Journal of Medicinal Plants Research Vol. 6(25), pp.4193-4199, 5 July, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.069
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Investigation of antioxidant activity of alcoholic extract of *Globularia alypum* L.

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Accepted 21 March, 2012

Hydroethanolic extract from leaves of *Globularia alypum* L. (HEGA), Globulariaceae, was screened for its content on total polyphenols, flavonoids and anthocyanins. Its antioxidant activity was also assessed in chemical and biological systems. Analysis of the antioxidant compounds reveals that the HEGA contains high amounts of polyphenols, flavonoids and anthocyanins. Besides, it shows a significant antioxidant activity. When tested in HeLa cells, the extract exerts an antiproliferative effect. The concentration leading to a 50% inhibition was estimated at 1.53 mg/ml. The pretreatment of HeLa cells with non cytotoxic concentration of the leaf extract protects them from Fe^{2+} and hydrogen peroxide induced oxidative stress. A good protection against lipid peroxidation was obtained and evidenced by a decrease in the level of two lipid peroxidation (LPO) products, Malodialdehyde (MDA) and conjugated dienes (CD). Besides, a decrease in catalase and superoxide dismutase (SOD) activities was obtained. This suggests a recovery of the oxidative balance in the treated cells.

Key words: *Globularia alypum*, oxidative stress, cell culture, malondialdehyde, superoxide dismutase, catalase.

INTRODUCTION

The over production of reactive oxygen species (ROS) occurred during internal cell metabolism, as well as in response to environmental factors. These ROS compounds have been implicated in a variety of human disorders, such as cardiac ischemia and neurodegenerative disorders (Sasidharan et al., 2007). This harmful effect is due to their high reactivity with vital biomolecules (DNA, lipids, proteins) in cells and body fluids (Diplock et al., 1998). Recent studies showed that antioxidants could help in prevention against these diseases because they have the capacity to quench free radicals (In-Kyoung et al., 2007). ROS could also affect the quality of foods promoting the development of food rancidity and off-flavours (Ladikos and Lougovois, 1990). To prevent the damages of ROS, some synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertiobutylhydroxytoluene (t-BHT), were used for their potent free radical scavenging effects (Sherwin, 1990; Gülçin et al., 2010). Nevertheless, it was reported that these antioxidant species are suspected of being responsible for vital organ damages and carcinogenesis on laboratory animals (Grice, 1986; Wichi, 1988). For these reasons, the development and utilization of antioxidants from natural origin for food or medical

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Abbreviations: DM, Dry matter; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **FCS,** foetal calf serum; **GAE,** gallic acid equivalent; HEGA, hydroethanolic extract of *Globularia alypum;* MDA, malodialdehyde; **GA,** *Globularia alypum*; **PBS,** phosphate buffer saline; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **TBA,** thiobarbuturic acid; **TBARs**, thiobarbuturic acid reactive species; **TEAC,** trolox equivalent antioxidant capacity; **TEP,** 1,1,3,3-tetraethoxypropane.

applications in replacement of synthetic antioxidants became subject of interest for many research groups (Oktay et al., 2003; Djeridane et al., 2010; Es-Safi et al., 2007; Vagi et al., 2005).

Medicinal plants have received special attention. *Globularia alypum* is a wild plant belonging to the Globulariaceae family. It is a perennial shrub found throughout the Mediterranean area. It is known for its uses in traditional medicine for a variety of purposes (Jouad et al., 2001). It is used as a hypoglycemic agent, laxative, cholagogue, stomachic, purgative, and sudorific, as well as in the treatment of cardiovascular and renal diseases (Jouad et al., 2001; Bellakhdar et al., 1991). In northwest Tunisia, an ethnobotanical inquiry conducted in 2003 with elders practicing traditional medicine has shown the use of 100 plant species against a wide range of ailments through 279 traditional recipes (Ben Haj Jilani et al., 2011). Among these plants, *G. alypum* was known in treating skin diseases notably eczema by applying on the skin dried ground powder of leaves with some “samne”, rancid butter made from cow milk, melted and salted. The decoction prepared from the leaves can also be drank with some honey to treat digestive troubles including stomach and intestinal pains, high blood pressure, heart disorders, renal colic and diabetes (Ben Haj Jilani et al., 2007, 2011). Previous studies have focused on the analysis of antioxidant molecules and the evaluation of the antioxidant activity of *G. alypum* extracts. For example, Djeridane et al (2010, 2006) have evaluated the antioxidant potency of aqueous ethanolic extract of GA using two chemical free radicals: DPPH and ABTS. In the same way, Es-Safi et al. (2007) isolated and assessed the antioxidant potency of isolated compounds from the aqueous methanolic extract from GA leaves and stems.

In this study, the cytotoxic effect of hydroethanolic extract of *G. alypum* (HEGA) was firstly determined by MTT assay on HeLa cell culture. Secondly, the chemical composition of this extract was performed. The antioxidant activity of HEGA was finally determined using chemical (DPPH and ABTS scavenging activity) and biological (HeLa cell culture) tests.

MATERIALS AND METHODS

Plant materials and extraction procedure

The choice of *G. alypum* L. as a plant material in this work has been done following the results of the ethnobotanical survey carried out in the Southwest of Kef (Northwest of the Tunisian territory), which have shown that this plant species is potentially valuable crop. Eleven spontaneous taxa, including *G. alypum* L., which arouse a great interest owing to their polymorphism, as well as their recognized therapeutic indications, were selected (Ben Haj Jilani et al., 2007; Ben Haj Jilani, 2008).

The leaves of *G. alypum,* used as plant materials in this work, were harvested in 2004. A voucher specimen of the taxon has been collected in the field, given a reference number (1288) and kept in the Herbarium of National Agronomic Institute of Tunisia. Leaves were dried at room temperature and stored at 4°C until extraction. When required, 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 as reported by Singleton and Rossi (1965).

Total flavonoid determination

Total flavonoids were measured by a colorimetric assay as described by Zhishen et al (1999).

Total anthocyanins measurement using pH differential method

Total anthocyanins were measured according to a modification of the methods described earlier (Fuleki and Francis, 1968; Lee et al., 2005).

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. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging effect was evaluated following the procedure described in a previous study (Bouaziz et al., 2010).

Trolox equivalent antioxidant capacity (TEAC*)*

The Trolox equivalent antioxidant capacity (TEAC) measures the reduction of the radical cation of ABTS by antioxidants. This assay was performed as previously described (*Re* et al., 1999).

HeLa cell culture

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

MTT cell proliferation assay

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) 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 MTT compound (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 for 72 h before addition of 20 µl of a MTT solution (5 mg/ ml in PBS). The plate was incubated for 4 h at 37°C in a CO_2 incubator

Table 1. Concentration of total phenolics, flavonoïds and anthocyane in hydroethanolic extract of *G. alypum* (HEGA) leaves.

Extract of *G. alypum* HEGA) leaves	Concentration
Total phenol content (mg GAE/g dray matter)	247.24±5.09
Flavonoides (mg QE/ g dray matter)	78.82 ± 2.09
Anthocyane (mg Cy-3-glu E/ g dray matter)	0.53 ± 0.12

and 180 µl of medium were then removed from each well and 180 µl of DMSO/methanol (50:50) were added to each sample. The solutions were mixed thoroughly 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).

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 to the cells of Fe^{2+} (as Fe_2SO_4 100 µM). The oxidation was performed for 1 h 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 enzymes activities were assessed in cell lysates.

Malondialdehyde (MDA) determination

The evaluation of MDA production rate was performed using thiobarbituric acid-reactive species (TBARs) assay. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 500 µL of deionized water and lysed by five cycles of sonication for 20 s at 35% (Sonisc, vibracell). One millilitre of TBA solution (15% trichloroacetic acid, 0.8% thiobarbituric acid, 0.25 N HCl) was added. The mixture was heated at 95°C for 15 min to form MDA–TBA adduct and the absorbance was measured at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane (1.1.3.3 TEP).

Conjugated dienes (CDs)

After sonication, cell lysates were extracted with 3 mL chloroform: methanol (2:1 v/v). After centrifugation at 3000 revolutions per min (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 aforementioned markers were evaluated. Different controls were used: (i) HeLa cells without any treatment; (ii) HeLa cells with 100 µM Fe^{2+}; (iii) 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]*(1000/50 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 in Tris–cacodylic acid diethylenetriaminepentaacetic acid (DTPA) 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.

Statistical analysis

Values of different parameters were expressed as the mean of three replications ± standard deviation (x±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

The levels of total phenolic, flavonoïd compounds and anthocyanins in the hydroethanolic extract of *G. alypum* (HEGA) were determined using pH differential method, Follin-Ciocatleau colorimetric and $AlCl_3$ methods, separately. The obtained results are given in Table 1.

Antioxidant potential

The HEGA was screened for its antioxidant capacity by DPPH and ABTS radical scavenging assays (Table 2). It exerted an antioxidant activity, which was comparable to that of BHT as shown by ABTS assay. However, the

Table 2. ABTS and DPPH IC_{50} values for the hydroethanolic extract of *Globularia alypum* (HEGA) leaves and BHT.

Sample	ABTS values (TEAC)	DPPH IC_{50} values (µg/ml)
HEGA	3.02±0.09	6.45±0.19
BHT	2.81±0.13	8.31±0.2

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

Enzymes	Catalase (U/ml)	SOD (% of inhibition)
C-	44.5±7.77	26.5±4.94
C-ox	126.5±9.19	90±7.07
HEOA (µg/ml)		
500	70*±3.8	42.5**±9.35
750	42.5*±6.3	32.5**±2.47

*:<0.01; **: <0.001.

DPPH assays showed that the plant extract exhibited higher antioxidant activity than BHT.

Cytotoxicity effect of *G. alypum* extract

To investigate the cytotoxic effect of HEGA on HeLa human cell line, cells were treated with various concentrations of HEGA ranging from 0 to 3.5 mg/ml for 72 h, and then submitted to the MTT test (Figure 1). Data showed that HEGA displayed inhibition effects on human cell growth at a dose dependent manner. The IC_{50} of HEGA was evaluated to 1.53 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, which induce less than 20% cytotoxicity.

Biological antioxidant activity in human cell culture

Lipid peroxidation

The biological antioxidant activity of HEGA was investigated in the HeLa human cell line. Cells were cultured with or without the addition of HEGA for 72 h. Oxidative stress was induced by adding 100n µM Fe^{2+} solution (as Fe_2SO_4) in PBS for one hour. In Malondialdehyde and conjugated diene production, two lipid peroxydation markers were evaluated.

The oxidative treatment induced in at least 23 fold increases in TBARs concentration compared with control cells. As shown in Figure 2A, a significant protection against ROS inducing damage was obtained with both used concentrations in a dose dependent manner. Indeed, a significant decrease in TBARs level was obtained with both concentrations as compared to Fe^{2+} treated cells ($p<0.05$).

Concomitantly, HeLa cells treatment with HEGA exhibited an antioxidant effect evidenced by a decrease in conjugated diene level with both used concentrations in a dose dependent manner as compared with Fe^{2+} treated cells control. In contrast to TBARs data, a reestablishment of conjugate diene level was obtained with the concentration of 750 µg/ml of HEGA (Figure 2B).

Antioxidant enzyme activities

The effect of HEGA 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 SOD and catalase activities that can be explained by an adaptation of the cell enzymatic antioxidant system to the ROS production. Interestingly, cells treatment with both concentrations of HEGA induced a significant decrease in the catalase and SOD activities ($p<0.05$).

DISCUSSION

In this study, we assessed the total polyphenols, anthocyanins and flavonoïds composition of aqueous ethanolic extract from *G. alypum* (HEGA) species. As compared to other studies (Djeridane et al., 2010; Djeridane et al., 2006), the HEGA extract produced here showed higher levels of flavonoids and total phenols contents. This difference may be due to the difference of plant tissue used. Indeed, Djeridane et al. (2006)

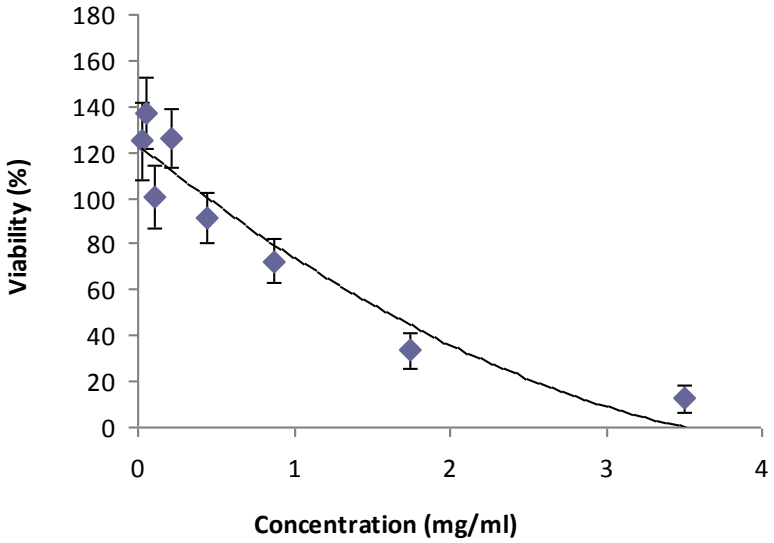

Figure 1. Cytotoxic effect of HEGA on HeLa cell line. The inhibitory effect of different doses on cell growth was determined by MTT assay. Cells were treated with HEGA at concentration ranging from 0 to 4 mg/ml. the percent growth reduction was calculated from the extinction difference between treated cell culture and the control. Results are the means of three repetitions.

described the entire plant content, while in this study, the leaf extract was used. Leaves may contain more antioxidant molecules than other parts of the plant. Moreover, extraction from leaves is generally easier than from stems and therefore yields may be better. These results also suggest an antioxidant effectiveness of the HEGA evaluated in a dynamic way using a biological test (HeLa cell culture), and through a chemical method (DPPH and ABTS assays). 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 display an ability to scavenge the stable DPPH free radical reaching 50% of reduction with an IC_{50} values of 6.45 ± 0.19 µg/ml, which is comparable to the IC_{50} determined by the BHT as a positive control (8.31 µg/ml). The same result was obtained using the ABTS radical. Indeed, the IC_{50} of this study's extract was evaluated at 3.02 mM TEAC versus 2.81 for BHT. This antioxidant activity could be explained by the high content of polyphenolic, flavonoid and anthocyanins in HEGA. Moreover, new active molecules were isolated from the hydromethanolic extract of stem and leaves of GA identified as new phenolic compounds named as 6-hydroxyluteolin 7-O-laminaribioside, eriodictyol 7-O-sophoroside and 6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl)ethyl]-β-D-glucopyranoside as well as two flavonoid glycosides identified as 6-hydroxyluteolin 7-O-β-D-glucopyranoside and luteolin 7-O-sophoroside. These molecules exhibit an antioxidant activity using the DPPH assay as described by Es-Safi et al. (2007, 2006, 2005) and Khlifi et al. (2005). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metallic chelating potential (Rice Evans et al., 1995).

Our results are in agreement with those of Djeridane et al. (2010) who reported that the hydromethanolic extract of *G. alypum* has an antioxidant activity evaluated by the DPPH assay. However, using the ABTS assay, our crude extract showed a higher antioxidant activity compared to the aqueous ethanol extract of *G. alypum* (70:30; v/v) (Djeridane et al., 2006).

The biological antioxidant activity of HEGA was also investigated on the HeLa cells. Considering that the chemical composition of the HEGA is not well determined previously, we initially carried out control experiments to assess the cytotoxicity of our extract on HeLa cell line using MTT assay. The results showed that the extract had a cytotoxic effect at a dose dependent manner with an IC_{50} value evaluated as 1.53 mg/ml. In order to investigate the antioxidant activity of HEGA on model cells, the two concentrations chosen induced less than 20% of toxicity (Figure 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) to assess lipid peroxidation. They were also treated by hydrogen peroxide to assess antioxidant enzymes activities. 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 HEGA led to the reduction of

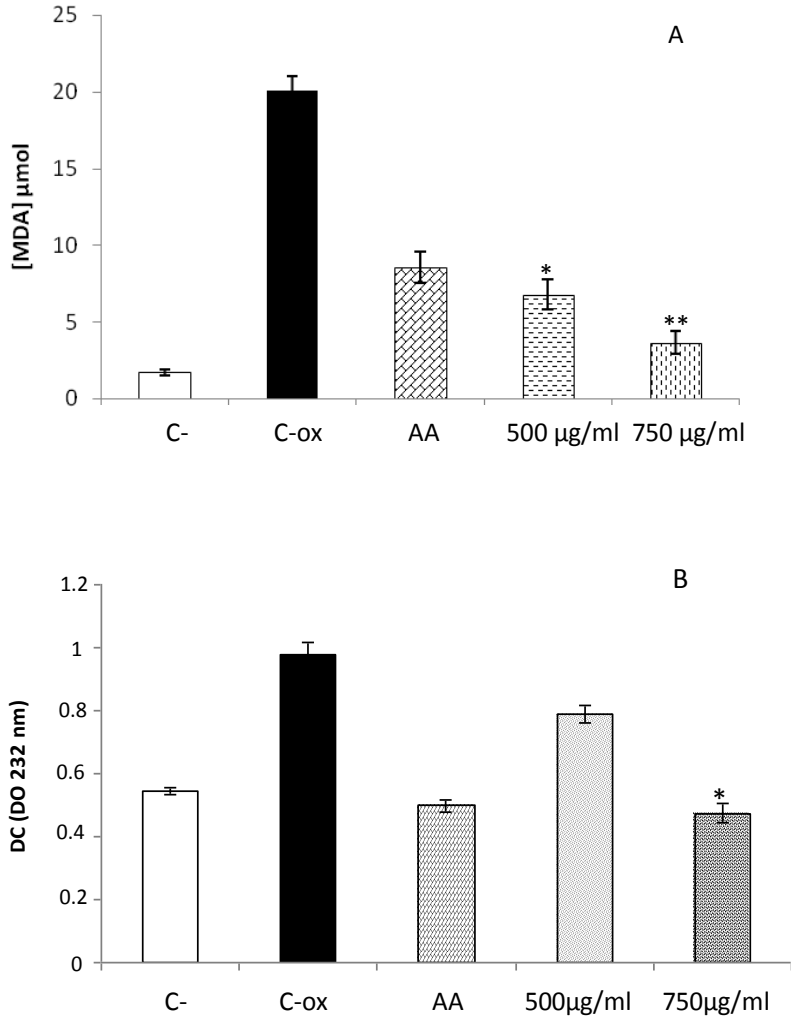

Figure 2. MDA (A) and conjugated diene (B) levels in HEGA supplemented HeLa cell line. Cells were cultured in 25 cm² flasks with 500 and 750 µg/ml of HEOA for 72 hours. Oxidative stress was induced by addition of Fe^{2+} to the cells for 1 hour at a final concentration of 100µM. TBARs and conjugated diene (CD) were compared to untreated cells (C-), cells treated with Fe^{2+} alone (C-ox) and cells treated with 100µM ascorbic acid (AA). (*:<0.05; **:<0.01).

the production rate of both considered markers (Figure 2). Moreover, the addition of H_2O_2 in the culture medium resulted in an increase of catalase and superoxide dismutase activities. The increase of catalase activity, that converts hydrogen peroxide into oxygen and water, can be considered as an adaptation response of cells to H_2O_2 treatment. Although the standard substrate of SOD is the superoxide anion, it was recently reported that at high level of H_2O_2, the MnSOD can show a reverse reaction yielding to superoxide anion (MacMillan and Crow, 2011). This can explain the increased SOD activity after H_2O_2 treatment. The addition of the HEGA in the culture medium significantly decreased the catalase and SOD activities (Table 3). This result could be explained by the reestablishment of the oxidant/antioxidant balance in the cell line and confirms the antioxidant property of HEGA extract. These data are in agreement with those of Djeridane et al. (2010) who reported the ability of *G. alypum* extract in protecting the whole blood against free radical aggression and cell lysis.

These results confirmed that the hydroethanolic extract of *G. alypum* contains important amounts of polyphenol, flavonoid and anthocyanin compounds that explain the significant antioxidant activity as determined by chemical and biological assays. The leaves of *G. alypum* could be used as a potential source of natural antioxidants and bioactive molecules in pharmaceutical, as well as food industries due to the low cytotoxic behavior of this ex-

tract.

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