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Antioxidant activity and cytotoxic profile of *Chuquiraga spinosa* Lessing on human tumor cell lines: A promissory plant from Peruvian flora

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ABSTRACT

Objective: To determine the phytochemical content, antioxidant activity *in vitro* and cytotoxicity of crude ethanol extract (CEE), *n*-hexane fraction (NHF), petroleum ether fraction (PEF), chloroform fraction (CLF) and ethyl acetate fraction (EAF) of aerial parts of *Chuquiraga spinosa* (*C. spinosa)* Lessing.

Methods: Phytochemical screening was developed by color and precipitated formation. The evaluation of antioxidant activity was assessed using hydroxyl and nitric oxide radical. Total phenolic content (TPC) and total flavonoids content (TFC) were measured by using standard methods by spectrophotometry. The cytotoxic effect was determined on human tumor cell lines including MCF-7, H-460, HT-29, M-14, HUTU-80, K-562 and DU-145.

Results: Phytochemical analysis confirmed the presence of phenols, flavonoids in crude extract and its all fractions. The CEE showed the highest antioxidant activity, for OH and NO radical scavenging tests (IC_{50} = 15.16 ± 3.45 μg/mL and IC_{50} = 18.91 ± 1.13 μg/mL, respectively). TPC was found to be the highest in the CEE (121.36 mg of gallic acid equivalent/g of dried extract) compared to other fractions. The ranking order of NHF, PEF, CLF, EAF and CEE for TFC was 21.17 < 35.20 < 62.19 < 70.25 < 78.25 mg quercetin equivalent/g of dried extract. The crude ethanolic extract (μg/mL) showed a high cytotoxicity on MCF-7 (IC_{50} = 9.25 ± 0.81), K-562 (IC_{50} = 7.34 ± 1.00), HT-29 (IC_{50} = 8.52 ± 2.69), H-460 (IC_{50} = 5.32 ± 1.05), M-14 (IC_{50} = 8.30 ± 0.60), DU-145 (IC_{50} = 7.09 ± 0.09), HUTU-80 (IC_{50} = 6.20 ± 0.50).

Conclusions: The study showed that CEE of the aerial parts of *C. spinosa* can be measured as a natural source of antioxidant which might be effective towards preventing or slowing oxidative stress related to chronic diseases as well as cytotoxic agent.

1. Introduction

Cancer, is one of the leading causes of death in the world, responsible for 7.6 million deaths in 2008 with approximately 70% occurring in low- and middle-income countries, generating the highest cost for its treatment. Only in the United States, these costs will be $173 billion in 2020[1].

Recently, various biochemical and physiological carcinogenics have been linked to cancer, such as tobacco smoking (lung, pancreas and breast cancer), virus (cervix and liver cancer), bacterial infections (*Helicobacter pylori*-stomach cancer), parasites, mycotoxins (liver cancer) and endogenous imbalance of redox systems that affects biomolecules like proteins, lipids and DNA[2]. In addition, chemotherapy is the main treatment for cancer. However, side effects and non-selectivity to difference malignant cells could be some disadvantages to improve the quality of life and survival rate in those patients, as well as the resistance to anticancer agents[3].

New products of natural sources have focused on anticancer activity[4]. Therefore, alternative treatments for cancers with medicinal plants represent a promising alternative, according to the World Health Organization (WHO), almost 80% of the population of

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developing countries use the traditional medicine based on plants for their primary health care[5].

Antioxidants are chemical substances that inhibit oxidative damage of other molecules in biological entities and exert its action by slowing or preventing the oxidation process that can damage cells[6]. In case of countering free radicals mediated oxidative stress, antioxidants are considered as crucial while the human body has its endogenous antioxidants defenses against oxidative stress[7]. The antioxidant activity of medicinal plants are due to the presence of phytocompounds such as flavonoids and tannins to prevent the oxidative stress caused by reactive species oxygen (ROS) [8]. Complementary and alternative medicine of different parts of the world lead to finding therapeutically effective antioxidant and antitumor compounds from medicinal plants[9].

Chuquiraga spinosa Lessing (Family: Asteraceae) (*C. spinosa*) is called "huamanpinta" that is a species with therapeutic potential from Peruvian flora. The stem and leaves infusion of this plant is used for its anti-inflammatory properties and for the treatment of urinary infections. Previous studies have reported that *C. spinosa* presented anti-inflammatory and antimicrobial effects[10]. Despite their widespread use, cytotoxic effect has not been studied. The main objective in this research was to determine the phytochemical screening, antioxidant activity and cytotoxic effect of crude ethanol extract (CEE), *n*-hexane fraction (NHF), petroleum ether fraction (PEF), chloroform fraction (CLF) and ethyl acetate fraction (EAF) of aerial parts of *C. spinosa*.

2. Materials and methods

2.1. Chemicals

2-Deoxy-2-ribose, EDTA (ethylenediaminetetraacetic acid), thiobarbituric acid (TBA), trichloro aceticacid (TCA), Folin-Ciocalteu (FC) reagent, ascorbic acid (AA), gallic acid (GA) and quercetin (QR) were purchased from Sigma–Aldrich, USA. Unless otherwise specified, remaining chemicals were of analytical grade and obtained from native sources.

2.2. Plant material

C. spinosa was collected, in January 2016 from Tambo, Huancayo, Peru, and identified by Hamilton Beltran. A voucher specimen (152-USM-2016) was deposited at the National Herbarium of National University of San Marcos (UNMSM), Lima, Peru.

2.3. Extraction and fractionation of plant materials

The aerial parts of *C. spinosa* (1 000 g) were dried at room temperature and pulverized at the Chemical Laboratory, Faculty of Pharmacy and Biochemistry, Universidad Nacional San Luis Gonzaga de Ica (UNICA). The powder material was exhaustively soaked with 96% ethanol and intermittent shaking every day for 7 days. The extract was filtered and evaporated by using a rotavap. The crude ethanolic extract (CEE) obtained (20 g) was subjected to fraction, by using *n*-hexane (NHF), petroleum ether (PEF), chloroform (CLF) and ethyl acetate (EAF) respectively. Then fractionated solvents were evaporated to produce 1.20 g, 2.12 g, 2.53 g and 2.15 g fractions, respectively and kept until antioxidant and cytotoxic tests.

2.4. Phytochemical screening

The fractioned extracts obtained were screened to determine the presence of phytochemical constituents, such as alkaloids, terpenoids, quinone, flavonoids, tannins, saponins, steroids and phenolic compounds, with the standard qualitative phytochemical methods described[11].

2.5. Hydroxyl radical scavenging assay

The method of Kunchandy *et al*.[12] was used to determine hydroxyl (OH) radical scavenging activity of *C. spinosa*. In this test, 100 μL of sample (extract/fractions) at various concentrations (10 to 400 μg/mL) was added to 1 000 μL of reaction mixture [500 μL of 2.8 mmol/L 2-deoxyribose in a 50 mmol/L phosphate buffer (pH 7.4), 200 μL of premixed 100 μmol/L ferric chloride and 100 μmol/L EDTA (1:1; v/v), 100 μL of 200 mmol/L hydrogen peroxide and 100 μL 300 μmol/L AA] into the test tubes. After an incubation period of 1 h at 37 ˚C, 500 μL of the reaction mixture was added to 1 000 μL 2.8% TCA followed by addition of 1 000 μL 1% TBA solution and then the reaction mixture was incubated at 90 ˚C for 15 min. Then at 25 ˚C test tubes were cooled and the absorbance was measured at 532 nm. Methanol was used as blank by using UV spectrophotometry. For this test as a standard AA was used. The following equation was used to calculate the percent scavenging of the OH free radical:

OH radical scavenging (%) = [1 – (A/Ao)] × 100

where, A symbolizes the absorbance of the sample/standard solution and Ao symbolizes the absorbance of the control.

2.6. Nitric oxide radical scavenging assay

The method of Rai *et al*.[13] was examined to determine nitric oxide (NO) radical scavenging activity of the *C. spinosa*. In this test, 500 μL of sample (extract/fractions) at various concentrations (10 to 400 μg/mL) was added to 500 μL of 10 mmol/L sodium nitroprusside in phosphate buffered-saline into the test tubes. After an incubation period of 150 min at 25 ˚C in the dark, 1 000 μL of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) was added to 500 μL of the reaction mixture. Then the test tubes were again incubated for 5 min followed by addition of 1 mL of 0.1% naphthyl ethylene diamine dihydrochloride and again incubated for 30 min at 25 ˚C. Then the absorbance was measured at 540 nm, using methanol as blank with an UV spectrophotometer. For this test as a standard AA was used. The following equation was used to calculate the percent scavenging of the NO free radical:

NO radical scavenging (%) = [1 – (A/Ao)] × 100

where, A symbolizes the absorbance of the sample/standard solution and Ao symbolizes the absorbance of the control.

2.7. Total phenolic content (TPC)

In according to Singleton and Rossi[14] with minor modifications, TPC of aerial partsof *C. spinosa* was examined. In this test 100 mL of sample (extract/fractions) with a concentration of 1 000 μg/mL was mixed with 750 μL of FC reagent that was previously diluted 1 000-fold by using distilled water into the test tubes. Then the test tubes were incubated at 22 ˚C for 5 min followed by the addition of 0.06% sodium carbonate solution and again incubated for 90 min at 22 ˚C to complete the reaction. Then the absorbance was measured at 760 nm, using a reagent blank by spectrophotometry. For this test to estimate TPC, gallic acid standard curve was used and results were expressed as mg of gallic acid equivalents (GAE)/g of dried sample.

2.8. Total flavonoid content (TFC)

In line with the method as stated by Chang *et al.*[15], TFC of *C. spinosa* was examined by spectrophotometry. In this test 100 μL of sample (extract/fractions) with a concentration of 1 000 μg/mL was mixed with 3 000 μL of methanol, 200 μL of 10% aluminum chloride, 200 μL of 1 mol/L potassium acetate and 5.6 mL of distilled water into the test tubes. Then the test tubes were incubated at 25 °C for 25 min to complete the reaction and the absorbance was measured at 420 nm by spectrophotometry. For this test to estimate TFC quercetin standard curve was used and results were expressed as mg of quercetin equivalents (QRE)/g of dried sample.

2.9. Cytotoxicity effect

2.9.1. Cell culture

The HUTU-80 (duodenum adenocarcinoma), MCF-7 (human breast adenocarcinoma), M-14 (human amelanotic melanoma), HT-29 (human colon adenocarcinoma), H-460 (human lung large cell carcinoma), DU-145 (human prostate carcinoma) and K562 (human chronic myelogenous leukemia), 3T3 (non-tumorogenic, BALB/c mouse embryo cells) cell lines were obtained from the Laboratory "Abraham Vaisberg Wholach", Universidad Peruana Cayetano Heredia (UPCH). The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/mL gentamycin in humidified 5% CO_2/95% air at 37 °C.

2.9.2. Cytotoxicity assay

In according to Hossain *et al.*[16], 3 000–5 000 cells were inoculated in each well of 96-well tissue culture plates and incubated at 37 °C with their corresponding culture medium during 24 h. The ethanolic extract and fractions (0–250 μg/mL) and 5-FU (0–62.5 μg/mL) were mixed with dimethyl sulfoxide (DMSO) and incubated at 37 °C with 5% CO_2 and 95% air for 48 h. Next, cell monolayers were fixed with 10% trichloroacetic acid (TCA) and stained for 20 min using the sulforhodamine B (SRB) dye. The excess dye was removed by washing with 1% acetic acid, subsequently a solution 10 mmol/L Tris buffer (pH 10.5) was used to solubilize the protein-bound dye in order to read at 510 nm by using a microplate reader. The results were expressed as inhibitory concentration (IC_{50}) which meant the concentration of a test sample resulting in a 50% reduction of absorbance compared with control sample and was determined by linear regression analysis.

2.10. Statistical analysis

The results were expressed as mean ± SD from three observations. For *in vitro* antioxidant tests, student's *t* test was used to find the significance of standard and sample in case of IC_{50}. The statistical and graphical analysis was performed by using SPSS 21.0 (Chicago, IL, USA) and Microsoft Excel 2010 (Roselle, IL, USA). The value of $P < 0.05$ was considered as significant.

3. Results

3.1. Determination of phytochemical constituents

Table 1 shows the phytochemical constituents of the *C. spinosa* extract and fractions based on the intensity of the characteristic color. Preliminary phytochemical screening of *C. spinosa* indicated the presence of various classes of secondary metabolites except quinone, furthermore, alkaloids and saponins were not found in fractions such as NHF, PEF, CLF, and EAF.

Table 1
Phytochemical constituents of ethanolic extract of *C. spinosa* and fractions.

Constituents	Test	NHF	PEF	CLF	EAF	EEC
Alkaloids	Mayer	–	–	–	–	+
	Dragendorff	–	–	–	–	+
	Wagner	–	–	–	–	+
Flavonoid	Shinoda	+	+	+	+	+
Quinone	Bornträger	–	–	–	–	–
Phenols compounds	Ferric chloride	+	+	+	+	+
Saponins	frothing	–	–	–	–	+
Tannins	Gelatin	–	–	–	+	+
Terpenes and steroids	Liebermann–Burchard	+	+	+	+	+

+: Positive; –: Negative; NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract.

3.2. Determination of OH radical scavenging activity

The OH radical scavenging effect of the *C. spinosa* extract and fractions are given in Figure 1. The radical scavenging activity was in the following order: NHF < PEF < CLF < EAF < CEE < AA. The IC_{50} values were shown in Table 2. Compared to AA the IC_{50} value of CEE was statistically significant ($P < 0.01$).

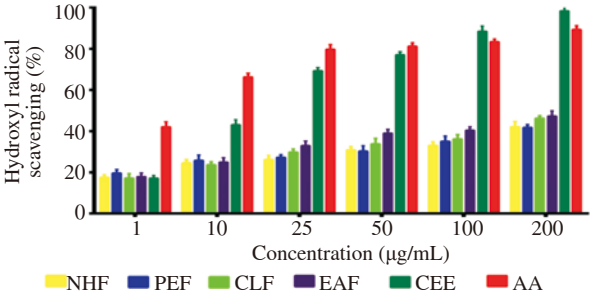

Figure 1. OH radical scavenging activity of the aerial parts of *C. spinosa* extract and fractions at various concentrations.
Values were expressed as mean ± SD ($n = 3$). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract; AA: Ascorbic acid.

Table 2
IC_{50} values of the aerial part of *C. spinosa* extract and fractions for OH and NO radical scavenging activity (μg/mL).

SAMPLES	OH Radical	NO Radical
NHF	112.21 ± 2.14	98.76 ± 2.08
PEF	88.91 ± 2.50	91.11 ± 2.12
CLF	48.91 ± 2.31	52.69 ± 2.25
EAF	31.66 ± 1.76*	38.77 ± 1.43*
CEE	15.16 ± 3.45**	18.91 ± 2.70**
AA	4.91 ± 1.26	6.18 ± 1.13

Values were expressed as mean ± SD ($n = 3$). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract; AA: Ascorbic acid. *: $P < 0.05$, **: $P < 0.01$ significant difference as compared to standard.

3.3. Determination of NO radical scavenging activity

In Figure 2, NO radical scavenging activity of the *C. spinosa* extract and fractions are stated in the following order: NHF < PEF < CLF < EAF < CEE < AA. The CEE showed the highest nitric oxide scavenging activity compared to other fractions and the IC_{50} value (18.91 ± 1.13 μg/mL) of this extract was statistically significant ($P < 0.05$) compared to AA.

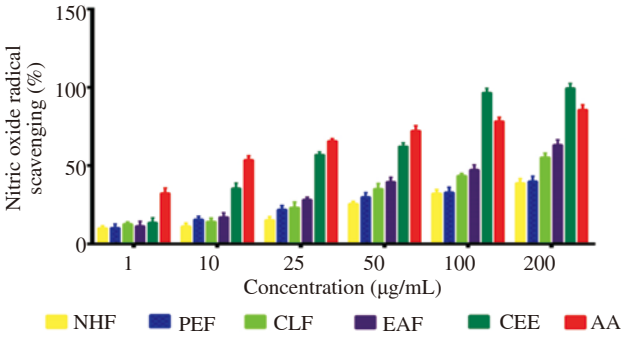

Figure 2. NO radical scavenging activity of the aerial parts of *C. spinosa* extract and fractions at various concentrations.

Values were expressed as mean ± SD (n = 3). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract; AA: Ascorbic acid.

3.4. Determination of TPC

TPC of the *C. spinosa* extract and fractions were calculated from the standard curve of gallic acid ($y = 0.0152x + 0.0445$; $R^2 = 0.992$). Among the extract and fractions, the highest TPC was found in CEE compared to the remaining fractions given in Figure 3. The following order was founded based on the outcomes of the aforementioned figure: NHF < PEF < CLF < EAF < CEE.

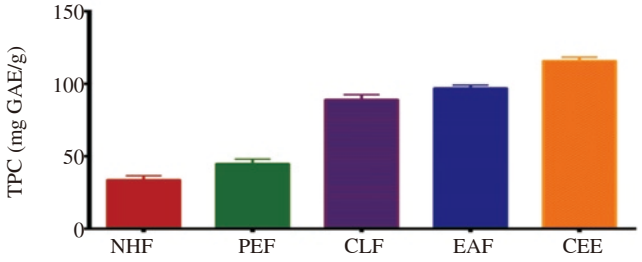

Figure 3. TPC of the aerial parts of *C. spinosa* extract and fractions at various concentrations.

Values were expressed as mean ± SD (n = 3). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract.

3.5. Determination of TFC

TFC of the *C. spinosa* extract and fractions were calculated from the standard curve of quercetin ($y = 0.0098x + 0.1177$; $R^2 = 0.9908$). Figure 4 represents that the TFC was in the following order: NHF < PEF < CLF < EAF < CEE. According to this sequence, CEE exhibited the highest flavonoid contents.

3.6. Cytotoxicity assay

Table 3 shows IC_{50} values of CEE and fractions as well as 5-FU on tumor cell lines. The extract showed IC_{50} values below of 10 μg/mL for all tumor cell lines, and above of 10 μg/mL for 3T3 cells that was used as standard cell line to evidence cytotoxicity. The correlation response-doses was calculated by using Rho Spearman test, and these values were between –0.95 and –0.99 ($P < 0.05$) for CEE and –0.99 and –0.98 ($P < 0.05$) for 5- FU.

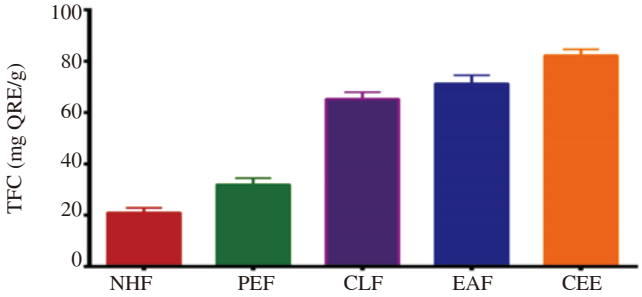

Figure 4. TFC of the aerial parts of *C. spinosa* extract and fractions at various concentrations.

Values were expressed as mean ± SD (n = 3). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract.

4. Discussion

The results confirmed the presence of alkaloids, phenols, flavonoids, saponins, tannins, terpenoids and steroids in CEE. Phenols, flavonoids, terpenoids and steroids were present in NHF, PEF, CLF and EAF. The results found in CEE were similar in according to Landa *et al.*[17].

The OH radical is highly reactive short-lived (approximately 10^{-9} seconds) and shows a significant role in the pathogenesis of biological systems and contributes to neurodegeneration, mutagenesis and carcinogenesis[18]. The source of this radical is mainly the decomposition of hydroperoxides, however byproduct of immune action may also contribute. In this test the formation of low intensity of red color solution indicates OH radical scavenging power connected to antioxidant capacity. CEE showed highest percentage of scavenging activity compared to remaining fractions. The OH radical scavenging activity of plant extract is responsible for reduction of lipid peroxidation which is considered as leading causative factor for numerous diseases[19].

NO is a free radical generated endogenously in several types of cells. It serves as an important biological messenger (cellular signaling molecule) involved in many physiological plus pathological processes. A high concentration of NO displays neurotoxicity and can induce apoptotic cell death in different types of neuronal cells[4]. It has been suggested that NO-facilitated neuronal injury is involved in several neuronal disorders such as Parkinson disease. The results of this study showed the CEE has substantial NO radical scavenging activity, compared to remaining factions in a dose-dependent manner.

Many reports based on polyphenol contents of medicinal plants

Table 3

Cytotoxicity of *C. spinosa* on different human tumor cell lines.

Cytotoxic samples	Tumor cell lines							Mouse embryo
	MCF-7	K-562	HT-29	H-460	M-14	DU-145	HUTU-80	3T3
NHF	59.25 ± 3.50	99.44 ± 2.48	66.54 ± 1.32	65.13 ± 1.25	45.30 ± 1.60	27.03 ± 3.20	119.20 ± 3.50	28.12 ± 3.10
PEF	49.44 ± 2.85	87.30 ± 1.00	65.12 ± 2.60	51.78 ± 2.23	25.43 ± 1.43	33.10 ± 2.50	77.20 ± 3.50	38.21 ± 2.11
CLF	39.25 ± 1.65	67.34 ± 1.88	55.52 ± 2.50	43.12 ± 2.00	53.30 ± 1.45	24.19 ± 1.10	66.22 ± 1.30	23.11 ± 1.14
EAF	44.12 ± 1.89	57.34 ± 1.05	43.12 ± 2.00	23.12 ± 2.15	74.18 ± 1.60	54.12 ± 2.19	82.17 ± 1.80	42.14 ± 2.10
CEE	9.25 ± 0.81*	7.34 ± 1.00**	8.52 ± 2.69*	5.32 ± 1.05*	8.30 ± 0.60*	7.09 ± 0.09*	6.20 ± 0.50*	18.80 ± 2.10*
5-FU	0.645 ± 0.050	4.08 ± 0.54	0.33 ± 0.01	0.35 ± 0.02	1.17 ± 0.09	> 15.63	0.27± 0.01	< 0.24

Values were expressed as mean ± SD (n = 3). NHF: *N*-hexane fraction; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethyl acetate fraction; CEE: Crude ethanol extract; 5-FU: 5-Fluorouracil. *: $P < 0.05$, **: $P < 0.01$ significant difference as compared to standard.

are determined mainly the soluble free phenolics. However, recent researches have evidenced that in addition to the soluble free phenolics, there are bound phenolics, which are mainly in the form of β-glycosides usually release and absorb in the colon. Plant phenolics include phenolics acids, flavonoids and tannins which have been connected with multiple biological roles such as free radical scavenger, anti-inflammatory, anticancer, hypoglycemic, hypocholesterolemia antibacterial, antifungal, antiviral activities[20]. Otherwise, numerous studies related flavonoids with antioxidant, anticancer, anti-inflammatory and cardiovascular effects[21].

According to the U.S. National Cancer Institute (NCI) plant screening program, plant extracts with IC_{50} values $\leqslant$ 20 μg/mL and for isolated compounds $\leqslant$ 4ug/mL following incubation between 48 and 72 h are recognized as potential cytotoxic agents[22]. The cytotoxic effect showed for CEE could be linked to secondary metabolites such as phenolic compounds, flavonoids, tannins, saponins, alkaloids; furthermore, many reports attributed that flavonoids like quercetin, rutin and kaempferol have chemoprotective effect on induced neoplasia in experimental animals. Kaempferol, a flavonoid isolated by Landa *et al*.[17] from *C. spinosa* possesses various typeof this molecule: Kaempferol 3-O-β-D-glucuronide, kaempferol 3–O–rutinoside y el kaempferol 3–O–glucoside, which have many properties to decrease the inflammatory mediators and reduce high probabilities to develop some pathologies related with cancer[23,24]. Tannins are polyphenolics compounds found in many plants, vegetables and beverages (tea, wine, dry fruits, *etc.*) could be avoiding the onset and promotion of tumor cells. Furthermore, it has been demonstrated that gallic acid could cause cancer cell death in various cancer cell lines, including the breast cancer (MCF-7), gastric cancer (MKN-28), cervix cancer (Ca Ski), human esophageal cancer (TE-2), colon cancer (HT-29), and malignant brain tumor (CGNH-89 and CGNH-PM)[25].

This study evaluated that the CEE of the aerial parts of *C. spinosa* exhibited significant antioxidant and cytoxicity activity on human tumor cell lines with a substantial amount of phenols and flavonoids in regard to remaining fractions. Aerial parts of *C. spinosa* can be a good source of natural antioxidant alternatives and anticancer phytomedicine, further studies will be required to isolate more bioactive compound(s).

Conflict of interest statement

We declare that we have no conflict of interest.

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