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Free radical-scavenging activity of sequential leaf extracts of *Embothrium coccineum*

Abstract: The aim of this study was to evaluate the influence of phenolic, flavonoid, and anthraquinones from sequential extracts (n-hexane, dichloromethane, ethyl acetate, and ethanol) of *Embothrium coccineum* leaves on the antioxidant capacity, cell viability, and toxicity of the same, in order to find possible sources for novel antioxidants for food and pharmaceutical formulations. Antioxidant potential of sequential extracts was analyzed by five different assays: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay (DPPH), hydrogen peroxide scavenging activity (H_2O_2), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), and total reactive antioxidant potential (TRAP). An *in vitro* growth inhibition assay was performed using sulphorhodamine dye to quantify cell viability, while an *in vivo* brine shrimp lethality test was used to quantify toxicity. The dichloromethane extract has a greater efficiency in scavenging free radicals, combined with low toxicity, and no effect exhibited on healthy cells, compared to observations for the other extracts tested. Further research is in progress to identify and separate the active compounds of active extracts and investigate the protective effect of extracts on human dermal fibroblast injury induced by hydrogen peroxide.

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1 Introduction

Excessive concentrations of reactive oxygen species (ROS) in the human body are usually involved in a number of processes that cause cellular damage. The role of free radical reactions in cellular damage is well established and known to be involved in many acute, chronic diseases in humans, such as cancer, neurodegeneration, aging, atherosclerosis, ischemic injury, inflammation, and diabetes [1,2]. An imbalance between ROS and the inherent antioxidant capacity of the body calls for the use of dietary and /or medicinal supplements, particularly during the symptomatic periods of these diseases. The free radical reactions can be effectively quenched by synthetic antioxidants, such as BHA and BHT, which are known as ROS scavengers. In addition, these compounds are commonly used in food processing to prolong the storage stability of fats, oils, and lipid-containing foods; however, these synthetic antioxidants have also been reported to be involved in a number of physiological disorders and diseases [3]. In the last decade, natural phenolic compounds have been used as functional and nutraceutical ingredients and natural alternatives to synthetic antioxidants in the food industry [4-6]. These compounds not only have profound effects on food preservation, but also play an important role in preventing chronic diseases by reducing the oxidative damage caused by highly reactive molecules such as the radical $OH^{\bullet}$, $O_2^{\bullet}$ and radical peroxil ($ROO^{\bullet}$) [7,8]. A number of antioxidative sources of natural origin have been explored so far, but there is still a great need to search for new sources, which may be safer, more economical, and preferably from medicinal plants.

Embothrium coccineum J.R. Forst. & G. Forst., belonging to the Proteaceae family and more colloquially known as *notro*, *ciruelillo*, or *fosforito*, is an evergreen tree, 3-15 m high, with leathery, lance-shaped leaves and clusters of narrow, tubular scarlet flowers in late spring and early summer, found in the south of Chile and Argentina [9,10]. *E. coccineum* is a typical temperate forest tree, occurring also in southern Patagonia. It is grown as an ornamental in Great Britain and the United States, and as far north as the Faroe Islands [11]. *E. coccineum* has been used in various cultures, for example, the Huilliche and Yagan people, who recognized *E. coccineum* as a 'multipurpose medicinal tree', and used it for its antiseptic properties, in treatment of glandular conditions, wound healing, treatment of neuralgia, and tooth pain [12-14]. As such, this species has a long tradition as an important medicinal plant.

As previously mentioned, the search for novel natural antioxidants of plant origin is of increased interest to researchers, especially because of their free radical scavenging abilities and the protection they offer against diseases. *E. coccineum* is a tree that could potentially offer a good source of antioxidants. However, there are no reports regarding its chemical composition, the antioxidant capacity, or toxicity of leaf extracts. To fill this gap in the literature and applied sciences, the present study was designed to evaluate the radical scavenging capacity, cell viability, and toxicity of sequential extracts from leaves of *E. coccineum*. These extracts may serve as a potential source of new drugs for the cosmetic and pharmaceutical industry.

2 Methods

2.1 Plant collection

Leaves were collected in November 2013 from Malleco, Chile. The plant material was botanically identified by Forest Engineer Patricio Novoa, Botanical Expert, Horticulture Department Chief, "Jardín Botánico Nacional", Viña del Mar, Chile. A voucher specimen (accession #Ec11113) was deposited at the Herbarium of "Dr. Herbert Appel A.", Natural Products Laboratory, Department of Chemistry, Universidad Técnico Federico Santa María, Valparaíso, Chile.

2.2 Extract preparation

In brief, leaves of *E. coccineum* (200 g) were air-dried in shade at room temperature. The dry plant was powdered, and then mixed with 500 mL hexane. The extraction was carried out by using an orbital shaker (150 rpm) at 25°C for 24 h. The resulting mixture was filtered through Whatman No 1 filter paper and the hexane was removed from the filtrate under reduced pressure with a rotary evaporator. The residue was further extracted with dichloromethane, ethyl acetate, and ethanol, sequentially and serially. Finally, each extract was weighed and the yield was calculated. *E. coccineum* extracts were kept at 0°C prior to further analyses.

2.3 Chemicals

Aluminum chloride ($AlCl_3$); 2,2'-azo-bis(2-amidino propane) (ABAP); 2,2'-azinobi(3-ethylbenzothiazoline-6-sulfonic acid (ABTS); 2,2-diphenyl-1-picrylhydrazyl (DPPH); ferric chloride ($FeCl_3$); Folin-Ciocalteu; sulforhodamine B; trichloroacetic acid; 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (HAc); butylatedhydroxytoluene (BHT); emodin; gallic acid; hydrogen peroxide (H_2O_2); quercetin; sodium acetate (NaAc); sodium chloride (NaCl); sodium carbonate anhydrous (Na_2CO_3); Potassium dichromate ($K_2Cr_2O_7$); Trolox™ and all solvents n-hexane (99.8%); chloroform (99.8%); ethyl acetate (99.8%) and ethanol (99.8%) used were of analytical grade and purchased from Merck Co. (Darmstadt, Germany).

2.4 Estimation of total phenolic content

The total phenolic content was determined by the spectrophotometric method [15] with small modifications [16]. In brief, 0.5 mL of sample (1 mg/mL) was mixed with 2.5 mL of Folin-Ciocalteu's phenol reagent (0.2 N). After 5 min, 2 mL of a 7.5% Na_2CO_3 solution was added to the mixture and mixed thoroughly. The mixture was kept in the dark for 2 h at room temperature, after which the absorbance was read at 700 nm. The total phenolic content was determined from extrapolation of the calibration curve, which was made by preparing a gallic acid solution. The estimation of the phenolic compounds was carried out in triplicate. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per g of dried extract.

2.5 Estimation of total flavonoid content

Total flavonoid content was determined following a method by Arvouet-Grand et al. (1994) [17] with small modifications [16]. In brief, 5 mL of extract solution (1.0 mg/mL) was taken and mixed with an equal quantity

of a solution of 2% aluminum chloride in ethanol. After 10 min, the absorbance was measured against the reagent blank at 415 nm. The standard curve for total flavonoids was made using quercetin standard solution under the same procedure described earlier. The total flavonoids were expressed as milligrams of quercetin equivalents (QE) per g of dried extract.

2.6 Estimation of total anthraquinone content

The total anthraquinone content was estimated according to Mellado et al. (2012) [18]. In brief, 5 mL of extract solution (1.0 mg/mL) was taken and mixed with an equal quantity of a solution of 2% aluminum chloride in ethanol. After 10 min, the absorbance was measured against the reagent blank at 485 nm. The standard curve for total flavonoids was made using emodine standard solution under the same procedure as earlier described. The total anthraquinone content was expressed as milligrams of emodin equivalents (EE) per g dried extract.

2.7 Antioxidant assays

Each sample was dissolved in 95% ethanol to make a concentration of 1 mg/mL and then diluted to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparison in all assays.

2.7.1 DPPH radical scavenging activity assay

The free radical scavenging activity of the extracts was measured *in vitro* by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier [19]. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. A 2.9 mL aliquot of this solution was mixed with 100 µL of the sample at various concentrations (2.0–0.2 mg/mL). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance (A) was recorded at 517 nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = [(A_{control}\text{-}A_{sample})/A_{control}] \times 100$$

From this, the obtained percent radical scavenging capacity gives the IC_{50} value, which represents the concentrations of extracts that caused 50% neutralization, as determined by linear regression analysis. All measurements were performed in triplicate.

2.7.2 Hydrogen peroxide scavenging activity

The ability of extracts to scavenge hydrogen peroxide can be estimated according to the method described earlier [20]. Hydrogen peroxide solution (40 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 mL) of different extracts were transferred into the test tubes and their volumes were made up to 0.4 mL with phosphate buffer. After addition of 0.6 mL hydrogen peroxide solution, tubes were vortexed and, after 10 min, absorbance of the hydrogen peroxide was determined at 230 nm against a blank. The abilities to scavenge the hydrogen peroxide were calculated using the following equation:

$$\text{Hydrogen peroxide scavenging activity (\%)} = [(A_{control}\text{-}A_{sample})/A_{control}] \times 100$$

From this, the obtained percent radical scavenging capacity gives the IC_{50} value, which represents the concentrations of extracts that caused 50% neutralization, as determined by linear regression analysis. All measurements were performed in triplicate.

2.7.3 Oxygen Radical Absorbance Capacity (ORAC)

Oxygen radical absorbance capacity was determined using the ORAC assay according to an earlier described method [21], with some modifications. Briefly, a solution containing pyrogallol red (PGR) (5 µM), Trolox™, 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) (10 mM) with or without the extract sample, was incubated at 37°C in phosphate buffer 75 mM, pH 7.4. PGR consumption was evaluated from a decrease in absorption intensity (A) at 540 nm. Control solutions (in the absence of extracts, A_0) showed a fast consumption of the probes. Values of (A/A_0) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (A/A_0) reached a value of 0.2. The ORAC values are calculated using the following equation:

$$\text{ORAC} = [(\text{AUC-AUC}_0)/(\text{AUC}_{Trolox}\text{-AUC}_0)] \times f[\text{Trolox}]$$

Where AUC is the area under the curve in the presence of tested extracts, integrated between time zero and that which corresponds to 80% of the probe consumption; AUC_0 is the area under the curve for the control (PGR plus AAPH solution); AUC_{Trolox} is the area under the curve for Trolox; f is dilution factor, equal to the ratio between the total volume of the AAPH-Trolox solution and the added extract volume; [Trolox] is Trolox millimolar concentration. The ORAC values of the extracts were expressed as μM of Trolox equivalent antioxidant capacity (μM TEAC) based on a trolox standard curve. All experiments were carried out in triplicate.

2.7.4 Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing power of extracts was determined using the FRAP assay [22] with minor modifications. The FRAP reagent consisted of 10 volumes of 300 mM acetate buffer, 1 volume of 20 mM $FeCl_3$ and 1 volume of 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution. 100 mL of the extracts (1.0 mg/mL) in distilled water (300 μL) was added to FRAP reagent and then shaken for 15 s. After incubation for 30 min at 37 °C in a water bath, the absorbance was measured at 593 nm. The reducing capacity FRAP of extracts was expressed in mM Trolox™, using a standard curve of Trolox™ (0-120 mg/L). All measurements were replicated three times.

2.7.5 Total Reactive Antioxidant Potential (TRAP)

The total reactive antioxidant potential of extracts was determined using the TRAP assay [23], slightly modified in this experiment. ABAP (2,2'-azo-bis(2-amidino propane) solution (10 mM) was mixed with $ABTS^{\bullet+}$ (2,2'-azinobi(3-ethylbenzothiazoline-6-sulfonic acid) solution (150 μM) in PBS (phosphate buffered saline, pH 7.4) solution (100 mM). The mixture was incubated at 45°C for 30 min. 10 μL of extract solution was added to 990 μL of the resulting blue-green ABTS radical solution. The decrease of absorbance of TRAP solutions and ABTS as blank were recorded after 50 s at room temperature. Then, the absorbance of the samples was measured at 734 nm. The total antioxidant capacity TRAP of extracts was expressed in mM Trolox™ equivalent antioxidant capacity (TEAC μM), using a standard curve of Trolox™ (0-120 mg/L). All measurements were replicated three times.

2.8 Cytotoxic analysis

2.8.1 Cell lines

The experimental cell cultures used were obtained from American Type Culture Collection (Rockville, MD, USA). Non-tumor cell line, human colon epithelial cells CCD 841 CoN (CoN), and human dermal fibroblast (DHF) were grown in DMEM-F12 medium containing 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1 mM L-glutamine. Cells were seeded into 96 well microtiter plates in 100 μL at a plating density of 3×10^3 cells/well. After 24 h incubation at 37°C (under a humidified 5% carbon dioxide atmosphere to allow cell attachment), the cells were treated with different concentrations of sequential extracts (0-150 μg/mL) and daunorubicin (0.05-50 μM) and incubated for 72 h under the same conditions. Stock solutions of compounds were prepared in ethanol and the final concentration of this solvent was kept constant at 1%. Control cultures received 1% ethanol alone.

2.8.2 *In vitro* growth inhibition assay

The sulforhodamine B assay was determined using the method of Madrid et al. (2011) [24]. Briefly, the cells were set up, $3{\times}10^3$ cells per well, in a 96-well, flat-bottomed 200 μL microplate. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and treated with the compounds at different concentrations for 72 hours. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid at 4°C (TCA final concentration 10%). After washing with distilled water, cells were stained with 0.1% sulforhodamine B, dissolved in 1% acetic acid (50 μL/well) for 30 min, and subsequently washed with 1% acetic acid to remove unbound stain. Protein-bound stain was solubilized with 100 μL of 10 mM unbuffered Tris base. The cell density was determined using a fluorescence plate reader (wavelength 540 nm). Values shown are the mean + SD three independent experiments in triplicate.

2.8.3 Brine shrimp lethality test

Toxicity was determined using the *A. salina* nauplii lethality assay developed by Meyer et al. with some modifications [25]. One gram of *A. salina* cysts (brine shrimp eggs) were allowed to hatch and mature as nauplii in 1000 mL filtered artificial seawater (3.8% w/v salt in distilled water) for

48 h at 25°C under continuous aeration and light regimen. Highly active nauplii were concentrated to a suitable density by placing an artificial light at one end of their incubation beaker and the nauplii-rich water closest to the light was removed with a pipette for biological assays. About ten nauplii were transferred to each of the two-fold serially diluted test (extracts) solutions (1000-10 mg/mL extract in 4 mL artificial seawater). Potassium dichromate ($K_2Cr_2O_7$) was prepared and serially diluted in artificial sea water to get concentrations between 1000-1.00 µg/mL to serve as positive control. Artificial sea water was used as negative control. After the 24 h incubation at 25°C, a magnifying lens was used to count the number of dead nauplii, and the mortality percentage was calculated [26]. Larvae were considered dead only if they did not move their appendages for 10 s during observation. The concentration killing fifty percent of the larvae (LC_{50}) was calculated using the probit analysis method [27].

2.9 Statistical analysis

The data were reported as mean values ± standard deviation (SD). Kruskal-Wallis ANOVA was used with a confidence level of 95%. Values representing the concentrations of investigated extracts that caused 50% of inhibition (IC_{50}) were determined by linear regression analysis of Radical Scavenging Capacity (%); likewise for the ORAC, FRAP, and TRAP assays (STATISTICA 7.0 program). For LC_{50} values, probit analysis was used with the software MINITAB 15, with a confidence level of 95%. Significant difference was considered at the level of $P < 0.001$.

3 Results and discussion

3.1 Extraction yields

The sequential method adapted to obtain different *E. coccineum* extracts was as follows: hexane, dichloromethane, ethyl acetate, and ethanol. The highest yields were achieved with ethanol (9.92%), followed by hexane (3.45%), dichloromethane (3.17%), while the lowest was ethyl acetate (1.54%).

3.2 Total phenolics, flavonoids and anthraquinones contents

This is the first study to record the phenolic, flavonoid, and anthraquinone contents of extracts from *E. coccineum* and from the genus *Embothrium*. All results are presented in Table 1. The amount of total phenolics varied in the different extracts, ranging from 9.8 ± 0.29 to 44.9 ± 1.44 mg GAE/g d.e. The highest amount of phenolics was in ethyl acetate extract (44.9 ± 1.44 mg GAE/g extract d.e.), while hexane was poor in phenolics (9.8 ± 0.29 mg GAE/g d.e.). This variation was due to the polarity of phenolics: the polar fractions had more phenolics than those non-polar fractions [28]. For the amount of flavonoids, a small quantity was in ethyl acetate extract (5.02 ± 0.09 mg QE/g d.e.). Hexane, dichloromethane, and ethanol extracts did not contain flavonoids. For anthraquinones, a small amount was obtained with dichloromethane extract (2.05 ± 0.03 mg EE/g d.e.); the others extracts did not contain anthraquinones. To our knowledge, no other study has investigated the quantities of phenolics, flavonoids, or anthraquinones for this species.

3.3 *In vitro* antioxidant activity

Table 2 presents the antioxidant capacity of sequential extracts obtained from leaves of *E. coccineum*, examined using five different assays.

In this study, five complementary antioxidant testing methods, based on different chemical reaction mechanisms, were used.

The DPPH free radical method determines the antiradical power of antioxidants. From all evaluated extracts, the extract of ethanol shows a significant difference ($p = 0.016$), indicating sharp activity (Table 1)

Table 1: Chemical composition of *E. coccineum* extracts.

Leaf extract	Polyphenols (GAE)[a]	Flavonoids (QE)[a]	Anthraquinones (EE)[a]
Hexane	9.84 ± 0.29	n.d.	n.d.
Dichloromethane	24.01 ± 1.30	n.d.	2.05 ± 0.03
Ethyl acetate	44.89 ± 1.44	0.15 ± 0.06	n.d.
Ethanol	30.13 ± 0.52	n.d.	n.d.

Standard deviations (SD) did not exceed 5%. n.d.: not detected.

[a]mg/g dry extract.

Table 2: Antioxidant capacities of sequential extracts from leaves of *E. coccineum*.

Sample	IC_{50} DPPH• (mg/mL)	IC_{50} H_2O_2 (mg/mL)	ORAC-PGR (μM TEAC)	FRAP (mM Trolox)	TRAP (μM Trolox)
Hexane	21.78 ± 2.46	0.43 ± 0.01	270.61 ± 24.88	0.40 ± 0.01	95.61 ± 15.2
Dichloromethane	15.51 ± 0.85	0.28 ± 0.01	390.98 ± 48.04	0.42 ± 0.01	167.19 ± 29.06
Ethyl acetate	8.79 ± 0.14	17.67 ± 1.62	405.21 ± 12.73	0.53 ± 0.02	96.96 ± 7.30
Ethanol	5.27 ± 0.06	12.38 ± 1.91	-16.78 ± 1.545	0.50 ± 0.01	98.31 ± 18.38
Trolox	0.11 ± 0.01	2.86 ± 0.06	n.d	n.d.	n.d.
BHT	n.d.	2.56 ± 0.05	583.90 ± 42.38	1.52 ± 0.07	1055.93 ± 20.03
Gallic Acid	0.06 ± 0.00	n.d.	695.33 ± 62.71	1.72 ± 0.02	1131.17 ± 8.47

n.d.: not detected.

with an IC_{50} value of 5.27 ± 0.03 mg/mL, followed by ethyl acetate extract (8.79 ± 0.14 mg/mL); meanwhile, the hexane extract has the lowest activity (21.78 ± 2.46 mg/mL). We can deduce that extracts obtained using high polarity solvents were considerably more effective radical-scavengers than were those using low polarity solvents. This sharp activity is correlated with the distribution of phenolic compounds ($r^2 = 0.638$). This variation was due to the polarity of phenolics, where the polar fractions had more phenolics than non-polar fractions [26]. In addition, the difference for IC_{50} between samples might be described by variation in polyphenolic composition of analyzed extracts [29].

Notwithstanding, the differentiation of the scavenger activity of free radicals of all extracts is of lower activity than the reference antioxidants (0.11 and 0.06 mg/mL Trolox and Galic acid, respectively).

Hydrogen peroxide (H_2O_2), when present in excess, is one of the many compounds that can be injurious for cells [30]. This damage increases as this reactive oxygen species (ROS) is combined with Fe^{+2}, producing hydroxyl radicals through the Fenton reaction, which involves lipid peroxidate [31]. With regards to the evaluated extracts, there is a significant difference ($p = 0.015$), indicating that the extracts related to dichloromethane and hexane are the most active (0.28 ± 0.01 and 0.43 ± 0.01 mg/mL, respectively). This result is consistent with the distribution of anthraquinones in the different extracts ($r^2 = 0.888$). This correlation is supported by the existence of some anthraquinone compounds, possibly alizarin and emodin, which have an inhibitory activity on lipid peroxidation and reduce H_2O_2 in cells [32,33]. In contrast to reference antioxidants used, hexane and dichloromethane extracts exhibited far higher activity than Trolox and BHT, at an average of 8-fold more active.

ORAC (oxygen radical absorbance capacity) is one of the most used antioxidant analyses in recent years, where the "antioxidant power" of food and chemical substances is evaluated [34]. Also, the ORAC rate has been widely used to evaluate concoction antioxidants. This method measures the protection supplied by an antioxidant to the objective molecule, which is oxidized by peroxyl radicals [21]. According to the obtained data, there is a significant difference ($p = 0.024$), indicating that the ethanol extract presents lower activity. In reference to the correlation with the phenolic compound contents (phenols, flavonoids, and anthraquinones), there is little correlation ($r^2 < 0.5$). In this sense, due to complex extract composition, the total antioxidant capacity ORAC is the result of the combination of activity and interaction of a wide range of compounds and is not correlated with the determination of phenolic compounds [35]. In contrast to the reference antioxidants used, there are significant differences ($p = 0.006$), for which only the ethanol extract is less active, so that the extracts have similar antioxidant activity to those of the references.

The measured antioxidant power, in correlation to the reduction of ferric cation to ferrous, is a simple and reliable colorimetric method. This assay is commonly used in routine "simple antioxidant" analysis and for the total antioxidant activity of plants extracts. The main part of the methods applied to the metabolite antioxidant evaluation and/or extracts does not directly measure scavenger capacity of the samples, which is an important parameter for effective antioxidants [36,37]. This assay showed that the ethyl acetate extract (0.53 ± 0.02 mM TEAC) had significant differences ($p = 0.016$) - this phenomenon can be explained due to the distribution of phenolic compounds ($r^2 = 0.872$, see Table 3), which is in direct accordance with literature [29]. However, in comparing all the extracts with the reference antioxidant, this extract has a lower reducing capacity. The total radical-trapping antioxidant potential (TRAP) assay is based on the protection given by the antioxidant during peroxidation reactions [34]. The data obtained do not show significant differences between the evaluated extracts ($p = 0.100$); however, there is a trend in the dichloromethane, which has the highest activity (167.19 ± 29.06 μM TEAC). This activity is related to the distribution of anthraquinones

Table 3: Correlation (r^2) of estimated compounds and antioxidant assays.

Compounds	DPPH	H_2O_2	ORAC	FRAP	TRAP
Phenols	0.638	0.756	0.020	0.756	0.019
Flavonoids	0.113	0.505	0.193	0.390	0.060
Anthraquinones	0.058	0.888	0.187	0.174	0.795

in the different extracts (r^2 = 0.795). This phenomenon allows for consideration of anthraquinone derivatives as multipotent antioxidants since this TRAP activity is in addition to their antioxidant activity [38].

3.4 Cytotoxic analysis

Free radicals caused alternation in the structure of biological membranes and further interfered with cellular integrity and metabolism, which leads to cellular toxicity [39]. The evaluation of viability of sequential extracts from *E. coccineum* on normal cells was dose-dependent (µg/mL), and determined by *in vitro* cytotoxicity assay and cell viability expressed as % vs. control vehicle (ethanol 0.1%) for the extracts. The IC_{50} values of sequential extracts of *E. coccineum* on normal cells were above 100.0 µg/mL.

These results indicate the non-toxic effect of sequential extracts of *E. coccineum* on normal cell lines, an interesting feature due to recent studies suggesting the toxic potential of synthetic antioxidants such as BHA in mammalian cells, in which low doses of a compound exerted a significant cytotoxic effect associated with loss of mitochondrial function. In parallel, BHA induced an irreversible loss of cell proliferative capacity, preceding apoptosis induction [40].

From this point of view, it can be presumed that the cytotoxic activity of components contained in the sequential extracts of *E. coccineum* is low against normal cells, so that these extracts may be applicable as dietary supplement in food industries with minimized adverse reaction.

3.5 Toxicity analysis

The brine shrimp lethality bioassay is an efficient, rapid and inexpensive test that requires only a relatively small amount of sample. This bioassay has good correlation with antioxidant activity and cytotoxic activity in some human cancer cells [41]. The results obtained in the bioassay with *Artemia salina* indicate that the ethanol extract is more toxic in comparison with the others, because a lower concentration is required to achieve 50% mortality (Table 4).

Extract is considered toxic only if LC_{50} is less than 1.0 mg/mL [25]. As these herbal substances have been shown to be nontoxic at very high doses in experimental *A. salina*, it might be a good candidate antioxidant agent for human consumption.

4 Conclusion

There are numerous reports on bioactive extracts in plants, but to date, there have been no reports regarding the antioxidant effects of leaves from *E. coccineum*. We first evaluated *E. coccineum* leaf extracts, obtained through sequential solvent extraction, for phenolic contents and *in vitro* radical scavenging activities using various methods. Second, the sequential extracts were tested for cell viability in two lines of normal cells, and toxicity *in vivo* against *A. salina* nauplii. We have shown that the dichloromethane extract has a greater efficiency in scavenging free radicals, combined with low toxicity, and no effect exhibited on healthy cells, compared to observations for the other extracts we tested in this study. This study thus proposes an alternative source of natural antioxidant for nutraceutical and functional food applications, which would strongly enhance the utilization of *E. coccineum*. Further research is in progress to identify and separate the active compounds of active extracts and investigate the protective effect of extracts on human dermal fibroblast injury induced by hydrogen peroxide.

Table 4: LD_{50} of sequential extracts of leaves of *E. coccineum* against *A. salina* nauplii.

Leaf Extract	LD_{50} (µg/mL)	X^2	Confidence Limit
Hexane	1.420	0.97	± 0.06
Dichloromethane	1.521	0.90	± 0.011
Ethyl acetate	1.449	0.98	± 0.032
Ethanol	1.369	0.96	± 0.029

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