 70% ethanol (12.5 mL) at room temperature for 1 hour in an ultrasound bath. Following extraction, the mixture was centrifuged, and the supernatant was evaporated under low pressure to produce HERP, which was prepared at 5% (w/v) with 70% ethanol.

Ultra-fast Liquid Chromatography (UFLC)

The chemical composition of HERP was determined using UFLC. Reversed phase columns (XP-ODS 50 x 3 mm; particle size, 2.2 micrometers) and a diode array detector (Shimadzu Co.) were used according to the previously described method[9], with modifications. HERP was dissolved in methanol (50 mg/mL) and filtered with a 0.45 μm filter (Millipore). Aliquots of 2 μL of 1% HERP (w/v) were injected into the UFLC system, and the column was eluted using a linear gradient of water (solvent A) and methanol (solvent B). This gradient initially consisted of 40% B and was increased to 60% B

(after 22.5 min), maintained at 90% B (37.3- 42.3 min), and decreased to 30% B (after 42.3 min), with a solvent flow rate of 0.4 mL/min. Chromatograms were recorded at 260 nm and processed using specific software and LC solution. The following authentic standards of flavonoids and phenolic acids were used: formononetin, quercetin, kaempferol, pinocembrin, 3-hydroxy-7- methoxyflavone, catechin, epicatechin, rutin, propyl gallate, ferulic acid and p-coumaric acid.

Carcinogenesis induction

Carcinogenesis was induced the backs of the mice via the topical application of 10 µL of 0.5% DMBA (Sigma-Aldrich, St. Louis, USA) diluted in acetone (P.A.). The procedures were carried out when the animals were immobilized but not sedated. DMBA was applied three times per week for five weeks, and saline solution was applied to animals in the control groups (CTR) using an identical procedure.

Oral administration of HERP

The dry extract was suspended in 2% Tween 80 at 10 mg/mL prior to administration by gavage to the animals. The doses applied in this study included 10, 50 and 100 mg/kg. Distilled water was given to animals in the CTR group, and 1 mL of diluent (2% Tween) was administered to the animals in the TUM group. These substances were administered every other day (alternating with the application of DMBA), and the gavage procedure was performed for one week at the same dosages prior to the induction of carcinogenesis to assess potential adverse reactions to the natural product. Gavage was then performed for five weeks following the induction of carcinogenesis.

Macroscopic analysis of the DMBA-induced lesions

The time at which the dermal lesions first appeared on the animals was recorded, and the tumors were then classified according to their clinical appearance (ulcerative/verrucous), color (leukoplastic/erythroplastic) and bleeding index (bleeding/not bleeding).

Procedures for the histomorphological analysis of the specimens

After five weeks, the animals were euthanized in a CO_2 chamber for the post-mortem surgical removal of the treated area, regardless of the presence or absence of tumors. Tissue specimens were fixed in buffered formaldehyde (10%, pH 7.4) for 24 h, dehydrated in increasing concentrations of ethyl alcohol, and diaphanized in xylol for embedding in paraffin. Subsequently, 5 µm-thick histological sections were obtained and stained with hematoxylin-eosin, and the samples were analyzed using a light microscope (Olympus CX31 optic microscope) by three trained observers.

Assessment of histological tumor malignancy grading

The diagnosis of the epithelial tumors that developed in the animals was carried out according to their histological features, as previously described[10], and the histological malignancy grading was assessed using the criteria established by the guidelines for the diagnosis and treatment of cutaneous squamous cell carcinoma and precursor lesions[11]. The four broadest classification grades were based on the ratio of differentiated to undifferentiated cells (grade 1 = 3:1; grade 2 = 1:1; grade 3 = 1:3 and grade 4 = no tendency toward differentiation).

Statistical analysis

The average number of tumors that developed in the animals of each group was expressed as the mean ± standard error of the mean, and these results were analyzed using an analysis of variance (with *post-hoc* Tukey's test). All of the other data were expressed as values of absolute (n) and relative (%) frequency, and the groups were compared using Fisher's exact test. Differences between groups were considered to be significant when $p<0.05$.

Results

UFLC analysis was used to determine the chemical profile of HERP, and a representative UFLC chromatogram of HERP is presented in Figure 1. Peak 1 was identified as propyl gallate, peak 2 was identified as catechin, peak 3 was identified as epicatechin, and peak 4 was identified as formononetin. The concentrations of propyl gallate and catechin in HERP were 5.46 $mg.g^{-1}$ and 17.69 $mg.g^{-1}$, respectively. Formononetin appeared to be a significant compound in this propolis extract, although neither this compound nor epicatechin could be quantified because they were co-eluted with other compounds. In addition, peak 5 (a major concentration) was unable to be identified.

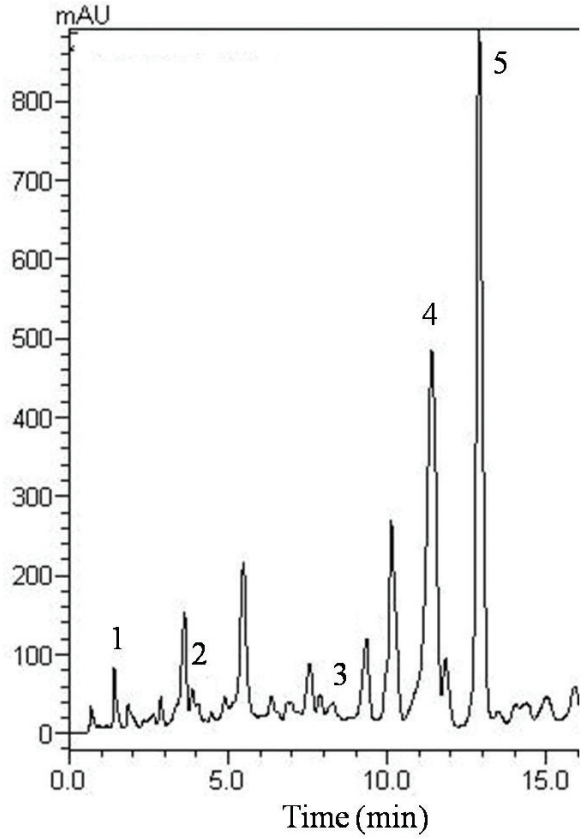

FIGURE 1 - UFLC chromatogram of HERP. (1) Propyl gallate; (2) Catechin; (3) Epicatechin; (4) Formononetin; (5) UV λ 234, 261 nm; RT = 12-13 min.

The oral administration of HERP did not delay the appearance of tumors, as skin tumors developed during the third week of tumor promotion following the initial carcinogen application in all of the DMBA-treated groups, regardless of HERP treatment. However, the highest dose of HERP (100 mg/kg) significantly decreased the tumor multiplicity throughout the five weeks of tumor promotion ($p<0.05$) (Figure 2).

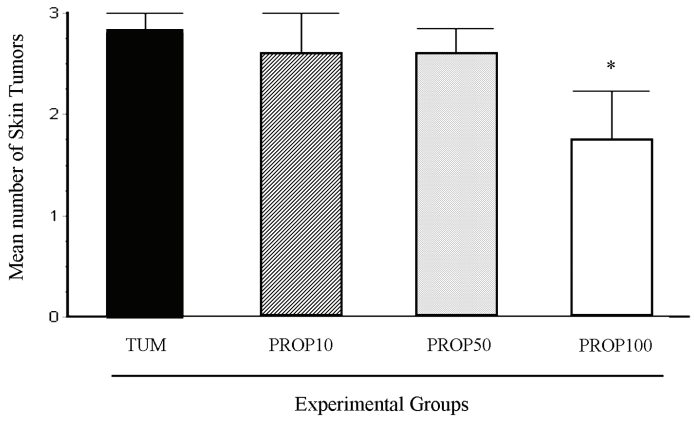

FIGURE 2 - Mean number of skin tumors that developed in the dermal tissues of DMBA-treated mice (mean ± standard error mean). *$p<0.05$.

The administration of 100 mg/kg HERP significantly altered the clinical presentation of the tumors ($p<0.05$), as the emergence of verrucous lesions was increased in PROP100-treated animals compared with the ulcerative pattern observed in the other DMBA-treated groups (Figures 3 and 4).

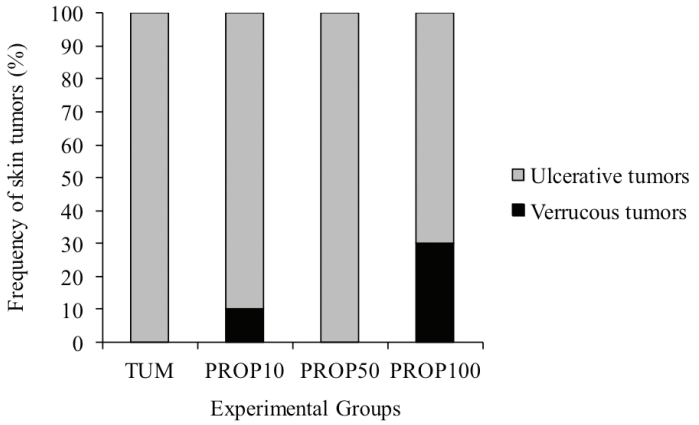

FIGURE 3 - Frequency of skin tumors that developed in DMBA-treated mice according to their clinical appearance. *$p<0.05$.

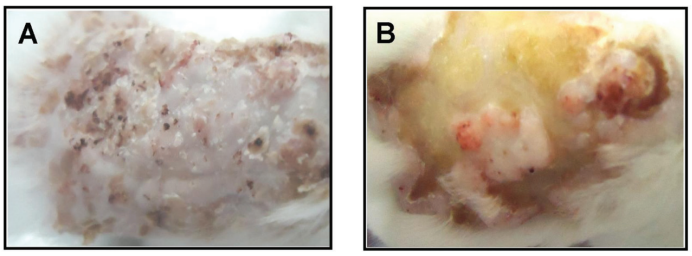

FIGURE 4 - Clinical appearance of DMBA-induced skin tumors. (A) Multiple ulcerative skin tumors were observed in TUM animals, and (B) Solitary verrucous tumors were observed in PROP100-treated animals.

All of the tumors that developed in the DMBA-treated animals were regarded as squamous cell carcinomas and were diagnosed either as non-invasive verrucous carcinomas (VCs) or invasive squamous cell carcinomas (SCCs). Moreover, these carcinomas were classified as grade 1, grade 2 or grade 3 tumors. Histologically, VCs appeared as a well-differentiated proliferation of squamous cells, consisting of the combination of high papillomatosis and hyperorthokeratinization with mild cytological or architectural anomalies. Moreover, the proliferative pattern seemed to push back rather than invade the underlying tissue. Grade 1 SCCs were characterized by the proliferation of well-differentiated squamous cells containing only slightly enlarged, hyperchromatic nuclei with abundant amounts of cytoplasm. Moreover, these tumors often produced large amounts of keratin and led to the formation of extracellular

keratin pearls. Grade 2 SCCs were characterized by moderately differentiated squamous cells with variable nuclear atypia and limited keratinization. Grade 3 SCCs presented as poorly differentiated tumors with greatly enlarged, pleomorphic nuclei and a high degree of atypia and frequent mitoses. Moreover, keratin production in these cells was markedly reduced or absent (Figure 5).

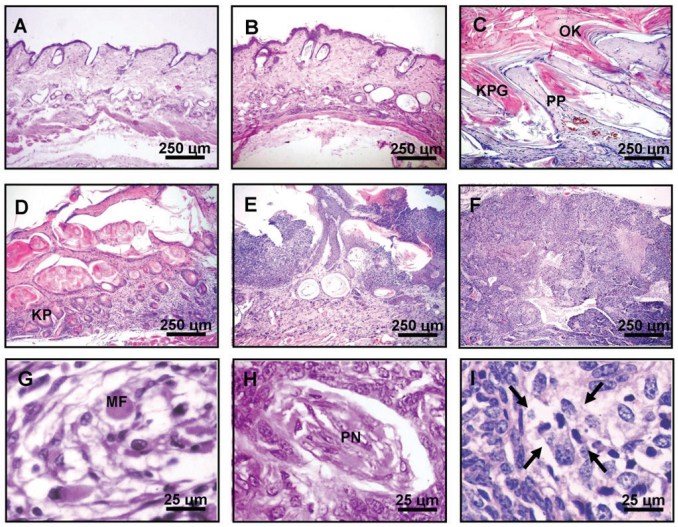

FIGURE 5 - The morphological features of H/E-stained histological sections from DMBA-induced squamous cell tumors of the skin. (A) and (B) Normal dermal tissue showing regular squamous epithelial lining with fibrous connective tissue below, as shown for CTR1 and CTR2, respectively. (C) Verrucous tumor showing intense orthokeratinization (OK), exophytic papillomatous projections (PP) and the formation of keratin plugs within the tumor crypts (KPG). (D) Grade 1 infiltrating SCC showing nests of well-differentiated squamous cells with abundant keratin pearl formation (KP). (E) Grade 2 SCC demonstrating proliferation of moderately differentiated squamous cells and few keratin pearls. (F) Grade 3 SCC showing the proliferation of poorly differentiated squamous cells and the lack of keratin formation. (G) Foci of intense cytological atypia and the disruption of muscular fibers (MF). (H) Invasion of the perineural space of the peripheral nerve (PN). (I) Tumor emboli within a lymphatic vessel (arrows).

No grade 4 SCCs (highly atypical sarcomatoid carcinomas) were detected in this study. The average scores for malignancy grading were significantly lower in PROP100-treated animals than TUM animals ($p<0.05$), but there were no differences between the other groups ($p>0.05$) (Figure 6).

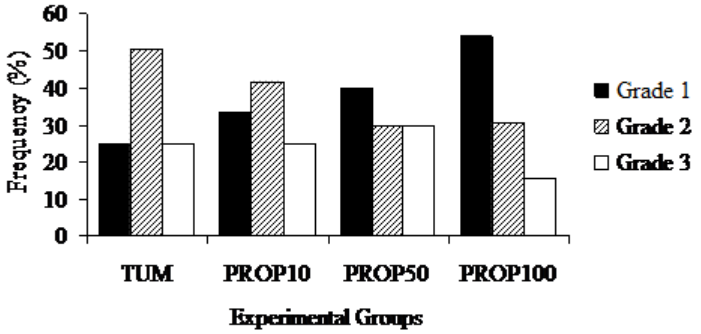

FIGURE 6 - Frequency of DMBA-induced squamous epithelial tumors in the experimental groups according to histological malignancy grading.

The frequency of tumor involvement in notable dermal anatomical structures, such as the invasion of the perineural space, the dissociation of muscle fibers and the formation of tumor emboli within lymphatic vessels (Figure 5), was evaluated. As shown in Figure 7, no significant difference in either the frequency of muscle invasion and dissociation or the infiltration of peripheral nerve sheathes was observed between groups ($p>0.05$).

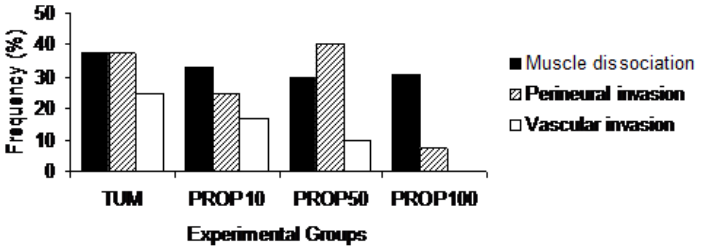

FIGURE 7 - Frequency of involvement of notable dermal anatomical structures by tumor cells in the experimental groups.

However, tumor emboli formation was significantly less frequent in the HERP-treated groups compared with the untreated groups, particularly at the dose of 100 mg/kg ($p<0.01$).

Discussion

According to Daugsch *et al.*[4], formononetin is one of the major components present in Brazilian red propolis, and several studies have used formononetin as a chemical and/or biological marker. Yang *et al.*[12] found that formononetin and its derivatives exhibited potent antiproliferative activities against two human tumor cell lines *in vitro*. Therefore, the identification of formononetin in our sample highlights the potential use of HERP as a chemoprotective agent.

The low number of DMBA-induced tumors observed in animals treated with the highest dose of HERP suggests that Brazilian red propolis had a chemopreventive effect against skin carcinogenesis. Furthermore, the number of verrucous tumors was also significantly increased in animals treated with 100 mg/kg HERP compared with the more ulcerative/infiltrative tumors that developed in the other treatment groups. These data suggest that Brazilian propolis may affect the growth pattern of tumors by stimulating the emergence of more exophytic (and less invasive) variants of SCC. Moreover, these morphological findings may influence the prognosis of cutaneous SCC, as it has been shown that exophytic malignant epithelial tumors display a lower tendency to generate lymph node metastases and therefore often lead to increased survival rates[13].

In the current study, significantly lower histological malignancy scores were observed for the tumors in HERP-treated animals, which supports the potential modulatory effect of Brazilian red propolis on the dynamics of tumor differentiation. These biological effects may be related to the chemical composition of the bee product, as it has been recently demonstrated that formononetin represents the major chemical constituent of Sergipe-derived Brazilian red propolis[4], and previous reports have also stated that this isoflavonoid possesses potent antioxidant activity[14]. However, studies have demonstrated that following consumption by mammals, formononetin is metabolized into daidzein, an aglycon isoflavonoid with distinct antitumor activities against breast[15] and ovarian cancer-derived cell lines[16]. These antitumor cytotoxic effects of daidzein are thought to be related to the inhibition of enzymes, such as DNA topoisomerase III, S6 ribosomal kinase, phosphoinositide 3-kinase and protein kinase C, which are proteins involved in the biochemical regulatory processes of cell proliferation and differentiation, and the generation of reactive oxygen species[17].

In this study, the epithelial tumors developed in animals treated with 100 mg/kg HERP exhibited better differentiation and high keratinization rate values compared with the other groups, and these histological findings may also be associated with the biological effects of daidzein. It has been reported that daidzein induces mitochondrial disruption and promotes apoptosis in tumor breast cells *in vitro* (MCF-7) by inhibiting the *bcl-2 gene*, stimulating bax transcription, and promoting the release of cytochrome C into the cytosolic environment[18]. As keratinization is an apoptosis-related phenomenon that occurs in fully differentiated keratinocytes, it is possible that the development of more keratinized tumors in HERP-treated animals could be related to dietary propolis-derived daidzein. However, although we identified formononetin in our HERP samples, we were unable to assess how much of this compound was metabolized into daidzein. Thus, further investigations are necessary to clarify whether this metabolite is involved in the modulation of cell proliferation and the differentiation of experimental cutaneous SCC.

No significant difference was observed between groups regarding the frequency of muscular and perineural invasion. However, the frequency of tumor cell emboli formation was significantly decreased in HERP100-treated animals. Moreover, lymph node metastases resulting from lymphatic vascular dissemination of tumor cells are regarded as the most relevant histological feature for predicting the biological behavior of SCCs[19]. Although no previous studies have investigated the antimetastatic properties of red propolis, the results of the current study suggest that such an antimetastatic effect may be related to the type of tumor differentiation and the less invasive tumors observed in HERP-treated animals. In fact, the invasiveness of SCCs is closely related to the metastatic potential of the tumor[20], but further studies are required to clarify the precise mechanisms underlying the red propolis-induced downregulation of vessel infiltration by squamous tumor cells.

In conclusion, oral administration of HERP at a dose of 100 mg/kg reduced the number of DMBA-induced skin SCCs, stimulated the formation of less invasive and more differentiated tumors, and reduced tumor emboli formation. These data suggest that Brazilian red propolis may exert an important modulatory effect on chemically induced dermal carcinogenesis.

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Acknowledgements

To Tiradentes University for the fellowship and the beekeepers, especially Jucilene Santana dos Santos, for the propolis sample.

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Received: Oct 15, 2013
Review: Dec 16, 2013
Accepted: Jan 20, 2014
Conflict of interest: none
Financial source: Foundation for Research and Technological Innovation Support from Sergipe (FAPITEC/SE)

[1]Research performed at Laboratory of Morphology and Structural Biology (LMBE), ITP (Science and Technology Institute), Tiradentes University (UNIT), Aracaju-SE, Brazil. Part of Master degree thesis, Postgraduate Program in Health and Environment. Tutor: Prof. Dr. Ricardo Luiz Cavalcanti de Albuquerque Junior.