



Preclinical Evaluation of the *Encelia canescens* Lam Extract: Medicinal Properties useful for Cancer Treatment

Juan P. Cayún¹, Javier Zamorano¹, Luis Quiñones^{1*}, Nelson M. Varela^{1,2},
Valentina Squicciarini¹, Héctor D. Larenas¹, Dante D. Cáceres^{1,3}

¹Laboratory of Chemical Carcinogenesis and Pharmacogenomics, IFT, Program of Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine, University of Chile, P.O. Box 701 11, Santiago, Chile

²School of Medical Technology, University of Chile, Santiago, Chile

³Environmental Health Program, School of Public Health, Faculty of Medicine, University of Chile, Santiago, Chile

Abstract

Encelia canescens Lam (nv: mancapaqi, mataloba, matalobo, mucle, or “coronilla de fraile”) is a 30-80-cm high shrubby perennial plant that grows in the Atacama and Coquimbo regions of Chile up to 1,700 m above the sea. The present research was performed to examine the toxicological, antioxidant and antitumoural properties of aqueous and ethanol *Encelia canescens* Lam extracts. Our results revealed the presence of saponins, terpenes, flavonoids, coumarins and tannins. The total phenolic compound contents were 19 ± 1.14 mg/g tannic acid in the aqueous extract and 23 ± 1.60 mg/g tannic acid in the ethanol extract. The total flavonoid contents were 0.15 ± 0.02 mg/g quercetin in the aqueous extract and 0.13 ± 0.06 mg/g quercetin in the ethanol extract. Based on 2,2-diphenyl-1-picrylhydrazyl assays, the antioxidant capacities were 354 ± 23 μ mol trolox/100 g of the dry ethanol extract and 303 ± 15 μ mol trolox/100 g of the dry aqueous extract. Using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid, the antioxidant capacities were $2,354 \pm 568$ μ mol trolox/100 g of the dry ethanol extract and $1,856 \pm 453$ μ mol trolox/100 g of the aqueous extract. Neither of the extracts exhibited toxicity in the acute topical and oral toxicity tests on the rodents. Antiproliferative studies with cultures of SaOS-2 cells revealed substantial inhibitory effects of both extracts. Moreover, an *in vivo* antitumour assay revealed that the aqueous extract at a dose of 16 mg/kg x day and the lyophilized extract at a dose of 4 and 8 mg/kg x day efficiently increased the survival of mice with tumours, and the lyophilized extract was able to significantly reduce tumour size at a dose of 8 mg/kg x day. Therefore, the results of the present study established that extracts of *Encelia canescens* Lam have antitumour activities. Considering the properties of *Encelia canescens* Lam revealed in this preclinical evaluation, clinical studies approved by the health authorities should be performed to examine the efficacy and safety of extracts of this plant in cancer patients.

Keywords: *Encelia Canescens* Lam, Antineoplastic, Cancer, Preclinical

1. Introduction

Through the years, humans have used agents from natural sources to cure their pathological conditions¹. In this sense, plants have formed the basis of traditional systems of medicine that have been in use for hundreds of years. Currently, these systems are still developing roles in healthcare. The World Health Organization

(WHO) has estimated that approximately 80% of people worldwide have used traditional medicine for health care². Moreover, many secondary metabolites of plants, including alkaloids, glycosides, polyphenols, coumarins, saponins, terpenes and terpenoids, which have interesting medicinal properties, including antineoplastic activity, are known to be potentially useful for protecting the human body from diverse pathological conditions^{3,4}.

*Author for correspondence

Email: lquinone@med.uchile.cl

Currently, phytopharmacology and ethnomedical ancestral knowledge is being combined with the modern basic and clinical pharmacological knowledge to fight pathological conditions. The present tendency retains the use of the whole medicinal plant in the form of standardized extracts combined with the use of numerous pharmaceutical technologies to create products that do not differ in appearance or quality from traditional allopathic medicines.

The WHO defines a medicinal plant as any plant species that contains substances that can be used for therapeutic purposes or whose active ingredients can act as catalysts for the synthesis of new drugs. Folk knowledge about medicinal plants is based on efficiency or is accepted and adopted based on observations of previously used plants; however, a problem in popular phytotherapy is the difficulty of controlling the dose and the product quality, and this problem can elicit risks and damage to health. Despite the low toxicity of the active ingredients of some plant species, they can elicit health problems due to factors such as microbiological contamination and the presence of pesticide residues, heavy metals or herbicides.

In contrast, cancer is a complex disease and the leading cause of death worldwide, and its treatment, control and prevention requires a multidisciplinary approach. Therefore, researchers are constantly searching for new compounds that can fight this disease and improve patient quality of life. In this search, plants are the main potential source of anticancer agents⁵.

Although the development of cancer treatments has been spectacular and there are many potential drugs for fighting the proliferation of tumour cells⁵⁻⁹, these drugs that are created by chemical synthesis and/or derived from plants do not have the expected clinical effects. Therefore, more effective and less toxic antineoplastic agents are needed, and their modes of action require further active research.

Among the natural products with anticancer activities, the best-known examples are the vinca alkaloids (vinblastine and vincristine) isolated from Madagascar periwinkle, *Catharantus roseus* which is used in many cultures for the treatment of diabetes. During an investigation of this plant, these compounds were discovered to exhibit strong potential as hypoglycaemic agents, but their discovery was indirectly

attributed to the observations based on an unrelated use. Other good examples include the epipodophyllotoxins (etoposide, etoposide phosphate, and teniposide), the taxanes (paclitaxel and docetaxel), and the camptothecin derivatives (irinotecan and topotecan). Several other plant-derived compounds are currently in preclinical and clinical trials³. Thus, research that is conducted on a natural product and progresses until the agents are obtained may be considered to be a complete cycle for a plant that was originally used for the treatment of cancer.

The new interesting and potential antitumoural plant reported here is *Encelia canescens* Lam (nv: mancapaqi, mataloba, matalobo, mucle, or “coronilla de fraile”), which is a 30-80 cm high shrub by perennial plant that grows in the Atacama and Coquimbo regions of Chile up to 1,700 m above sea level. The plant infusion has been used locally for at least 100 years to treat cancer symptoms and other pathologies in northern Chile. Until now there is not literature evidence for its anti-cancer properties. Based on this observation, the present study was conducted to gather information about the properties of *Encelia canescens* Lam extracts (ECEs) to examine their potential clinical applications for cancer treatment.

2. Materials and Methods

2.1 Chemicals

The analytical standards and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stain were purchased from Sigma-Aldrich (USA). Sodium hydrogen phosphate, potassium dihydrogen phosphate, sodium acetate, ibuprofen, sodium naproxen, acetic acid, dimethyl sulphoxide, methanol, ethanol, acetonitrile, and other solvents, as well as silicagel GF25460, nutrient broth, nutrient agar and Sabouraud dextrose agar were purchased from Merck A. G. (Darmstadt, Germany). Freshly deionized, ultrafiltered water was obtained with a Milli-Q system (Molsheim, France).

2.2 Plant Material

The aerial parts of *Encelia canescens* Lam. (Asteraceae) were collected in the Huasco River Valley Region III, Chile, firstly in 2006 (July) for preliminary physicochemical characterization and the study of antioxidant properties, and secondly in 2010 (July) for pre-clinical studies. A control sample of the plant identified by the School of

Chemical and Pharmaceutical Sciences of the University of Chile is preserved in the international Herbarium of the School of Chemistry and Pharmacy (SQF), Faculty of Chemical and Pharmaceutical Sciences, University of Chile under No. 22259.

2.3 Extraction and Identification

Encelia canescens Lam leaves were pulverized, boiled in distilled water or ethanol at 100 g/L for 10 min, and clarified by filtration through Whatman #1 paper. The filtered extracts were lyophilized and designated as aqueous *E. canescens* extract (A-ECE) and ethanol *E. canescens* extract (E-ECE). A solution of defined concentration was prepared in distilled water to test its biological activities (6.4 mg/mL). Identification screening was performed by thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance spectroscopy (H^1 RMN). For the HPLC identification, the extraction was performed in a methanol 50 ml – water 50 ml solution at 25° C in an ultrasonic extraction device for 60 min. The extract was filtered and injected into the HPLC column with an autosampler. Two-solvent gradient elution was used (solvent A: 0.32% phosphoric acid; solvent B: acetonitrile). Detection was performed at 280 nm and 40° C with a C8 VP ODS Shimadzu column (250 mm x 4.6 mm I.D. x 5 μ m). For H^1 RMN identification, fifty-milligrams of *Encelia canescens* Lam extracts were used; A-ECE and E-ECE in nanopure water/ethanol (20%), 1 mL sample to 10 mL aqueous or ethanolic sample. The undissolved remains were removed by centrifugation, and the samples were loaded onto C-18 Sep-pack disposable columns (Waters). Each fraction was collected separately and dried in vacuo using a CentriVap Console (Labconco) at 75° C. The samples were resuspended in deuterated chloroform and analysed by nuclear magnetic resonance spectroscopy on a Bruker Avance 400 MHz machine. For TLC identification, the chromatograms on silica gel GF254 were analysed with UV light (wavelengths of 254 and 366 nm). The compounds in the extracts dissolved in distilled water were examined with different developing systems.

2.4 Cell Line and Culture Media

The SaOS-2 cell line, which is a non-transformed cell line derived from a human osteogenic sarcoma (American

Type Culture Collection ATCC®: HTB-85 German Collection of Microorganisms and Cell Cultures DSMZ: ACC 243), was used for the antiproliferative studies. The cells were cultured in bovine foetal serum supplemented with McCoy's 5A media with 100 U/mL of penicillin, streptomycin (0.07 M), amphotericin (0.5 μ M) and L-glutamine (2mM; Gibco®). Incubation was performed at 37 °C for 48 hours in 95% air and 5% CO₂. A protocol for assessing the growth profiles of individual cell groups under study was applied to perform cell counts from the three plates for each group at regular intervals of 24 hours over a total of 5 days (i.e., the cell count times were 0, 24, 48, 72, 96 and 120 hours). The medium for each cell group was replaced every 48 hours (i.e., at 48 and 96 hours).

2.5 Animals

Wistar rats (150-200 g; 30-35 days old), Sprague Dawley rats (200-250 g; 30-35 days old) and albino CF-1 mice (25-35 g; 30-35 days old) of either sex were selected for the experiments and procured from the Biotery of the Faculty of Pharmaceutical and Chemical Sciences and the Laboratory of Biological Control of the Faculty of Medicine of the University of Chile. The A/J strain of the albino mice (30-35 g; 90 days old) was kindly supplied by Dr. Jorge Ferreira Parker of the Biomedical Science Institute, Faculty of Medicine, University of Chile. The animals were maintained for two weeks in our laboratory environment prior to the experiments while housed in polypropylene cages in standard laboratory conditions (20°C \pm 2°C; 55-70% humidity; and on a 12-12-h light-dark cycle). The animals were fed with standard diet and provided water *ad libitum*. The principles of the NIH's Guide for Care and Use of Laboratory Animals (2011) and the instructions given by the Animal Bioethical Committee from the Faculty of Medicine, University of Chile, were followed throughout the experiments. For the oral analgesia and toxicity tests, the minimum dose was extrapolated from the traditional human use of the herbal infusion; people typically drink 2 g of the plant, which resulted in 80-150 mg of the water extract and 10-40 mg of the lyophilized extract.

2.6 Antimicrobial Activity (Bioautography)

The following clinically isolated microorganisms were used in this study: *Staphylococcus aureus*, *Bacillus*

subtilis, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, *Myotis flavus*, *Saccharomyces cerevisiae*, and *S. aviatum*. The isolates were maintained on agar slants at 4°C in a refrigerator.

The bioautography technique^{10,11} was employed to evaluate the antimicrobial activities of the extracts. 20 milligrams samples of the plants were dissolved in 1 mL of methanol and applied in volumes of 10 µL to two glass plates coated with silica gel 60 (5 x 7 cm) that had previously been UV sterilized. The solvent solution used for elution was hexane:ethyl acetate (6:4). A Thin Layer Chromatography (TLC) plate was placed into a sterile petri dish and incubated at 37°C for 24 h for the bacteria or at 28°C for 48 for the fungi. The TLC plate was then stained with a solution of tetrazolium bromide dye at 5 mg/mL for 1 h. Purple or colourless halos indicated growth inhibition.

2.7 Determination of the Total Phenolic Compounds

Using a modified Folin-Ciocalteu method¹², the total phenol contents of the extracts were determined. One-millilitre aliquots of the extracts were mixed with 5 mL of water-diluted (1:10) Folin-Ciocalteu reagent and 4 mL (75 g/l) of sodium carbonate. The tubes were vortexed for 10 sec and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using a UNICAM UV-VS spectrophotometer. The samples of the extracts were evaluated at a final concentration of 0.1 mg/mL. The total phenolic contents are expressed as mg/g tannic acid equivalents.

2.8 Determination of Total Flavonoids

The estimations of the total flavonoids in the plant extracts were performed using the method of¹³. One-half millilitre of 2% AlCl₃ ethanol solution was added to 0.5 mL of the sample. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoids. The extract samples were evaluated at a final concentration of 0.1 mg/mL. The total flavonoid contents were calculated as quercetin equivalents (mg/g) using the following equation, which was based on the calibration curve: $y = 0.0255 (X)$, $R^2 = 0.99812$, where X is the absorbance in quercetin equivalents (mg/g).

2.9 Antioxidant Activity

2.9.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) Test

For the proper measurement of the antioxidant potentials of the tested extracts, the DPPH free radical scavenging method was utilized¹⁴. The scans were run against pure ethanol or distilled water in a UNICAM spectrophotometer at 517 nm. The measurements in the measuring cuvettes were performed 30 min after the addition of DPPH in darkness to allow sufficient time for the reactions of the antioxidants with DPPH. All measurements were performed in triplicate. The results are presented as trolox equivalent antioxidant capacities (µmol trolox/100 g dry extract).

2.9.2 ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Assay

The method of¹⁵ was adopted for the ABTS radical scavenging assay. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL of the ABTS solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm on the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) were allowed to react with 1 mL of the ABTS solution, and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The results are presented as trolox equivalent antioxidant capacities (µmol trolox/100 g dry extract).

2.10 Oral Toxicity

Albino CF-1 mice were used to evaluate the oral toxicities¹⁶ of the ECEs extracts following the administration of 10 mg to 2 g/kg of body weight of the extracts suspended in gum arabicin 5% saline. For this study, we generated 12 groups of 5 animals per dose plus a control group (1 mL of tap water). Parameters including convulsions, sedation, hyperactivity, grooming and accelerated breathing were observed for ten days following treatment while food and water temperature were controlled. Upon the completion of testing, the animals were sacrificed by cervical dislocation. Necropsies were performed on the

main organs of the animals following the observation period to determine whether changes occurred.

2.11 Acute Dermal Toxicity

Sprague-Dawley (200-250 g) rats were used to evaluate dermal toxicity according to the guidelines of the Environmental Protection Agency¹⁷. Eight groups of five rats of both sexes were assayed. The administered ECE doses were 10mg/kg, 30mg/kg and 50mg/kg. A non-treated group was used as a control. Upon the completion of testing, the animals were sacrificed by cervical dislocation, and necropsies were performed to evaluate potential changes in the animals' internal organs.

2.12 Topical Analgesia

Individual male CF-1 mice were placed in acrylic boxes (30 x 30 x 30 cm) with mirrors on three sides to aid observation for approximately 15 minutes prior to the initiation of the test for habituation. The animals were pre-treated with the ECEs or ibuprofen as a standard analgesic for three minutes via the immersion of the tails of the animals in 5% w/v of the test sample in Dimethyl Sulfoxide (DMSO). Subsequently, 10% formalin (saline solution) was intradermally injected in the first third of the tail using a 29G needle, and the animals were immediately placed in the boxes. Pain-like behaviours (i.e., licking/biting and flinching) were recorded in 5-minute time bins using the Pocket Observer (Noldus) for 45 minutes¹⁸. The animals were euthanized immediately at the end of the study. All experiments were recorded using a video camera to create back-up information. The control mice were submitted to the same procedure, but the tails were immersed in a DMSO solution that lacked ECEs and ibuprofen.

Topical analgesic activity (AA) was calculated according to the following formula:

$$\%AA = 100 - (\text{median licking time for the test} / \text{median licking time for the control}) \times 100.$$

2.13 Oral Analgesia

The peripheral analgesic activities of the ECEs were measured with the acetic acid-induced writhing test as described earlier¹⁹. Briefly, the inhibitions of

writhing produced by ECEs that were administered via gastric feeding tubes (80 mg/kg mouse weight) were determined by comparison with the inhibition exhibited by the control group. Sodium naproxen at an oral dose of 50 mg/kg was used as the standard analgesic agent. Intraperitoneal injections of acetic acid (0.6%) at 1 ml/100 g of body weight were used to create pain sensations (algesic agent). The animals were individually placed in a glass chamber with mirrors on three sides. The numbers of writhings were calculated over 5 min following the application of acetic acid.

Oral analgesic activity (AA) was calculated according to the following formula:

$$\%AA = 100 - (\text{median writhing time for the test} / \text{median writhing time for the control}) \times 100.$$

2.14 Inhibition of the Growth of Tumour Cells in Culture

For the cell proliferation study, we generated five study groups of SaOS-2 cell line cultures according to the following treatments, 1. Control group, 2. 1% A-ECE, 3. Water, 4. 1% E-ECE, and 5. 0.5% ethanol. A protocol for assessing the growth profiles of the individual cell groups under study was applied. This protocol involved the performance of cell counts from three plates for each group at regular intervals of 24 hours over a total of 5 days (i.e., the cell counts occurred at 0, 24, 48, 72, 96 and 120 hours). After every 48 hours elapse of cultivation (i.e., at 48 and 96 hours in the protocol), the culture medium of each cell group was replaced with fresh media.

2.15 In vivo Inhibition of Tumour Growth in the Mice

A trial was conducted on the A/J strain mice according to the recommendations of the National Health Institute²⁰ for assessing the antineoplastic effects and safety and toxicity of chemical substances following acute exposure. This trial was based on the recommendations of the OECD provided in regulation No. 451²¹. Subcutaneous tumours were generated via the injection of TA3 oncogenic cells (10^6 cells/0.1 ml) mixed with 0.9% NaCl solution at a 1:1 ratio into the backs of the mice. The tumours were allowed to grow for seven days. The extracts were orally administered daily for 15 days beginning at day 7 of tumour cell injection. Fourteen

groups of 5 animals of both sexes per dose plus a control group without tumours were created. Tumour growth was monitored over time via daily measurements of the tumour volumes. The potential systemic toxicity was monitored based on assessments of the changes in body weight during the course of the treatment. On day 15, the mice were euthanized, and blood and tumour tissues were collected for *ex vivo* veterinary analyses. The oral doses that were administered daily for 15 days were 4 mg/kg, 8 mg/kg and 16 mg/kg of the aqueous stock solution of the extract and 6.4 mg/mL of the lyophilized *Encelia canescens*, and these groups were compared with the untreated controls with and without tumours. The animals were constantly observed for several minutes at 1, 24, 48, 72 and 96 hours after the application of the treatments to record any symptoms and/or toxic signs. The procedures were registered in record sheets, and alterations were observed and recorded via digital photography. The tumour sizes were measured using a calibrated Vernier callipers and recorded daily for each animal.

2.16 Statistical Analyses

The experimental results are expressed as the means \pm the standard errors (SEs) of three replicates. When applicable, the data were subjected to one-way Analyses of Variance (ANOVA), and the differences between samples were determined with Kruskal-Wallis tests using STATA 10.0. P values < 0.05 were regarded as significant.

3. Results

3.1 Qualitative Screening

As a first approach, the qualitative physicochemical characterization for several extracts from *Encelia canescens* Lam was assessed with Thin Layer Chromatography (TLC). The results revealed the presence of several potentially relevant compounds, specifically, saponins, sterols, terpenes, flavonoids, coumarins and tannins (Table 1.), and some specific compounds representative of these families were detected and quantified with High-Performance Liquid Chromatography (HPLC) and nuclear magnetic resonance spectroscopy (^1H RMN) (Figures 1 and 2).

3.2 Antimicrobial Activity

The antimicrobial activities of the extracts were examined with bioautography assays. The results revealed that the non-polar and methanolic extracts exhibited antimicrobial activities, particularly against *E. coli*, *Klebsiella*, *M. flavus*, *S. aureus*, and *B. subtilis*, and the essential oil also exhibited activity against *E. coli*, *M. flavus* and *B. subtilis*. Therefore, neither the E-ECE nor A-ECE extracts exhibited relevant antimicrobial activity (data not shown).

3.3 Determination of the Total Phenolic Compounds and Total Flavonoids

The total phenolic compounds, expressed as mg (mean \pm SD) per g of tannic acid, were 19 ± 1.14 for the A-ECE and 23 ± 1.60 for the E-ECE. The total flavonoids, expressed

Table 1: Qualitative screening of compounds in several ECEs by Thin Layer Chromatography (TLC)

Secondary Metabolite	Developing System	Hexanol Extract	Dichloromethan Extract	Acetic Extract	Ethanol Extract	Water Extract
Alkaloids	Dragendorff	ND	ND	ND	ND	ND
Esterols and triterpenes	Liebermann Burchard	+	+	+	+	ND
Anthraquinone	Böntrager	ND	ND	ND	ND	ND
Flavonoids	NP/PEG	+	+	+	+	+
Coumarines	NP/PEG	+	+	+	+	+
Tanines	FeCl_3	ND	ND	+	+	+
Saponines	Foam	ND	ND	ND	ND	+

+: Present

ND: Not detectable levels

+: Present in low levels.

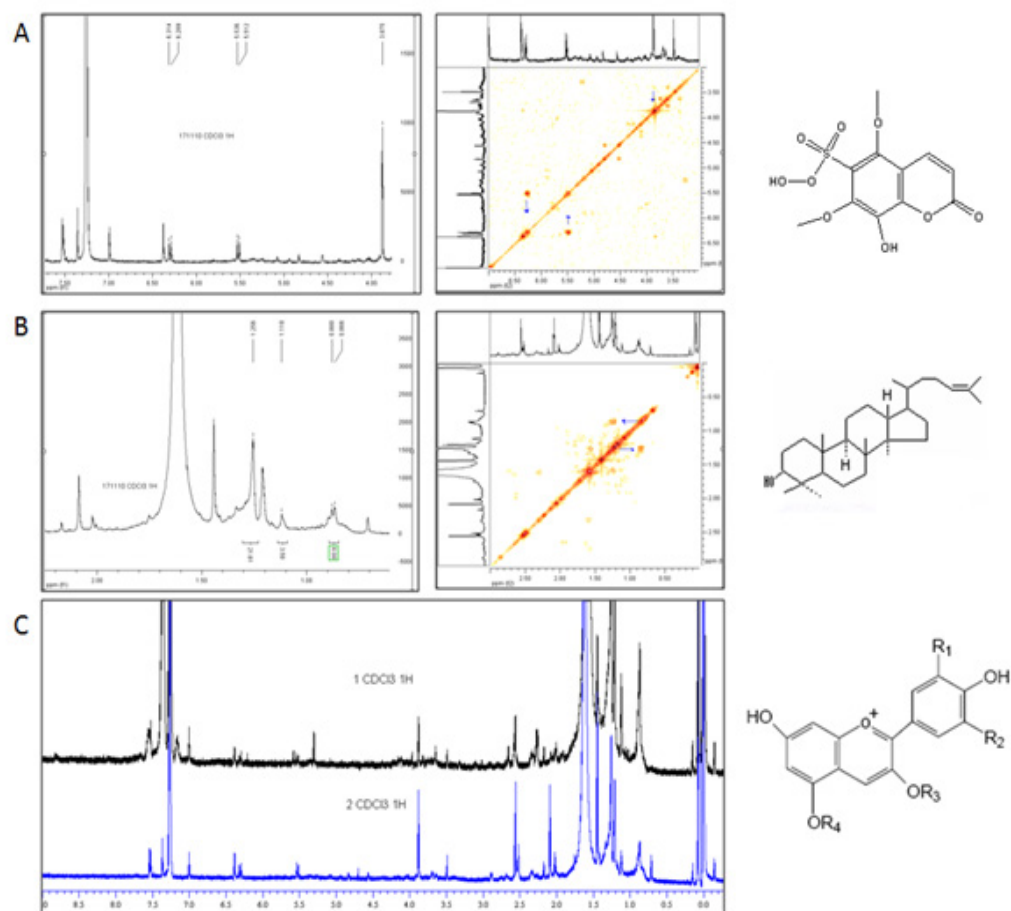


Fig. 1. Some structural families detected in the ECE extract and the ^1H NMR spectra (400 MHz) in deuterated chloroform. (a) Coumarin. (b) Anthocyanin. (c) Terpenoid.

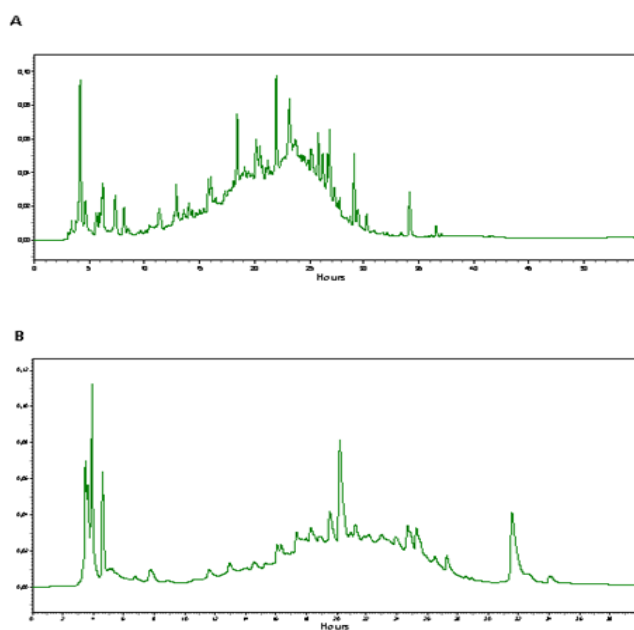


Fig. 2. Representative HPLC chromatograms of the aqueous. (a) And ethanolic. (b) Extracts.

as mg (mean \pm SD) per g of quercetin, were 0.15 ± 0.02 for the A-ECE and 0.13 ± 0.06 for the E-ECE (Table 2).

3.4 Antioxidant Activity

The antioxidant capacities of the extracts were evaluated as Trolox Equivalent Antioxidant Capacities (TEACs) using DPPH and ABTS assays. The results revealed both extracts exhibited good antioxidant activity, and the E-ECE was the slightly more powerful antioxidant extract; i.e., for DPPH and ABTS, the activities of E-ECE were 354 ± 23 μ mol trolox/100 g dry extract and $2,354 \pm 568$ μ mol trolox/100 g dry extract, respectively, compared with the DPPH and ABTS activities of 303 ± 15 μ mol trolox/100 g dry extract and $1,856 \pm 453$ μ mol trolox/100 g dry extract, respectively, for the A-ECE (Table 3).

3.5 Toxicity Evaluation

Assays of the acute dermal and oral toxicities were performed (data not shown). No dermal effects (oedema and/or erythaema) were observed. Furthermore, no changes in weight relative to the control group or alterations in the internal organs were observed at any of the tested doses. Similarly, no evidence of toxicity was observed in the oral toxicity assay; i.e., there were no signs of toxicity during or at the end of the treatments. Thus, the movements of animals were normal, and no hind drop, drowsiness, wheezing, cyanosis, diarrhoea, emesis, salivation or piloerection were observed. The

necropsies revealed no differences in the colours, sizes or textures of the internal organs of the extract-treated animals compared with the animals in the control group.

3.6 Analgesic Activity (%AA)

The results displayed in Table 4(a) revealed topical analgesic activities of 51.9 ± 10.92 for the E-ECE and 56.4 ± 8.73 for the A-ECE, whereas the analgesic activity of the ibuprofen reference was 76.5 ± 6.3 . Similarly, the oral analgesic activities as evaluated with the acetic acid-induced writhing test revealed AA% of 40.5 ± 9.9 for the E-ECE, 45.2 ± 3.5 for the A-ECE (Table 4(b)) and 70.0 ± 4.0 for naproxen sodium.

3.7 Antiproliferative Activity in Cell Culture

The comparative analysis of the cell counts for the groups of cells treated with the two extracts (aqueous and ethanolic) in relation to the control cells and the cells treated with the aqueous or ethanolic phases (vehicles) revealed statistically significant differences in the reductions in the potentials for the proliferative growth of the SaOS-2 cells ($p < 0.001$) (Figure 3). The E-ECE extract was slightly more efficient in terms of this antiproliferative effect.

3.8 In vivo Antitumour Activity

The tumour incidences were 100% in the control and treated groups of mice prior to extract treatment. There

Table 2: Total amount of total phenolic and total flavonoids in *Encelia canescens* Lam Extracts

Extract	Total phenolic (mg/g tannic acid equivalents, mean+ SD)	Total flavonoids (mg/g quercetin equivalents, mean + SD)
A-ECE*	19 + 1.14	0.15 + 0.02
E-ECE**	23 + 1.60	0.13 + 0.06

*aqueous *E. canescens* Lam. extract (A-ECE)

**ethanol *E. canescens* Lam. extract (E-ECE)

Table 3: Antioxidant capacity of ECEs extracts (trolox equivalent antioxidant capacity, TEAC)

Sample	DPPH* μ mol trolox/100 g dry extract	ABTS** μ mol trolox/100 g dry extract
E-ECE	354 + 23	2,354 + 568
A-ECE	303 + 15	1,856 + 453

*DPPH: 2,2-diphenyl-1-picrylhydrazyl

**ABTS: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

were no significant changes in the weights of the mice during the course of treatment, which corroborated the lack of toxicity of the ECEs that was previously observed (data not shown). Analyses of the changes in the tumour sizes of the control and ECE treatment groups revealed a lower tumour growth rate and a significant ($p < 0.05$)

Table 4: Analgesic effect of ECEs (a) Topical: Formalin induced tail licking. Results are reported as tail licking time (seg) (b) Oral: Acetic acid-induced writhing test. Results are reported as number of writhings.

Group	Median + SEM	%AA
Control (n=8)	48.9 + 10.30	Ref
E-ECE (n=16)	23.5 + 5.35*	51.9 + 10.92
A-ECE (n=16)	21.3 + 3.64*	56.4 + 8.73
Ibuprofen 5% w/v (n=8)	11.49 + 4.67**	76.5 + 6.3

(b)

Group	Median + SEM	%AA
Control (n=8)	18.49 + 9.4	Ref
E-ECE (n=16)	11.0 + 1.8*	40.5 + 9.9
A-ECE (n=16)	10.14 + 1.6 *	45.2 + 3.5
Sodium naproxen 20 mg/Kg (n=8)	5.56 + 2.5 **	70.0 + 4.0

*statistically different from control and from ibuprofen group ($p > 0.05$)

** statistically different from control and from E-ECE group ($p > 0.05$)

%AA = Percentage of analgesic activity.

A-ECE = Aqueous extract from *Encelia canescens*

E-ECE = Ethanol extract from *Encelia canescens*

decrease in tumour size in the mice treated with the lyophilized extract at the dose of 8 mg/kg x day (Table 5). In contrast, a between-group evaluation of survival performed with a Kruskal-Wallis analysis of variance test for independent samples revealed significant differences between the control mice and the mice treated with the lyophilized extract at the doses of 4 and 8 mg/kg x day and mice treated with A-ECE at the dose of 16 mg/kg x day (Table 6).

4. Discussion

The search for plants and active compounds with anticancer properties began in the 1950s following the discovery of vinca alkaloids and podophyllotoxins. Subsequently, plants became a prime source of highly effective conventional drugs for many cancer treatments²², and this process has created a list of many anticancer phytochemicals that includes saponins, sterols, terpenes, flavonoids, and alkaloids among others^{8,23}. However, the question presently posed is why some of these compounds are more toxic and occasionally less active than whole-plant extracts. Therefore, the study of extracts seems to be a promising area of research that has recently become a major concern across the world^{5-7,9}.

Considering that there are probably many anticancer plants in nature that have yet to be explored in terms of their antitumoural activities, there is an urgent need to develop physicochemical, preclinical and clinical studies

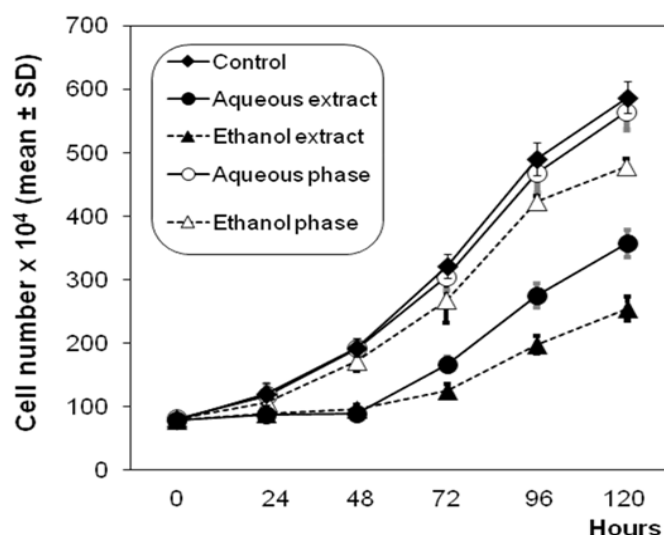


Fig. 3. Antiproliferative effects of the ethanol and aqueous extracts of *Encelia canescens* Lam.

Table 5: Tumor size effect in mice with tumors treated with lyophilized extract or aqueous extract

	n	Lyophilized extract	n	A-ECE
Dose group		Tumor size (cm)		Tumor size (cm)
Control	12	1.34 ± 0.12		
4mg/Kg	23	1.05 ± 0.06	12	1.87 ± 0.33
8 mg/Kg	16	0.57 ± 0.09*	12	1.72 ± 0.08
16 mg/Kg	17	1.58 ± 0.12	26	1.89 ± 0.09

*statistically different from control with tumor (Kruskall Wallis test, $p < 0.05$)

A-ECE= Aqueous *Encelia canescens* extract

Table 6: Comparison of mean survival to 15 days in mice with tumors treated with the aqueous or lyophilized extract

Group	n	Suvival (days)
Control without tumor	12	15.0 ± 0.5
Control whith tumor	12	3.0 ± 0.7
Lyophilized A-ECE 4mg/Kg	6	7.7 ± 1.5*
Lyophilized A-ECE 8 mg/Kg	6	6.0 ± 2.5*
Lyophilized A-ECE 16 mg/Kg	6	5.0 ± 3.6
A-ECE 4mg/Kg	6	4.0 ± 1.0
A-ECE 8mg/Kg	6	4.0 ± 0.6
A-ECE 16mg/Kg	6	6.5 ± 1.9*

*statistically different from control with tumor (Kruskall Wallis test, $p < 0.05$)

A-ECE= Aqueous *Encelia canescens* extract

to explore these activities. Therefore, in the present study, we examined the toxicological, antibacterial, antioxidant, analgesic, antiproliferative and antitumoural properties of extracts of *E. canescens* Lam, all of them useful in cancer patients.

The families of active compounds identified by TLC screening and HPLC and NMR analyses in the present work were saponins, terpenes, flavonoids, coumarins and tannins. Evaluations of the antioxidant capacities of the *E-ECE* and *A-ECE* extracts revealed that both extracts exhibited strong antireactive oxygen species properties. Moreover, neither extract exhibited toxicity in the acute topical or oral toxicity tests.

It is important highlight that at this stage we decided not to investigate antineoplastic effects of the individual components of the extracts to instead evaluate the potential utilities of the whole extracts. In this respect, there is growing evidence that whole extracts can be effective anticancer reagents that are occasionally

superior to the individual components. In²⁴ reported that whole cell extracts from *Urtica membranacea*, *Artemisia monosperma* and *Origanum dayi* post exhibit particularly strong anticancer capabilities. Similar findings have been reported in relation to other pathologies; for example, it is quite clear that mixed herbal components and plant extracts exhibited superior efficacies in the treatment of malaria compared with single compounds²⁵.

In contrast, patients with cancer are at a significant risk for infection due to chemotherapy, radiation or surgery treatments. Additionally, many researchers have proposed that bacterial infections can cause cancer^{26,27}. Therefore, we examined the antibacterial properties of *Encelia canescens* Lam extracts to analyse their usefulness for addressing this collateral factor. Unfortunately, we did not observe any antibacterial activity for the water or ethanol extracts.

The evidence for the anticancer effects reported here is mainly based on results related to the antiproliferative effects in tumour cell cultures and the antitumouractivities in mice. The antiproliferative study clearly demonstrated substantial inhibitory effects of both the *E-ECE* and *A-ECE* extracts (Figure 1). Moreover, the *in vivo* antitumour assays revealed that the *A-ECE* extract at the dose of 16 mg/kg x day and the lyophilized aqueous extract at 4 and 8 mg/kg x day efficiently increased the survival of the mice with tumours and that the lyophilized extract was able to significantly reduce tumour size at the dose of 8 mg/kg x day.

As has previously been reported by several authors, antioxidant activity seems to be directly related to anticancer potential^{23,28}; thus, the potent antioxidant activities observed for both the *E-ECE* and *A-ECE* extracts might reinforce this idea.

Finally, the observed analgesic effects are also interesting because analgesic use is an important issue for cancer patients (NCI, 2015).

5. Conclusion

The results presented here establish that the extracts of *Encelia canescens* Lam have specific antiproliferative and antitumour activities. Additionally, the observed antibacterial and analgesic effects of both extracts are positive findings of the present research that demonstrate additional features of this potentially antineoplastic plant. Moreover, considering the innocuous natures of the extracts and their interesting antioxidant activities, clinical and pharmaceutical studies should be approved by health authorities and performed to support the effectiveness and safety of the use of extracts of this plant in cancer patients.

6. Acknowledgements

This research was conducted with funding provided by Naturaleza y Fe Ltda and Grant CORFO-INNOVA 12IDL2-16208. We thank Drs. Ruben García and Carla Delporte who developed by contract some of the preliminary results for this research. We also thank Mr. Ramiro Perez for his excellent technical support.

7. References

1. Paolini M. Brussels sprouts: An exceptionally rich source of ambiguity for anticancer strategies. *Toxicol Appl Pharmacol.* 1998 Oct; 152(2):293–4.
2. Cragg GM, Nexman DJ. Discovery and development of antineoplastic agents from natural sources. *Cancer Investig.* 1999; 17(2):153–63.
3. Kinghorn. Plant secondary metabolites as potential anticancer agents and cancer chemopreventives. *Molecules.* 2000; 5:288.
4. Nirmala MJ, Samundeeswari A, Sankar PD. Natural plant resources in anti-cancer therapy-A review. *Research in Plant Biology.* 2011; 1(3):01–14.
5. Manju K, Jat R, Gupta A. A review on medicinal plants used as a source of anticancer agents. *Int J Drug Res Tech.* 2012; 2(2):177–83.
6. Kaur R, Singh J, Singh G, Kaur H. Anticancer plants: A review. *J Nat Prod Plant Resour.* 2011; 1 (4):131–6.
7. Takhore P, Mani R, Kavitha, Singh J. A brief review of plants having anticancer property. *IJPRD.* 2012; 3(11):129–36.
8. Fernando W, Rupasinghe HPV. Anticancer properties of phytochemicals present in medicinal plants of North America, using old solutions to new problems - Natural drug discovery in the 21st Century. Kulka M, ed. ISBN: 978-953-51-1158-0, InTech, Doi: 10.5772/55859. Available from: <http://www.intechopen.com/books/using-old-solutions-to-new-problems-natural-drug-discovery-in-the-21st-century/anticancer-properties-of-phytochemicals-present-in-medicinal-plants-of-north-america>
9. Rahman M, Khan A. Anti-cancer potential of south Asian plants. *Nat Prod Bioprospect.* 2013; 3:74–88.
10. Rahalison L, Hamburger M, Hostettmann K, Monod M, Frenk E. A Bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical Analysis.* 1991; 2(5):199–203.
11. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: Current methods and future trends. *African Journal of Biotechnology.* 2008; 7(12):1797.
12. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. *J Agric Food Chem* 2003; 51:609–14.
13. Ordon AA, Gomez JZ, Vattuone MA, Isla MI. Antioxidant activities of Sechiumedule (Jacq.) Swart extracts. *Food Chem.* 2006; 97:452–8.
14. Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature* 1958; 181:1199–200.
15. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio Med.* 1999; 26(9-10):1231–7.
16. OECD. Organization for Economic Cooperation and Development. Guidelines for Testing of Chemicals. Guideline 423: Guideline For Testing of Chemical "Acute Oral Toxicity – Acute Toxic Class Method"; Adopted at 2001 Dec 17.
17. EPA. Health Effects Test Guidelines OPPTS 870.1200. Acute Dermal Toxicity. 712–C–98–192; 1998 Aug.
18. Kolesnikov Y, Cristea M, Oksman G, Torosjan A, Wilson R. Evaluation of the tail formalin test in mice as a new model to assess the local analgesic effects. *Brain Research.* 2004; 1029:217–23
19. Saha A, Masud MA, Bachar SC, Kundu JK, Datta BK, Nahar L, Sarker SD. The analgesic and anti-inflammatory activities of the extracts of *Phyllanthus reticulatus* in mice model. *Pharm Biol.* 2007; 45:355–9.

20. National Institute Health (NIH). Guide for the Care and Use of Laboratory Animals. 8th ed. Washington DC: Animals Institute for Laboratory Animal Research; 2011 Jan.
21. OECD. Guidelines for Testing of Chemicals. Division on Earth and Life Studies Organization for Economic Cooperation and Development. Guideline 451: Guideline For Testing Of Chemical: Carcinogenicity Studies; Adopted on 2009 Dec 7.
22. Cragg G, Newman D. Plants as a source of anticancer agents. J Ethnopharmacol. 2005; 100:72–9.
23. Shah U, Shah R, Acharya S, Acharya N. Novel anticancer agents from plant sources. Chinese J Nat Med. 2013; 11(1):16–23.
24. Solowey E, Lichtenstein M, Sallon S, Paavilainen H, Solowey E, Lorberboum-Galski H. Evaluating medicinal plants for anticancer activity. The Scientific World Journal. 2014.
25. Rasoanaivo P, Wright C, Willcox M, Gilbert B. Whole plant extract versus single compounds for the treatment of malaria: synergy and positive interactions. Malaria Journal. 2011; 10(1):54.
26. Parsonnet J. Bacterial infection as a cause of cancer. Environmental Health Perspectives. 1995; 103(8):263–8.
27. Correa P. Bacterial infection as a cause of cancer. Journal of the National Cancer Institute. 2003; 95(7):E3.
28. Tamokou J, Chouna J, Fischer-Fodor E, Chereches G, Barbos O, Damian G, Benedec D, Duma M, Efouet A, Wabo H, Kuiate J, Mot A, Silaghi-Dumitrescu R. Anticancer and antimicrobial activities of some antioxidant-rich Cameroonian medicinal plants. PlosOne. 2013; 8(2):1–14.
29. National Cancer Institute (NCI-NIH): Pain control: Support for people with cancer. Available from: <http://www.cancer.gov/publications/patient-education/pain-control>