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Anticancer Activity of *Rosmarinus Officinalis* Aqueous Extracts from Three Locations in Algeria

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ABSTRACT

The aqueous extracts of the aerial parts of Rosmarinus officinalis from three different geographic locations (humid, semiarid, and arid) were subjected to phenolic assessment and antiproliferative activity using appropriate assays, mainly ELISA and xCELLigence. The results of total phenolics and flavonoids revealed yields ranging from 58.26 to 114.10 mg GAE (gallic acid equivalent) /g and from 14.63 to 28.86 mg QE (quercetin equivalent)/g, respectively. Moreover, the extracts were tested against two cancer cell lines namely C6 (rat brain tumor) and Hela (adenocarcinoma of the cervix) to show an up-and-down efficiency ranging from good at 250 µg/mL for sample 3 to very weak mainly at 50 µg/mL. These disparities highlighted the effect of climatic factors as quality determinants of secondary metabolites, and hence as a key influence of the biological activity.

Keywords:*Rosmarinus Officinalis, Aqueous Extract, Phenolics, Flavonoids, C6, Hela, Anti-Proliferative Activity.*

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1. INTRODUCTION

The application of plant extracts in folk remediation has a long time records in many ethnicities throughout the history. Many traditional herbs have been widely known for healing trouble-free ailments to grave and dangerous diseases, including special kinds of cancers, due to their efficacy as good sources of bioactive compounds (Doudach et al. 2013; Fattahi et al. 2013).

Nowadays, various herbal preparations have fascinated many researchers' consideration for cancer treatments. A number of medicinal plants and their bioactive compounds have shown anticarcinogenic and antiproliferative effects on cancer cells. Currently, over 60% anti-cancer agents have been derived from natural sources, including plants, marine organisms, and micro-organisms. A variety of reports have shown the capability of phytochemicals to offer protection against free radical induced disorders due mainly to their content of flavonoids and phenolics in free or complex forms. These compounds have been identified and quantified in several fruits and vegetables, and have shown a high correlation with antioxidant activity (Ziech et al. 2012; Carmona-Ramirez et al. 2013).

Rosmarinus officinalis belonging to Lamiaceae family, known as Rosemary is very widespread in the Mediteranean region, and distributed throughout the world. Rosemary extracts display many biological activities, including antimicrobial, anti-mammary tumor genesis, anti-mutagenesis, antidepressant, anti-ulcerogenic, anti-inflammatory and antioxidant. The main components of Rosemary are rosmarinic acid, which has been reported to have anticarcinogenic, anti-allergic, antimutagenic, antibacterial and antioxidant activities (Machado et al. 2013; Amaral et al. 2013; Lucarini et al. 2013; Ma et al. 2013)

In the present study, the antiproliferative activity of water extract of the plant *Rosmarinus officinalis* collected from three different geographical origins (including: arid, semi-arid, and humid), was examined to highlight the impact of environmental conditions on the content of secondary

metabolites and consequently on the remedial potency of this species.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial parts of *R. officinalis* were collected from Oum El Bouaghi (site 1: semiarid), El Taref (site 2: humid) and Ouargla (site 3: arid). The plant was identified by Pr. A. zellagui, Oum El Bouaghi University, Algeria. Voucher specimens (RO1, RO2, RO3 for *R. officinalis* of the 3 sites respectively) were deposited in the Laboratory of Natural Resources and Management of Sensitive Environments, University of Oum El Bouaghi, Algeria.

2.2. Extraction and preparation of the different fractions

2.2.1. Water extract:

500 mL of boiling distilled water were added to 100 g of finely ground dry plant material of each sample. After 60 min, the water extract was filtered and dried under vacuum, weighed and prepared for HPLC analysis.

W1: Water extract of *R. officinalis* from Oum El Bouaghi

W2: water extract of *R. officinalis* from El Tarf

W3: water extract of *R. officinalis* from Ouargla

2.3. Total phenolic content

The TPC of the extract was determined using the folin-ciocalteau rGAEent method according to the method of Singleton (Singleton et al., 1999). 0.5 mL of the diluted solution of each extracts in methanol was added to 2.5 mL of FCR diluted 1/10 with distilled water), and they were mixed. after 5 min, 02 mL of sodium carbonate water solution Na_2CO_3 (75g/L) was added to the mixture, and incubated at 40 °C for 30 min. The results were represented as mg of gallic acid Equivalent (GAE)/g of dry extracts. Gallic Acid was considered as a standard. The analysis of all the samples was done in triplicates.

2.4. Total flavonoids content

The plant extract's total flavonoids content was assessed based on the aluminium chloride colorimetric method (Djeridane et al., 2006). This method was used according to the formation of a complex flavonoid-aluminium having the maximum absorbance at 430 nm. 01 mL of each extract was mixed with 1 mL of 2% $AlCl_3$methanolic solution, and then the absorbance was measured at 430 nm using UV-VIS spectrophotometer. Total flavonoids content was represented as mg quercetin QEuivalent/g of dry extracts. Quercetin was regarded as a standard. The analysis of all the samples was done in triplicates.

2.5. Determination of in vitro anti-proliferative activity

Anti-proliferative activity of extract was evaluated in vitro by estimation of the inhibitory effect of phenolics on the growth of cells on C6 rat brain tumor, and Helacell lines using proliferation BrdU ELISA and xCELLigence assays (Demirtas &Sahin-Yaglioglu, 2012).

2.5.1. Cell culture

The cells were grown in Dulbecco's modified GAEle'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.

2.5.2. Cell proliferation assays

- **ELISA assay:**

The cells were incubated in 96-well culture plates (COSTAR, Corning, USA) at a density of 30.000 cells in each well. The samples' activities were scrutinized on 250, 100 and 50 µg/mL. After that, the cells were incubated overnight before administrating the BrdU Cell Proliferation ELISA assay (rent Roche, Germany) in accordance with the manufacturer's procedure. The quantity of cell proliferation was identified at 450 nm by applying a microplate reader (Awareness Chromate, USA). The results were represented as the percentages of the inhibition of cell proliferation, where the optical density calculated from vehicle-treated cells was regarded to be 100% of proliferation. Dimethyl sulfoxide (DMSO) was used to prepare the stock solution of the extracts and DMEM was applied to dilute them. DMSO final concentration was below 0.1% in all tests. 5-FU was used as standard compounds. Percentage of inhibition of cell proliferation was measured as follows: 1 - $A_{treatments}$ /$A_{vehicle\ control}$)) x 100. The half maximal inhibitory concentration (IC_{50}) was a measure of the effectiveness of a compound in inhibiting a biological function. In this paper, IC_{50} and IC_{75} values were determined using ED50 in addition to V1.0 (Derango and Page 1996).

- **xCELLigence assay**

To assess the extracts, controls, and medium, the xCELLigence system was applied with the disposable E-plate 96. The measurements were done based on the impedance difference, which was resulted by the cells attached to the E-Plate 96.

The medium 100 µL) was added to each well of E-Plate 96, and inserted in the incubator. E-plate 96 was placed to the xCELLigence station, and the background impedance was assessed during 1 min. Then, 50 µL of each cell suspension was placed in medium containing wells, and adjusted to 20.000 HeLa cells mL^{-1}. To attach the cells to the E-Plate 96 wells, the plate was kept in a sterile cabinet at room temperature for 30 min.Lastly, the adhesion, growth and proliferation of HeLa cells were observed every 10 min for in a period of up to 3 h *via* the incorporated sensor electrode arrays of the E-Plate 96. The extracts were poured into the wells of the plate, and the crude water extract concentrations of 250, 100, 50 and 10 µgmL-1 were obtained. The plates were then instantly inserted into the incubator, and monitored every 10 min during 48 h. Using the standard deviation bars, the tests were replicated and repeated three times (Koldaş et al., 2015).

2.6. Statistical analysis

The results of in vitro investigation of anticancer activity were shown by means ± SD of six measurements. The differences between groups were tested with ANOVA. (They were analyzed by SPSS version 11.5 for Windows 2000, SPSS Inc p values of <0.01 were considered as significant.).

The results of scavenging activity and total phenolic compounds were performed from the averages of samples reading mean ±SD standard deviation (Exel 2003 was used). All analyses were carried out in triplicates.

3. RESULTS AND DISCUSSION

3.1. Total phenolic contents

Phenolic compounds are secondary metabolism components showing health promoting activities. The properties of the extracting solvents significantly affected the yields, total amount, and oxidant activity, as assessed by the folin-ciocalteu's method. The total phenolics contents in the extracts are presented as mg GAE/g DW of extracts in Table 1.

Table 1: Total phenolics in the water extract (mg GAE/gDW)

samples	Total phenolics content in water extracts of *R. officinalis*
W1	81.63±0.16
W2	114.10±0.15
W3	58.26±0.31

The results exposed important variations in total phenolics amount from one site to another. Site 2 (humid region) exhibits important phenolic contents (114.10 to 167.91 mg GAE/g DW**)**. This is certainly due to environmental factors especially water and nutrients favorable for the biosynthesis of such elements.

3.2. Flavonoids content

The flavonoids content, expressed in milligram of QEuivalent quercetin per grams of dry weight extract (mg QE / g DW), was determined from the regression curve whose QEuation is: y = 0.0299 X + 0.0979, R^2 = 0.9746 (table 2)

Table 2: Total flavonoids in the water extracts *(*mg QE / g DW)

samples	Flavonoids content in water extracts of *R. officinalis*
W1	28.86±0.31
W2	28.78±0.02
W3	14.63±0.08

3.3. Anti-proliferative activities

3.3.1. C6 cells

- **Water extract:**

The anti-proliferative activities of W1, W2, W3 and 5-FU were determined against C6 cells. The IC_{50} and IC_{75} values of the extracts are given in Table 3. The anti-proliferative activity of all the water extracts showed an increase in activities as a function of the dose increase against the C6 cells (Fig.1). Water extracts W2 and W3 were determined to have relatively high anti-proliferative activity against C6 cells at a concentration of 250 µg/mL. W2 has a good anti-proliferative activity compared to the standard compound at both 100 and 250 µg/mL. The potency of the inhibitions (at 100 µg/mL) against C6 cells was: 5-FU> W2> W3> W1.

Table3. IC50 and IC75 values of the water extracts against C6 cell

	W1	W2	W3
IC50	156.08	14.20	163.47
IC75	205.86	122.97	209.40

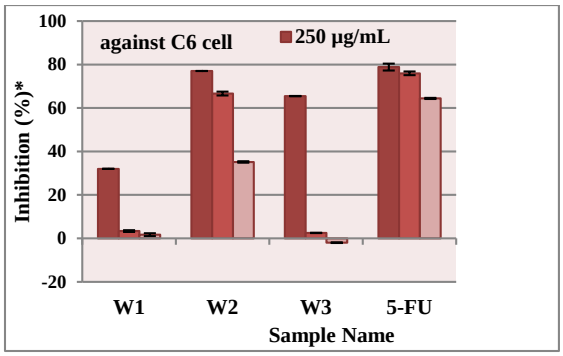


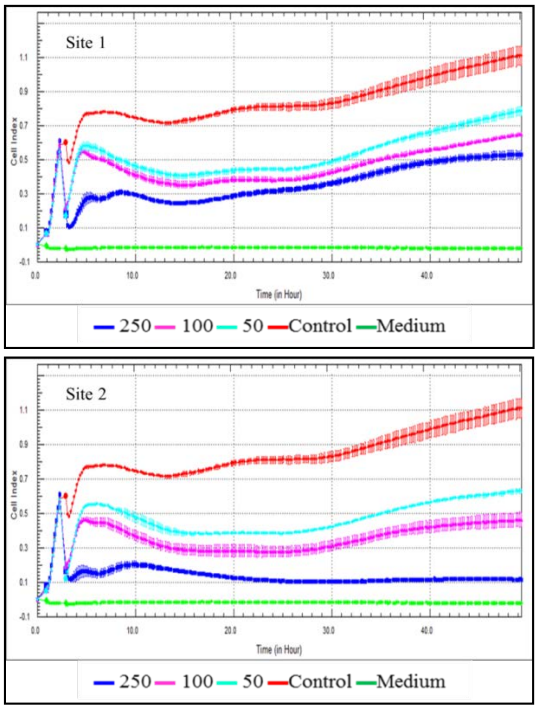
Figure 1. Antiproliferative activities of water extracts against C6 cells

* each extract was tested twice in triplicate against cell lines. The data showed the average of two individual experiments p <0.01).

3.3.2. Hella cells

The anti-proliferative effect of the extract was calculated on HeLa cell lines at 50, 100 and 250 µg/mL by means of real time cell analyzer xCELLigence procedure. The system quantified the impedance difference in order to determine the Cell Index (CI) values at time points whose periods could be set by the operator. These impedance dissimilarities and thus the CI values depended on the cell activity at the base of the wells (Isik at al., 2012). CI is a dimensionless parameter resulted as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows us that the cancer cells are dying. The records of this study showed that the extracts displayed anti-proliferative effect against HeLa cell line mainly at 250 µg/mL (Figure 2).

- ***R. officinalis* water extract:**



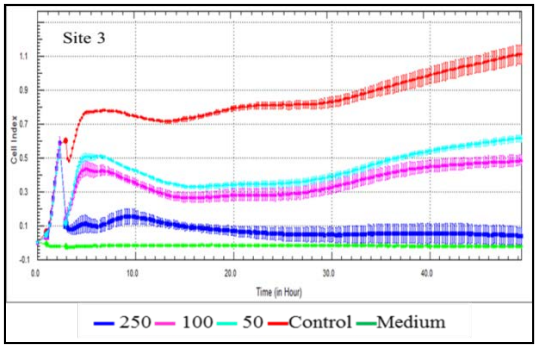


Figure 2. Antiproliferative activity of the water extract of *R. officinalis* againstHella cells

No effect was evident on the Hella cancer cells at 50 and 100µg/mL for the three extracts. At 250 µg/mL, there was a moderate effect for sample 2, whereas sample 3 showed the most important activity. This could be seen through the behavior of the blue curve vis-à-vis the medium.

4. CONCLUSION

The quantity of total phenolics displays a relationship with their anti-proliferative activity. It was made known through the tests used to weigh up the anti-proliferative activity that water extracts of the *R. officinalis* showed a good anti-proliferative activity at 250 µg/mL overall w3 when evaluated by xCELLigence. The geographical locations and hence abiotic factors seemed to have a significant role in affecting either the phenolic contents or the cytotoxic potency of the crude extract. Based on these results, the plant can be a potential source of natural cytotoxic agents, and *in vivo* studies are needed to extra corroborate the beneficial quality of these extracts, and further studies are defensible for the isolation and recognition of individual compounds to appreciate what kinds of compounds are in charge for bioactivities.

Conflict of Interest: The authors declare no conflict of interest

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