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Phenolic Content, Anti-Proliferative and Antioxidant Activities of Butanolic Extract of *Euphorbia Helioscopia* L. Growing in Humid Area in Algeria

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ABSTRACT

The objective of the work in hand was appraising the phenolic composition and the in vitro antioxidant activity by reducing power and lipid peroxidation inhibition essays as well as the anti-proliferative activity on C6 and Hela cancer cell lines of butanol extract of the aerial parts of Euphorbia helioscopia collected in humid areas in Algeria. The results revealed that butanol extract showed a good antioxidant activity in all the tested methods. The total phenolics and flavonoids were relatively high including 156.21±4.55 mg GAE (gallic acid equivalent)/g and 22.08±0.81 mg QE (quercetin equivalent)/g, respectively. Phenolics were analyzed by HPLC-TOF-MS, and identified by comparing their retention times, UV-Vis absorption spectra and mass spectra with authentic standards. It was shown that gentisic acid was the major component of the extract. The extract seemed to possess important antioxidant potencies and phenol contents. The ferric reducing activity was 1.396 at 1000 µg/mL, and the lipid peroxidation inhibition was 44.38 % at 500 µg/mL. The extract exerted a moderate inhibition of proliferation against Hela cell, and no inhibitory effect against C6.

Keywords: *Euphorbia Helioscopia, Phenolics, Reducing Capacity, Lipid Peroxidation, Anti-Proliferative Activity.*

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1. INTRODUCTION

Oxidative stress or excessive production of reactive oxygen species (ROS) has been implicated in many diseases such as cancer, atherosclerosis, aging, diabetes, etc (Finkel and Holbrook, 2000). Many studies stated that more than 20 % of malignancy diseases might be avoided by increased ingestion of fruits and vegetables in every day nutrition (Ruhul Amin et al, 2009). In the previous decades, there has been a considerable development in the folk medicine which has been getting more and more well-liked in both emergent and industrialized countries due to its natural origin and less significant secondary effects (Scartezzini and Speroni, 2000). Medicinal plants enclose bioactive substances that throughout the history have been employed in traditional remediation to cure a range of ailments. Many investigations have established the potential herbal components to protect human body from free radical stimulated ailments (Ziech et al, 2012). The major kinds of natural antioxidant substances in nature are flavonoids and phenolic acids in free or complex structures. These compounds have been recognized and measured in quite a lot of fruits and vegetables, and exhibited an arrogant relationship with antioxidant activity (Luo et al, 2002). The plants in the family of Euphorbiaceae hold the famous skin exasperating and tumor-promoting diterpenoids, tigliane, ingenane, and daphnane skeletons (Evans and Taylor, 1983). Euphorbia, the biggest genus of Euphorbiaceae, involving more than 2000 species is characterized by the presence of milky latex. Some varieties of Euphorbia have been employed as therapeutic plants for the management of skin infections, gonorrhea, migraine and intestinal vermin, and as lump therapy. As a remedial plant, *Euphorbia helioscopia* has been extensively used with a long history to treat diverse health disorders, such as ascites, edema, tuberculosis, dysentery, scabies, lung cancer, cervical carcinoma, and esophageal cancer. It is also believed to have antifungal and antibacterial properties (Uzair et al, 2009).

In this paper, different *in vitro* experiments have been presented, through which the antioxidant and antiproliferative activity of n-butanolic *E. helioscopia* extract was investigated. The HPLC-TOF-MS technique was used to elucidate the phenolic profile of the extract.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial part of E. helioscopia Linn was collected from Bejaia (Cap-Carbon) during May 2013 (flowering stage). The plant was recognized by Pr. Zellagui Amar, and a voucher specimen was placed in the Laboratory of Biomolecules and Plant Breeding, University of Larbi Ben Mhidi Oum El Bouaghi, Algeria (E. helioscopia voucher number ZA 141).

2.2. Extraction

The *Euphorbia* plant was air-dried and preserved at room temperature and then crushed into powder. 200 g of plant was weighed and soaked in 500 ml of 70% (methanol, water) for 1 week at room temperature, the extracts were filtered after one week by using a Whatman filter paper N°1, the solvent of the extraction was evaporated to dryness by rotary vapor, the extraction process was repeated three times in the same condition. The methanol extracts obtained were collected, filtered, and dried under vacuum. The deposit was dissolved in 200 mL of distilled water, and partitioned with *n*-butanol (3 X 150ml). The resulting solutions were concentrated in vacuum to dryness, to give fraction of 0.33 g. This fraction was kept at 4°C, in shadow for further analysis.

2.3. HPLC-TOF/MS analysis

Phenolic quantification of the extract was performed using Agilent Technology of 1260 Infinity HPLC System coupled with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x100mm, 3.5µm) column. Vector phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile. The flow rate was 0.6 mL min-1, and the column temperature was 35°C. The injection volume was 10 µL. The solvent program was as follow: 0. min 10 % B; 0-1.min 10% B; 1-20.min 50% B; 20-23.min 80% B; 23-25.min 10% B; 25-30. min 10 % B. The ionization mode was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min-1, nebulizer of 40 psi, capillary potential difference of 4000 V and finally, fragmentor potential difference of 175 V. For sample investigation, the desiccated crude extracts (200 ppm) were dissolved in methanol at room temperature. Samples were filtered passing through a PTFE (0.45 µm) filter by an injector to remove particulates.

2.4. Total phenolic content (TPC)

The TPC of n-butanolic extract was realized using the folin-ciocalteau according to the method of (Singleton and Rossi, 1965). 0.5 ml of the diluted solution of each extract in methanol was added to 2.5 ml of FCR (diluted 1/10 with distilled water) and mixed, after 05 min, 02 ml of sodium carbonate aqueous solution Na_2CO_3 (75g/L) was added to the mixture and incubated at 40 °C for 30 min. Results were conveyed as mg of acid gallic equivalent (GAE)/g of dry extracts. Acid gallic was used as a standard. All the samples were analyzed three times.

2.5. Total flavonoids content (TPC)

Total flavonoids were estimated according to the aluminum chloride colorimetric process (Djeridane et al, 2006), based on the formation of a complex flavonoid-aluminum having the maximum absorbance at 430 nm. 01 ml of methanol extract was assorted with 01 ml of 2% $AlCl_3$ methanol solution, and then the absorbance was determined at 430 nm using UV-VIS spectrophotometer. Total flavonoid content was expressed as mg quercetin equivalent/g of dry extracts. Quercetin was used as a standard. All the samples were analyzed three times

2.6. Reducing power assay

In this assay, the golden color of the analysis mixture shifted to green depending on the reducing power of the sample. The presence of reducing compounds in the solution causes the change of the Fe^{3+}/ ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be screened by the measurement of the absorbance at 700 nm. Briefly, 01ml of serial concentrations of the extracts (20-100 µg/mL) was assorted with 1 ml of sodium phosphate buffer (0.2M, pH= 6.6) and 1 ml of potassium ferricyanide (1%). Reaction mixture was protected at 50 °C for 20 min, and then 1 ml of trichloroacetic acid (10%) was supplemented and centrifuged for 10 min. From the upper layer, 1 ml was mixed with 1 ml of distilled water, and 0.3 ml of $Fecl_3$ (0.1%). Absorbance of resulting solution was measured at 700 nm. Quercetin, gallic acid and ascorbic acid were used as the standards. The values were presented as mg quercetin equivalent per g of extract.

2.7. The Inhibition of Linoleic Acid peroxidation

The antiperoxidation assays of the n-butanolic E. helioscopia extract were carried out using thiobarbituric acid (TBA) method based on the inhibition of linoleic acid peroxidation according to the slightly modified method of (Choi et al, 2002). This technique was selected for the assessment of lipid peroxidation test, with linoleic acid as the source of linoleic acid in an oxidation reaction catalyzed by Fe-ascorbate. The samples (50-500 µg/ml) were mixed with linoleic acid solution (0.28 mg linoleic acid and 0.28 mg tween-20) in 100 µM phosphate buffer (pH =7.4), 500 ml of phosphate buffer (100 µM, pH 7.4) and 150 µl of ascorbic acid (10 µM). The mixture was vortexed and sonicated to get a homogeneous emulsion solution. The linoleic acid peroxidation was set off by adding 0.1 mL $FeSO_4$ (10 µM) and incubated at 37 °C for 60 min. The mixture was refrigerated, and 1.5 ml trichloroacetic acid (10% in 0.5% HCl) was added. Then, 3 mL of TBA (1%, in 50 mM NaOH) was added. The reaction blend and TBA solution were heated in a water bath at 90 °C for 60 min. After cooling down, 2 ml aliquots were taken from each sample, and stirred with 2 ml butanol and centrifuged at 1000 x g for 30 min. The upper layer solution was separated for the spectrophotometrically measurement and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was read at 532 nm, and transformed into the percentage of the antioxidant potency. The percentage of linoleic acid peroxidation inhibition was defined as in the following equation: Linoleic acid peroxidation inhibition activity (%) = $((A_o-A_1)/A_o)$ x 100 where A_o is the absorbance of control reaction (containing all reagents except the sample) and A_1 is the absorbance of the sample extract or standard.

2.8. In vitro anti-proliferative activity

Anti-proliferative effect of the extract was evaluated in vitro by the estimation of the inhibitory effect of phenolic on the growth of cells on C6 (rat brain tumor) and Hela cell lines using proliferation BrdU ELISA assay (Demirtas and Yaglioglu, 2012).

2.9. Cell culture

The cells were grown in Dulbecco's adapted eagle's medium (DMEM, Sigma), supplemented with 10% (v/v) fetal bovine serum (Sigma, Germany) and PenStrep solution (Sigma, Germany) at 37°C in a 5% CO_2 humidified atmosphere.

2.10. ELISA assay

The cells were plated in 96-well culture plates (COSTAR, Corning, USA) at a density of 30000 cells per well. The activities of sample were investigated on 250, 100 and 50 µg/mL. The cells were then kept warm overnight prior to applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer's recommended procedure. The quantity of cell propagation was measured at 450 nm using a microplate reader (Awareness Chromate, USA). The results were given as the percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was set to be 100% of proliferation. The supply solution of the extracts was arranged in dimethyl sulfoxide (DMSO) and diluted with DMEM. DMSO's final concentration was below 0.1% in all the tests. 5-FU was used as the standard compound. The percentage of inhibition of cell proliferation was considered as follows: $(1 - (A_{treatments} / A_{vehicle\ control})) \times 100$. The half maximal inhibitory concentration (IC_{50}) was an assessment of the efficacy of a compound to restrain a biological function. In this paper, IC_{50} and IC_{75} values were determined.

2.11. xCELLigence assay

The xCELLigence technique was employed with the throwaway E-plate 96 for the calculations of the solvent extracts, controls and medium. The measurements based on the impedance difference were caused by the cells attached to the E-Plate 96. The figure 10 displayed the cell index (CI) according to the cell numbers against time. 100 µL of the medium was added to each well of E-Plate 96 and placed into the incubator. E-plate 96 was placed in the xCELLigence position, and the backdrop impedance was calculated during 1 min. Next, 50 µL of each cell suspension was put in medium holding wells, and accustomed to 20 000 HeLa cells mL-1. The plate was held in a sterile cabinet at room temperature for 30 min for affixing the cells to the E-Plate 96 wells. Finally, HeLa cells were checked every 10 min for attachment, growth and proliferation in a period of up to 3 h via the integrated sensor electrode arrays of the E-Plate 96. The extract was added to wells of the plate and attuned to the n-butanol extract concentrations of 250, 100, 50 and 10 µgmL-1. The plates were then immediately placed in the incubator, and observed every 10 min during 48 h. The tests were replicated and repeated three times given with the standard deviation bars (Koldaş et al, 2015).

Statistical analysis

The results of the in vitro investigation of the extracts' anticancer activity were means ± SD of six measurements. The differences between groups were tested by ANOVA. The p values of <0.01 were considered as significant and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). The results of scavenging activity and overall phenolicd were performed by the averages of samples' mean ±SD (standard deviation) using exel 2003. All the analyses were done in triplicates.

3. RESULTS

3.1. Extraction yield

The extract yield of the n-butanolic extract of E. helioscopia has been given in (table 1), and was conveyed as the ratio of the dry weight of the plant extract to the dry weight of the plant material used for the extraction yield of n-butanolic extract (E.h.B) which was 1.33 %.

Table 1: *E. helioscopia* extract yield

Extract	Yield (%)
n-Butanol extract (E.h.B)	1.33 %

3.2. Total phenolics (TPC), and flavonoids (TF)

Phenolic compounds are bioactive components of plants and show health promoting activities. The characteristics of the extracting solvents extensively affected the yields, total phenolics and oxidant activity. As estimated by the folin-ciocalteu's method, the total phenolics were presented as mg GAE/g extracts, and the amount of total phenolics (table 2), and the amounts found in the extract of n-butanol (E.h.B) were 156.21±4.55 mg GAE/g of extracts.

Table 2: phenolic compounds of *E. helioscopia* extract

Extract	TPC	TF
n-Butanol	156.21±4.55	22.08±0.81

3.3. HPLC analysis

To understand the active principles responsible for the activity of the extract, the recognition and quantification of phenolics were carried using analytical method HPLC -TOF-MS, and the findings are shown in table 3. By comparing their HPLC retention times and UV spectra with those of reference compounds, 05 constituents in *E. helioscopia* were identified in n-butanol extract. Gentisic acid was the major phenolic compound with an amount of 23.29 mg/kg of powder.

Table 3: HPLC-TOF/MS composition of n-butanol extracts

Compounds	RT	butanol
Gentisic Acid	4.301	23.29
Chlorogenic Acid	6.134	0.27
Vanillic Acid	7.553	0.25
Rutine	9.407	0.46
Ferulic Acid	10.324	0.11

3.4. Reducing power assay

The reducing ability of compounds may provide a noteworthy sign for its possible antioxidant action (Hsu et al, 2006). Fig 1 demonstrates the reducing control of the test samples using the potassium ferricyanide reduction method, and Fig 2 shows the reductive capabilities of the standards. N-butanol extract showed the presence of the reductive effects, and exhibited the reducing power in a concentration dependent manner. The antioxidant activity of the extracts of *E. helioscopia* was affected by the extraction solvent and the analysis method. As concentration increased from 0 to 1000 µg/ml, the reducting power of n-butanol extract increased rapidly from 0 to 1.396. Reducing power of n-butanol extract and standards at 100 µg/ml decreased in the following order: Quercetin > ascorbic acid > gallic acid >E.h.B.

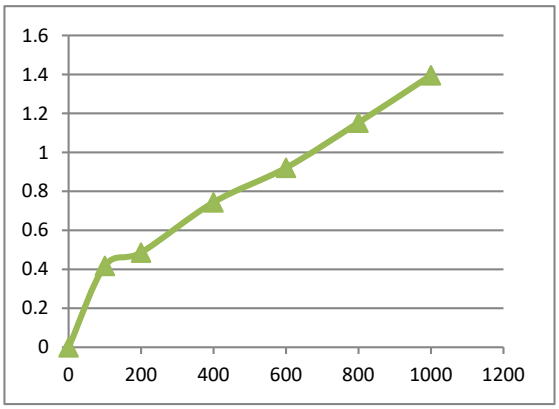

Figure 1: The reducing effect of n-butanol extract of *E. helioscopia* at different concentrations

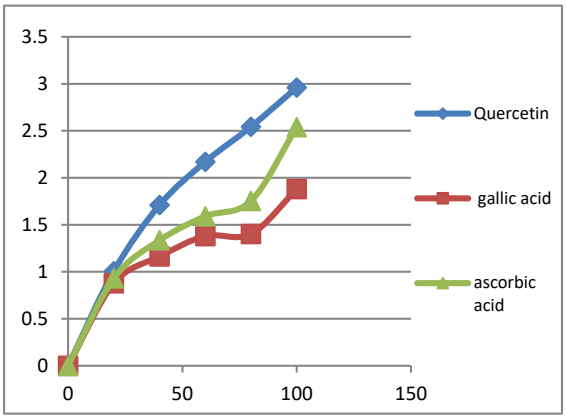

Figure 2: The reducing effect of standards at different concentrations

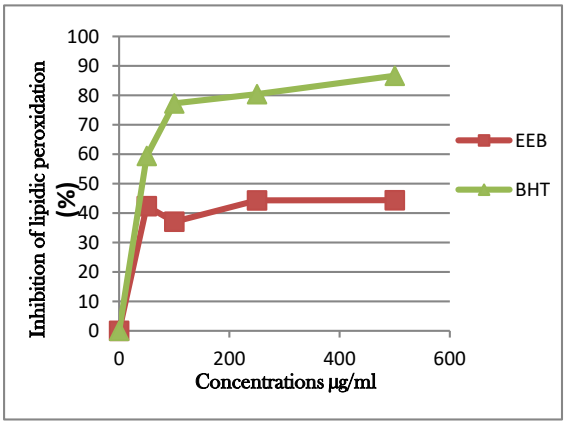

Figure 3: Inhibition percentage of linoleic acid peroxidation of *E. helioscopia* extract and the standard at different concentrations

3.5. Anti-proliferative activities

- **C6 cells**

5-FU was taken as positive control in this study. The E.h.B extract showed no inhibitory effect against C6 cell (fig 4). The IC50 and IC75 values of the extracts were given at Table IV, the cell proliferation inhibition of 5-FU was more pronounced at the concentration of 250 µg/ml against C6 cell fig 4. The potency of inhibitions at 250 µg/ml against C6 cells were: 5-FU > E.h.B.

- **Hella cells**

As a result of the activity test in various concentrations, as can be seen in figure 5, *n*-butanol extract (E.h.B) showed a moderate inhibition of proliferation against Hela cell in all the concentrations. The results indicated that the plant possesses the anti-cancer activity.

Table 4: IC_{50} and IC_{75} values of n-butanol extract against C6 cell.

	E.E.B
IC_{50}	171.98
IC_{75}	214.05

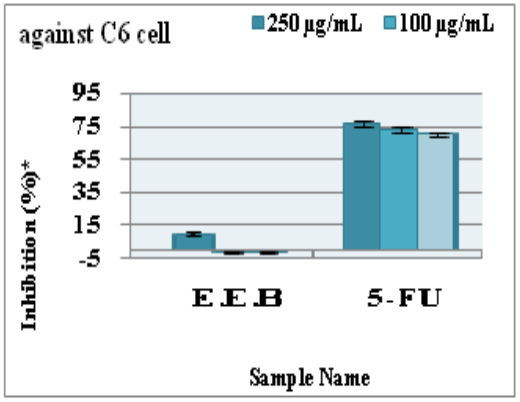

Figure 4: The anti-proliferative activities of the extracts against C6 cells. *each substance was tested twice in triplicates against cell lines. The data showed the average of two individual experiments (p<0.01).

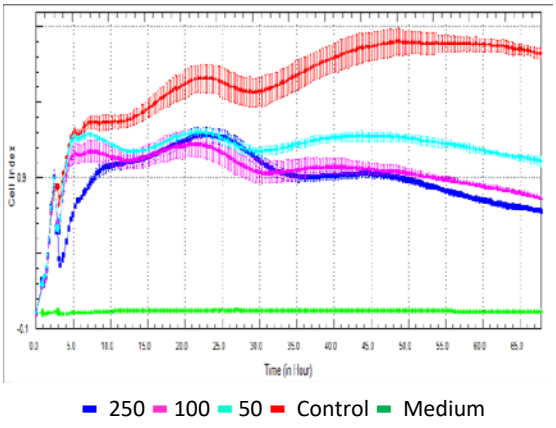

Figure 5: Anti-cancer activity against the Hela cell lines of E.h.B extract using xCELLigence RTCA instrument

4. DISCUSSION

The yields in this study were less than those obtained by Barla (Barla et al, 2007) on petroleum ether, acetone and ethanol

extract of three *Euphorbia spp* (*E. acanthothamnos, E. rigida , E. Macroclada*) in reported *E. macroclada* yields including (Petroleum ether (2.14%), Acetone (3.54%), Ethanol (11.14%), and were also less than those of Qaisar (Qaisar et al, 2012) of aerial parts of *E. milii, E. helioscopia, E. hirta* and *E. prostrata*,and the extractive values in *E. helioscopia* were n-hexane extract (5,89%), chloroform extract (35,55%), methanol extract (3,10%), and the non polar solvent which were rich intermediate type polar compounds such as alkaloids, triterpenes and steroids and non polar type hydrocarbons such triterpenes and fatty acids whereas the polar one was rich in polyphenols and flavonoids. The results of this study showed that the extraction solvents affected the phenolic contents of the extract. Flavonoids, tannins, catechins and other phenolics are the examples of common plant metabolites having prominent antioxidant activity (Laitonjam 2012). The results of this study on the *E. helioscopia* extract demonstrated that the plant extract was enriched in flavonoids, and n-butanol extract (E.h.B) had the highest content of 22,08±0,81 mg QE/g extracts because flavonoids are polar constituents and they can't be extracted using non-polar solvent system. As a consequence, few polar compounds would be present in those particular extracts. The reducing power of was relatively more pronounced than of extract at the same concentrations. The reducing power of the standard extract showed the same order of TPC. It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to the phenolic content (Rice-Evans et al, 1997). It has been known that phenolic compounds are related to antioxidant activity, and play an important role on stabilizing lipid peroxidation (Koleva et al, 2001). The percentage of inhibition of the extract and standard at different tested concentration have been shown in fig 06. In the present study, it was found that the alcoholic extract of *E. helioscopia* showed concentration dependent on lipid peroxidation inhibitory. E.h.B extract possessed the best effect (44.38 %) against lipid peroxidation at 500 µg/ml. The standard BHT showed better percentage of inhibition of lipid peroxidation (86.65 %) than the alcoholic extract of *E. helioscopia* at the same concentration (500 µg/ml). It could be observed that the content of phenolic in the extracts of *E. helioscopia* correlated with their inhibition of lipid peroxidation. Phenolic and flavonoid compounds seemed to have an important role in stabilizing lipid oxidation and be associated with the antioxidant activity which has been emphasized in several reports (Yanishlieva-Maslarova 2001). Although, some studies with similar results on the anti-proliferative effects of chloroform extract, ethyl acetate extract and n-butanol extract (the same extraction processes) from *E. helioscopia* on five human cancer cell lines (human hepatocellular carcinoma cell lines SMMC-7721, BEL-7402,HepG2, gastric carcinoma cell line SGC-7901 and colorectal cancer cell line SW480) were done by (Wang et al., 2012), and they found that E.h.B (n-butanol extract of E.helioscopia) exerted no inhibitory effects on all the cells, E.h.A (ethyl acetate extract) and E.h.C (chloroform extract) were very active : After treating with 150 and 200 µg/mL concentrations using SMMC-7721 cells for 72 h, EAE (ethyl acetate extract) inhibited cell growth by 77.47 and 80.91%, respectively, whereas CE (chloroform extract) inhibited cell growth by 40.91 and 51.76%, respectively.

5. CONCLUSION

The amount of total phenolic compounds in investigated plant extract correlated with its antioxidant activity, this study has shown that *n*-butanol extract showed the highest antioxidant activity when determined by reducing power and anti-peroxidation of lipid using two tests to evaluate the antioxidant and anti-proliferative activity, it can be concluded that the extract of the aerial part of *E. helioscopia* had good antioxidant and anti-proliferative activities. Based on these results, *E. helioscopia* plant can be a potential source of natural antioxidant, and *in vivo* studies are needed to further confirm the advantageous qualities of these extracts, and further studies are warranted for the isolation and identification of individual phenolic compounds to understand what kinds of compounds are responsible for the antioxidant and anti-proliferative activities.

Conflict of Interest: The authors declared no conflict of interest.

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