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ORIGINAL RESEARCH

Phenolic contents and antioxidant activity of ethanolic extract of *Capparis spinosa*

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Abstract Caper plant (*Capparis spinosa*) extracts have been associated with diverse biological activities including anti-oxidant properties. In this work, we characterized the hydro-ethanolic extract obtained from *C. spinosa* leaves [hydroethanolic extract of *C. spinosa* (HECS)] by analyzing the content in antioxidant compounds such as polyphenols, flavonoids and anthocyanins. Further, we evaluated HECS antioxidant activities in vitro using bleaching of 1,1-diphenyl-2-picrylhydrazyl radical and ABTS test as well as by pretreatment of HeLa cells exposed to Fe^{2+} or H_2O_2. Our findings indicate that HECS contains high amount of total phenolic compounds and high levels of flavonoids and anthocyanins. Furthermore, HECS exhibited antioxidant activity in both chemical and biological tests. Specially, pretreatment of HeLa cells with different concentrations of the extract conferred protection against lipid peroxidation and modulated activities of two antioxidant enzymes, SOD and catalase. These results revealed HECS antioxidant effects and suggest that *C. spinosa* leaves are a potential source of natural antioxidant molecules with possible applications in industry and medicine.

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Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DM	Dry matter
DPPH	1,1-Diphenyl-2-picrylhydrazyl
FCS	Foetal calf serum
GAE	Gallic acid equivalent
HECS	Hydroethanolic extract of *Capparis spinosa*
MDA	Malondialdehyde
CS	*Capparis spinosa*
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbuturic acid
TBARs	Thiobarbuturic acid reactive species

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TEAC   Trolox equivalent antioxidant capacity
TEP   1,1,3,3-Tetraethoxypropane

Introduction

Capparis spinosa L. is the most important economical species belonging to the *Capparidaceae* family (Tlili et al. 2010). Cappers, the common name of the genus *Capparis*, are typically not cultivated. The plants grow wildly at various regions of the world, especially in the Mediterranean basin, showing strong resistance to hard environmental conditions (Levizou et al. 2004). Besides its use in the Mediterranean diet, *C. spinosa* has been known since ancient times as a traditional herbal medicine for its diuretic, antihypertensive and tonic properties (Calis et al. 1999). Various extracts of *C. spinosa* have been shown to have many beneficial effects on human diseases with antidiabetic and hypolipidemic effects (Eddouks et al. 2004, 2005). For example, the methanol extract of *C. spinosa* showed a noteworthy antioxidant/free radical scavenging effectiveness in various models (Germanò et al. 2002; Bonina et al. 2002) and has been suggested as treatment to oxidative stress pathological conditions (Cao et al. 2010). Previous studies on the chemical composition of different extracts from *C. spinosa* have reported the presence of numerous beneficial compounds such as polyphenols, flavonoids and vitamins, known for their antioxidant properties (Matsuyama et al. 2009).

The purpose of this study was to characterize the chemical composition of the hydro-ethanolic extract from the plant leaves and to evaluate its antioxidant properties. First, the investigation of the flavonoid, anthocyanin and total polyphenol content of the extract was conducted. The antioxidant activity was then chemically determined by in vitro assays [1,1-diphenyl-2-picrylhydrazyl (DPPH) and ABTS scavenging activity] and biologically using the HeLa cell line. The cytotoxic effect of this extract was preliminary determined by MTT assay ensuring all subsequence concentrations used had no cytotoxic effect on the cell culture.

Materials and methods

Plant materials and extraction procedure

Plant material was kindly provided by Prof. Zeineb Ghrabi-Gammar and Dr. Imtinen Ben Haj Jilani. Plant material was dried at room temperature and 100 g of plant material were treated overnight with water:ethanol 20:80 (v/v) under gentle stirring. The hydroethanolic extract was filtered through a cellulose filter, lyophilized and frozen at -80 °C until use.

Total phenol determination

Total phenols were determined by using the Folin–Ciocalteu reagent (Sigma-Aldrich, France) according to the method of Singleton and Rossi (1965). Briefly, a 50 μL aliquot of the extract was assayed with 250 μL Folin reagent and 500 μL of sodium carbonate (20 %, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation of 30 min at room temperature, the absorbance was read at 765 nm. Total phenols were expressed as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution. For the gallic acid (Sigma-Aldrich), the curve absorbance versus concentration is described by the equation $y = 0.0012x - 0.0345$ ($R^2 = 0.9997$).

Total flavonoid determination

Total flavonoids were measured by a colorimetric assay developed by Zhishen et al. (1999). One millilitre of a diluted sample was added to 4 mL of water and 300 μL of $NaNO_2$ (5 %, v/v) in water was added. After 5 min of incubation, 300 μL of 10 % $AlCl_3$ was added. After 6 min, 2 mL of aqueous NaOH (1 M) was added to the mixture. Immediately, the mixture was diluted with water to 10 mL and the absorbance was read at 510 nm. Total flavonoids were expressed on a dry weight basis as quercetin equivalents, using a calibration curve of a freshly prepared quercetin solution. For quercetin, the curve absorbance versus concentration is described by the equation $y = 0.0049X$ ($R^2 = 0.9984$).

Total anthocyanins measurement using pH differential method

Total anthocyanins were measured according to a modification of the methods described previously (Fuleki and Francis 1968; Lee et al. 2005). Two dilutions of the sample were prepared, one for pH 1.0 using potassium chloride buffer (0.03 M, 1.9 g KCl into 980 mL distilled water) and the other for pH 4.5 using sodium acetate buffer (0.4 M, 54.4 g $CH_3COONa \cdot 3H_2O$ in 960 mL

distilled water). Samples were diluted 10 times to a final volume of 2 mL. The absorbance of each sample was measured at 520 nm against distilled water as blank. The samples had no haze or sediment and thus correction at 700 nm was omitted. The concentration (mg/L) of each anthocyanin was calculated according to the equation:

$$\frac{A \times MW \times DF \times 10^3}{\varepsilon}$$

and expressed as Cy-3-glc equivalents: where A is the absorbance $= (A_{\lambda vis\text{-}max})_{pH\ 1.0} - (A_{\lambda vis\text{-}max})_{pH\ 4.5}$, MW is the molecular weight (g/mol) $= 449.2$ g/mol for Cy-3-glc, DF is the dilution factor (0.2 mL sample is diluted to 2 mL, $DF = 10$), and ε is the extinction coefficient $(L \times cm^{-1} \times mol^{-1}) = 26{,}900$ for Cy-3-glc, where L is the path length $(=1$ cm).

For comparison, the same extinction coefficient was used for other standards to calculate the concentration of each anthocyanin and thus, results reported were expressed as Cy-3-glc equivalents.

Free radical scavenging activity

DPPH radical scavenging assay

The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of DPPH (Sigma-Aldrich, Saint-Quentin Fallavier, France). The DPPH radical-scavenging effect was evaluated following the procedure described in a previous study (Bouaziz et al. 2010). In succinct terms, aliquots (50 μL) of various concentrations of the test compound in methanol were added to 5 mL of a 0.004 % methanol solution of DPPH. After 30 min incubation at room temperature, the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated as following: $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Test compound concentration providing 50 % inhibition (IC_{50}, expressed in μg/mL) was calculated from the graph plotting inhibition percentage against extract concentration. Synthetic antioxidant butylated hyroxytoluene (BHT) (Sigma-Aldrich) was used as positive control and all tests were carried out in triplicate.

Trolox equivalent antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) measures the reduction of the radical cation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich) by antioxidants. This assay was performed as previously described (Re et al. 1999). Briefly, ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and by allowing the mixture to stand in the dark at room temperature for 12–24 h before use. For the study of phenolic compounds, the ABTS$^{\bullet+}$ was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. For the photometric assay 1 mL of the ABTS$^{\bullet+}$ solution and 100 μL antioxidant solution were mixed for 45 s and measured immediately after 5 min at 734 nm (absorbance did not change significantly up to 10 min). Compounds were assayed at five different concentrations determined within the linear range of the dose–response curve. A calibration curve was prepared with different concentrations of Trolox (0–20 μM). Results were expressed in mM of Trolox (Sigma-Aldrich, France).

HeLa cell culture

The continuous human cell lines HeLa (epithelial cervical cancer cell line) (ATCC, Manassas, VA, USA) were investigated for cytotoxicity and antioxidant effect of plant extracts. This adherent cell line was grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10 % (v/v) foetal calf serum (FCS) (Gibco) and 2 mM L-glutamin (Sigma-Aldrich) in tissue culture flasks (Nunc, Roskilde, Denmark). It was sub cultured twice a week and kept at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.

Induction of oxidative stress

Cells were adjusted to 5×10^5 cells/mL in 25 cm^2 flasks, and incubated at 37 °C. Oxidative stress was induced, after 72 h, by addition of Fe^{2+} (as Fe_2SO_4) to the cells at a final concentration of 100 μM, for 1 h. The oxidation was performed in phosphate buffered saline (PBS).

To evaluate superoxide dismutase (SOD) and catalase activities, oxidative stress was induced using

100 mM H_2O_2 during 1 h. The activities of catalase and SOD were assessed in cell lysates.

MTT cell proliferation assay

The MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide] (Sigma-Aldrich) cell proliferation assay measures the cell proliferation rate and conversely, the reduction in cell viability when metabolic events lead to apoptosis or necrosis. The yellow compound MTT (Sigma) is reduced by mitochondrial dehydrogenases to the water insoluble blue formazan compound, depending on the viability of the cells.

Cells (3×10^4 cells/mL) were grown on microtiter plates (200 µL of cell suspension/well) in 96 well microplates with serial dilutions of extract. 72 h later, 20 µL of a MTT solution (5 mg/mL in PBS) were added in each well. The plate was incubated for 4 h at 37 °C in a CO_2 incubator. Then, 180 µL of medium were removed from each well and 180 µL of DMSO/methanol (50:50) were added to each sample. The preparations were mixed vigorously on a plate shaker with the cells containing formazan crystals. When all the crystals were dissolved, absorbance was measured at 570 nm with a microplate reader (Elx 800 microplate reader).

Malondialdehyde (MDA) determination

For evaluation of MDA production rate, thiobarbituric acid-reactive species (TBARs) assay was used. Adherent cells were detached using trypsin/EDTA solution (Sigma-Aldrich) and centrifuged at 3,000 rpm for 10 min. The pellet was resuspended in 500 µL of deionized water and lysed by five cycles of sonication during 20 s at 35 % (Vibracell, Soniscs, Newtown, CT, USA). One millilitre of TBA solution (15 % trichloroacetic acid (Sigma-Aldrich), 0.8 % thiobarbituric acid (Sigma-Aldrich), 0.25 N HCl) was added. The mixture was heated at 95 °C for 15 min to form MDA–TBA adduct and then the optical density (OD) was measured by a spectrophotometer (Libra S32, Biochrom, Cambridge, UK) at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane (1.1.3.3 TEP) (Sigma-Aldrich).

Conjugated dienes (CDs)

After sonication, cell lysates were extracted with 3 mL chloroform: methanol (2:1 v/v). After centrifugation at 3,000 rpm for 15 min, 2 mL of the organic phase was transferred into another tube and dried at 45 °C. The dried lipids were dissolved in 2 mL of methanol and absorbance at 233 nm was determined. This corresponds to the maximum absorbance of the extracted compounds.

Antioxidant effect

To assess the capacity of plant extract to protect HeLa cells from ROS-mediated oxidative injury, cells were preincubated for 72 h in the presence of different concentrations of ethanol extracts. At the end of the preincubation time, the medium was changed before the addition of the oxidative stress-inducing agent (100 µM of Fe_2SO_4 or 100 mM of H_2O_2). Finally, the above mentioned markers were evaluated. Different controls were used: (1) HeLa cells without any treatment; (2) HeLa cells with 100 µM Fe^{2+}; (3) HeLa cells with 100 µM of ascorbic acid.

Determination of catalase activity

Catalase activity was measured as described previously by Aebi (1984). This method is based on the principle that the absorbance at 240 nm decreases because of dismutation of H_2O_2. The amount of H_2O_2 converted into H_2O and O_2 in 1 min under standard conditions is accepted as the enzyme reaction velocity. The number of catalase units was determined as follows: U/mL = [(3.45 × slope)/0.05] × (1,000/50 µL).

Determination of SOD activity

The SOD activity was determined by spectrophotometry (420 nm) using the pyrogallol assay as described previously (Jiang and Chen 1992) and modified as follows: the rate of autoxidation of pyrogallol (Sigma-Aldrich) in Tris–cacodylic acid (Sigma-Aldrich) diethylenetriaminepentaacetic acid (DTPA) (Sigma-Aldrich) buffer (pH 8–8.2) was determined (A1). The autoxidation of pyrogallol was evaluated under the same conditions after addition of 25 µL of extract (A2). The inhibition percentage of pyrogallol oxidation was determined using the formula: % Inhibition = [(A1 − A2)/A1] × 100.

Table 1 Concentration of total phenolics, flavonoids and anthocyane in hydroethanolic extract of *C. spinosa* (HECS)

Compounds	Concentration
Total phenol content (mg GAE/g dry matter)	427.27 ± 3.21
Flavonoids (mg QE/g dry matter)	57.93 ± 2.31
Anthocyanin (mg Cy-3-glu E/g dry matter)	4.81 ± 0.85

Table 2 ABTS and DPPH IC_{50} values for the hydroethanolic extract of *C. spinosa* (HECS and BHT)

Sample	ABTS values (TEAC)	DPPH IC_{50} values (µg/mL)
HECS	3.15 ± 0.18	7.41 ± 0.24
BHT	2.81 ± 0.13	8.31 ± 0.2

Fig. 1 Cytotoxic effect of HECS on HeLa cell line. The inhibitory effect of different doses on cell growth was determined by MTT assay. Results are the means of three repetitions

Statistical analysis

Each value is the mean of three replications. Values of different parameters were expressed as the mean $\pm$ standard deviation (x $\pm$ SD). The one-way analysis of variance (ANOVA) was performed at the level of $p < 0.05$ to evaluate the significance of differences between mean values. Statistical analysis was performed using SPSS (SPSS 13 for Windows) statistical software.
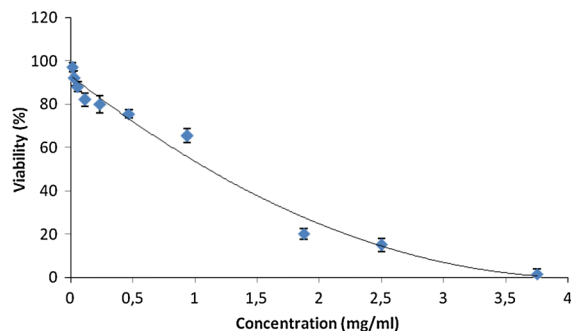
Results

Total phenolic, anthocyanins and flavonoid compounds

First, we evaluated the levels of total phenolic, flavonoid compounds and anthocyanins in the hydroethanolic extract of *C. spinosa* (HECS) using the pH differential method, Follin–Ciocatleu colorimetric and $AlCl_3$ methods, separately. The results show that HECS contains 4.81 ± 0.85 mg Cy-3-glu E/g DM, 427.27 ± 3.21 mg GAE/g DM and 57.93 ± 2.31 mg QE/g DM of the assessed compounds respectively (Table 1).

Antioxidant potential

The HECS was screened for its antioxidant capacity by DPPH and ABTS radicals scavenging assays (Table 2). It exerted an antioxidant activity which was comparable to that of BHT as shown by the ABTS assay (TEAC = 3.15 vs 2.81 mM). However, the DPPH assay showed that the plant extract exhibited higher antioxidant activity than BHT (IC_{50} = 7.41 vs 8.31 µg/mL).

Cytotoxicity effect of *Capparis spinosa* extract

To investigate the cytotoxic effect of HECS on human HeLa cell line, cells were treated with various concentrations of HECS ranging from 0 to 3.75 mg/mL for 72 h, and then submitted to the MTT test (Fig. 1). Data showed that HECS displayed an inhibition effects on human cell growth in a dose dependent manner. The IC_{50} of HECS was evaluated to 1.14 mg/mL. Hence doses under this concentration were used for biological antioxidant activity investigation. Two doses of the extract were chosen: 500 and 750 µg/mL.

Biological antioxidant activity in human cell culture

Lipid peroxidation

The investigation of the biological antioxidant activity of HECS was carried in the human HeLa cell line. Cells were cultured with or without addition of HECS for 72 h. Oxidative stress was induced by incubation with 100 µM Fe^{2+} solution (as Fe_2SO_4) in PBS for 1 h
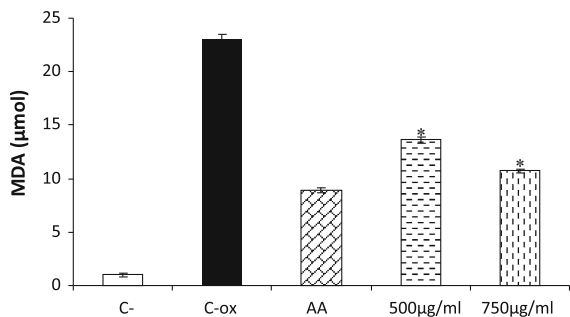
Fig. 2 Malondialdehyde (MDA) levels in HECS supplemented HeLa cell line. Untreated cells (C−), cells treated with Fe^{2+} alone (C-ox), cells treated with 100 μM ascorbic acid (AA) and cells treated with 500μg/ml and 750μg/ml of HECS respectively. Results are the means of three repetitions. ($*p < 0.05$)
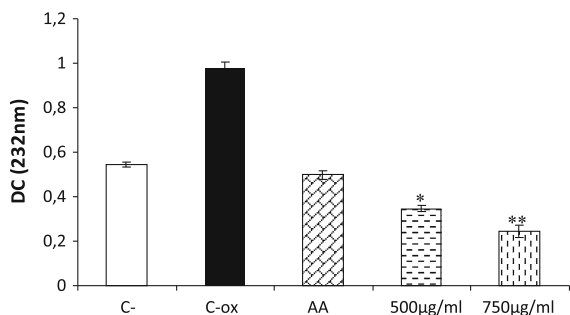

Fig. 3 Conjugated diene levels in HECS supplemented HeLa cell line. Untreated cells (C−), cells treated with Fe^{2+} alone (C-ox), cells treated with 100 μM ascorbic acid (AA) and cells treated with 500μg/ml and 750μg/ml of HECS respectively. Results are the means of three repetitions. ($*p < 0.05$; $**p < 0.01$)

and the production of two lipid peroxydation markers was measured, MDA and conjugated diene. The oxidative treatment resulted in at least 23-fold increases in TBARs concentration as compared to control cells. A significant protection against ROS inducing damage was obtained with HECS in a dose dependent manner (Fig. 2). In fact, a significant decrease in TBARs level was obtained with the two tested concentrations as compared to Fe^{2+} treated cells ($p < 0.05$).

Concomitantly, HeLa cells treatment with HECS exhibited an antioxidant effect evidenced by a decrease in conjugated diene level in HECS treated cells in a dose dependent manner as compared with Fe^{2+} treated cells alone. In contrast to TBARs data, a total re-establishment of conjugate diene level was obtained in HECS treated cells (Fig. 3).

Table 3 Effect of HECS cells pretreatment on catalase and superoxide dismutase activities

	Catalase (U/mL)	SOD (% of inhibition)
Enzymes		
C−	44.5 ± 7.77	26.5 ± 4.94
C-ox	126.5 ± 9.19	90 ± 7.07
HECS (μg/mL)		
500	66.5* ± 2.33	40.1** ± 7.31
750	50* ± 4.1	33.21** ± 5.22

* <0.01; ** <0.001

Antioxidant enzyme activities

Bioactivity of HECS on SOD and catalase antioxidant enzymes was measured in HeLa cells. As shown in Table 3, induction of oxidative stress with H_2O_2 led to an increase in the SOD and catalase activities which can be explained by an adaptation of the enzymatic antioxidant system of the cells to the ROS production/ addition. Interestingly, treatment of cells with given concentrations of HECS induced a significant decrease in the catalase and SOD activities ($p < 0.05$).

Discussion

Plants contain different groups of phenolic compounds, including simple phenols, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids. Several studies have linked plants rich in these compounds with their capacity to reduce the risk of several chronic and degenerative diseases including cancer, cardiovascular disorders as well as their free radical scavenging activity (Makris and Rossiter 2002; Mozaffarieh et al. 2003; Trombetta et al. 2005).

To our knowledge, no previous studies have reported the content on anthocyanins in caper extracts and our results show that the HECS extract was very rich in phenolic compounds, flavonoids and anthocyanins. Our results corroborate those reported by Tlili et al. (2010) who determined the content of phenolic compounds in leaves and buds of *C. spinosa*. Despite the use of the same technique to evaluate the amount of phenolic compounds, a significant variation was seen between the results that may be related to differences in the plants sampled. Indeed, other authors suggested that phenolic compounds are

relatively stable with respect to various modes of processing (Makris and Rossiter 2002; Shahidi and Naczk 2003).

These preliminary results, suggest an anti-radical property of the HECS. The free radical scavenging activity was determined through the DPPH test and the ABTS assay. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50 % (IC_{50}) is a parameter widely used to measure antioxidant activity. As the IC_{50} value of the extract decreases, the free radical scavenging activity increases. The investigated extract showed ability to scavenge the stable DPPH free radical with an IC_{50} value of 7.41 µg/mL. This is comparable to the IC_{50} determined by the BHT as a positive control (8.31 µg/mL). The investigated extract showed comparable antioxidant activity to BHT using the ABTS radical. In fact, the IC_{50} of our extract was evaluated at 3.15 mM TEAC versus 2.81 mM for the BHT. The free radical scavenging activity observed could be explained by the high content on phenolic compounds in this extract. Our results are in agreement with those reported by Germanò et al. (2002). Several studies have shown close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables (Shahidi and Naczk 2003; Bouaziz et al. 2005, 2009; Albayrak et al. 2010).

To investigate the biological antioxidant activity of hydroethanolic extract in HeLa cell cultures, we started by carrying out control experiments to assess cytotoxicity using the MTT assay. The results showed that the extract had a cytotoxic effect in a dose dependent manner with an IC_{50} value evaluated as 1,140 µg/mL. In order to investigate the antioxidant activity of our extract on a cell model, we choose two concentrations that induced <50 % of toxicity according to the results shown in Fig. 1. The concentrations used were 500 and 750 µg/mL in all experiments. HeLa cells were subjected to oxidation by Fe^{2+} solution (Fe_2SO_4) or hydrogen peroxide, to assess lipid peroxidation and antioxidant enzymes activities respectively. The oxidative treatment with 100 µM Fe^{2+} resulted in the increase of MDA and CD levels due to the enhancement of the lipid peroxidation reaction. The pretreatment of cells by the *C. spinosa* leaves extract resulted in the reduction of MDA and CD production rate as shown in Figs. 2 and 3. The addition of H_2O_2 in the culture medium resulted in an increase of the catalase and SOD activities. It is well

known that catalase converts hydrogen peroxide into oxygen and water. The increase of the activity of this enzyme could be considered as an adaptation response to the addition of H_2O_2 in the culture medium. The addition of the ethanolic extract in the culture medium restored the activity of catalase (Table 3). This result could be explained by the re-establishment of the oxidant/antioxidant balance in the cell line and confirmed the antioxidant property of our extract. This antioxidant activity could be explained by our extract's high content of polyphenol, flavonoid and anthocyanins. In the literature, few studies were interested in the antioxidant effect of caper extracts on cell culture. In a recent study, Cao et al. (2010) found that the use of ethanol extract from fruits of *C. spinosa* effectively minimized the loss of cell viability and apoptosis induced by hydrogen peroxide in cultured fibroblast. Moreover, using *C. spinosa* extract significantly reduced the production of reactive oxygen species in fibroblasts in a dose dependent manner.

This study revealed that the hydroethanolic extract of *C. spinosa*'s leaves contain a considerable amount of polyphenol, flavonoid and anthocyanin compounds, and had significant antioxidant activity as determined by chemical and biological assays. Thus, leaves of *C. spinosa* can be used as a potential source of natural antioxidants and bioactive molecules.

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