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Impact of Environmental factors on Phenolic Content and Anti-Proliferative Activity of *Rosmarinus Officinalis* Ethyl Acetate Extract

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Abstract

Recently, there has been a growing awareness in scientific circles regarding the use of ethnomedicines for treatment cancers. This attention has largely arisen due to the limited selectivity of current chemotherapy and radiotherapy usages. In this study, aerial parts of *Rosmarinus Officinalis* from three distinct geographic locations were extracted using ethyl acetate. The crude extracts underwent evaluation for polyphenolic content and antiproliferative activity using techniques such as ELISA and xCELLigence assay. Total phenolics and flavonoids were quantified, with total phenolic yields ranging from 73.75 to
167.91 µg EAG/mg, and flavonoid yields varying from 66.2 to 93.1 µg EQ / mg. Furthermore, the extracts were tested against two cancer cell lines, C6 (rat brain tumor) and Hela, revealing moderate activity, particularly at a concentration of 250 µg/mL. These findings underscore the impact of climatic factors on the production of secondary metabolites, thus influencing biological activity.

Keywords: Geographic Origin*; Rosmarinus officinalis;* phenolics; anti-proliferative activity.

1. Introduction

Cancer ranks as the second leading global cause of death, stemming from a combination of carcinogenic elements and genetic predispositions. Regarded as a preventable affliction, a significant portion of cancer cases result from environmental influences (ME et al., 2024).

The utilization of plant extracts has a rich historical tradition across numerous countries. Various traditional herbs have garnered recognition for their ability to address a spectrum of ailments, from minor illnesses to serious conditions like cancer, attributed to their potential as natural antioxidant reservoirs (Dhal et al., 2012; Fattahi et al., 2013; Rafat et al., 2010; Zhang et al., 2024).

The potential of herbal preparations for cancer treatment has apprehended the interest of various researchers. Many medicinal plants and their bioactive constituents have demonstrated anti-carcinogenic and antiproliferative properties against cancer cells. Presently, more than 60% of anti-cancer agents are sourced from natural reservoirs, including plants, marine organisms, and microorganisms. Numerous studies have highlighted the ability of phytochemicals to combat disorders induced by free radicals, primarily attributed to their abundance of flavonoids and phenolics in both free and complex forms. These compounds have been identified and quantified in different fruits and vegetables, exhibiting a strong correlation with antioxidant activity (Carmona-Ramírez et al., 2013; Ziech et al., 2012).

Rosmarinus officinalis, a member of the Lamiaceae family commonly known as Rosemary, thrives abundantly in the Mediterranean region. Extracts derived from Rosemary exhibit a diverse range of biological activities, encompassing antimicrobial, anti-mammary tumor genesis, anti-mutagenesis, antidepressant, anti-ulcerogenic, anti-inflammatory, and antioxidant properties. The primary constituents of Rosemary include rosmarinic acid, which has been documented to possess anticarcinogenic, anti-allergic, antimutagenic, antibacterial, and antioxidant properties (Amaral et al., 2013; Bernardes et al., 2010; Chunhui et al., 2013; Lucarini et al., 2013; Mbemi et al., 2020)

In our study we have carried out the antiproliferative activity of ethyl acetate extracts of *Rosmarinus officinalis* collected from three different geographical origins in Algeria [arid,

semi-arid and humid] so as to highlight the impact of environmental conditions on the content of secondary metabolites and consequently on the remedial potency of such species.

2. Materials and Methods

2.1. Plant material

The aerial parts of *R. officinalis* were collected from Oum El Bouaghi [site 1: semi-arid], 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 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

100 g of dry plant material of each sample was subjected to overnight extraction using ethyl acetate. After separation, the organic phase was evaporated and the crude extract was weighed and prepared for further analysis.

EA1: ethyl acetate extract of *R. officinalis* from Oum El Bouaghi

EA2: ethyl acetate extract of *R. officinalis* from El Tarf

EA3: ethyl acetate extract of *R. officinalis* from Ouargla

2.3. Total phenolic content (TPC)

The Total Phenolic Content (TPC) of each extract was determined using the Folin-Ciocalteu reagent method as outlined by Singleton (Singleton et al., 1999). To begin, 0.5 mL of the diluted solution of each extract in methanol was combined with 2.5 mL of Folin-Ciocalteu reagent (diluted to 1/10 with distilled water) and thoroughly mixed. Following a 5-minute incubation period, 0.2 mL of aqueous sodium carbonate solution (Na2CO3) at a concentration of 75g/L was added to the mixture, which was then further incubated at 40°C for 30 minutes. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extracts, with Gallic Acid serving as the standard. All samples were analyzed in triplicate to ensure accuracy.

2.4. Total flavonoids content

The Total Flavonoids Content (TFC) of the plant extracts was estimated utilizing the aluminum chloride colorimetric method, as described in reference (Djeridane et al., 2006). This method relies on the formation of a complex between flavonoids and aluminum, exhibiting maximum absorbance at 430 nm. To initiate the process, 0.1 mL of each extract was mixed with 1 mL of a 2% AlCl3 methanolic solution, and the absorbance was subsequently measured at 430 nm using a UV-VIS spectrophotometer. The total flavonoids content was quantified and expressed as milligrams of quercetin equivalent per gram of dry extracts, with quercetin serving as the standard reference. All samples were subjected to triplicate analyses to ensure precision and reliability.

2.5. Determination of in vitro anti-proliferative activity

The anti-proliferative activity of the extracts was assessed in vitro by determining the inhibitory effect on cell growth on C6 (rat brain tumor) and Hela cell lines. This evaluation was conducted using proliferation assays, specifically the BrdU ELISA and xCELLigence assays (Demirtas & Sahin, 2013).

2.5.1. Cell culture

The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma), supplemented with 10% fetal bovine serum (FBS, Sigma, Germany) and Penicillin-Streptomycin solution (Sigma, Germany). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.5.2. Cell proliferation assays

- ELISA assay

The cells were seeded into 96-well culture plates (COSTAR, Corning, USA) at a density of 30,000 cells per well. The samples were tested at concentrations of 250, 100, and 50 µg/mL. Following seeding, the cells were allowed to incubate overnight. Subsequently, the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) was applied to the cells according to the manufacturer's protocol.

The absorbance representing cell proliferation was measured at 450 nm using a microplate reader (Awareness Chromate, USA). The results were reported as the percentage of inhibition of cell proliferation, with the optical density from vehicle-treated cells considered as 100% proliferation. The stock solutions of the extracts were prepared in dimethyl sulfoxide (DMSO) and then diluted with DMEM, ensuring that the final concentration of DMSO remained below 0.1% in all tests. 5-FU was utilized as the standard compound.

The percentage of inhibition of cell proliferation was calculated as follows: [1 - [$A_{treatments}$ /$A_{vehicle\ control}$]] x 100. The half maximal inhibitory concentration **(IC_{50})** is 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** (Morales et al., 2024)**.**

- xCELLigence assay

The xCELLigence system was used with the disposable E-plate 96 for the measurements of extracts, controls and medium. The measurements base on the impedance difference was caused by the cells attached to the E-Plate 96.

The medium [100 µL] was added to each well of E-Plate 96 and placed into the incubator. E-plate 96 was inserted to the xCELLigence station, and the background impedance was measured 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}. The plate was held in a sterile cabinet at room temperature for 30 min for attaching the cells to the E-Plate 96 wells. Finally, HeLa cells were monitored every 10 min for adhesion, growth and proliferation in a period of up to 3 h *via* the incorporated sensor electrode arrays of the E-Plate 96. The extracts were added to wells of plate and adjusted the crude chloroform; ethyl acetate and n-butanol extract concentrations of 250, 100, 50 and 10 µgmL-1. The plates were then immediately placed in the incubator and monitored every 10 min during 48 h. The tests were replicated and repeated three times given with standard deviation bars (Jimoh et al., 2024).

2.6. Statistical analysis

The results of investigation in vitro of anticancer activity are means ± SD of six measurements. Differences between groups were tested with ANOVA. p values of <0.01 were considered as significant and analyzed by SPSS [version 11.5 for Windows 2000, SPSS Inc.].

The result of scavenging activity and total phenolic compounds were performed from the averages of samples reading mean ±SD [standard deviation] used exel 2003. All analyses were carried out in triplicates.

3. Results and discussion

3.1. Total phenolic contents

Phenolic compounds, which are bioactive constituents of plants, exhibit various health-promoting activities. The choice of extracting solvents significantly impacts the yields, total phenolics, and antioxidant activity. As stated by the Folin-Ciocalteu method, the total phenolic contents in the extracts are expressed as milligrams of equivalent gallic acid per gram of extracts, as shown in Table 1.

Table 1: Total phenolic in ethyl acetate extracts [mg EAG/g]

Sites	Total phenolic content
Site 1	123.22±0.31
Site 2	167.91±0.35
Site 3	73.75±0.07

The results exposed important variations in total phenolics amount from one site to another. Site 2 (humid region) exhibits important phenolic contents in both extracts (167.91 mg GAE/g**)**. This is certainly due to environmental factors especially water and nutrients favorable for the biosynthesis of such elements. Site 3, in turn displays the least content

3.2. Flavonoids content

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

Table 2: flavonoids content [mg EQ/g]

Sites	flavonoids content
Site 1	66.2±0.19
Site 2	93.1±0.07
Site 3	83.32±0.07

3.3. Anti-proliferative activities

3.3.1. C6 cells

The antiproliferative activities of the extracts against C6 cells were greater than those of 5-FU and showed an increase in activities as a function of the dose apart from EA-1 (Figure 1). The potency of the inhibitions was: EA-1> EA-2> EA-3 at 50 µg / mL, at 100 µg / mL it was EA-3>EA-2> EA-1. Whereas at 250 µg / mL it was EA-1> EA-2> EA-3.

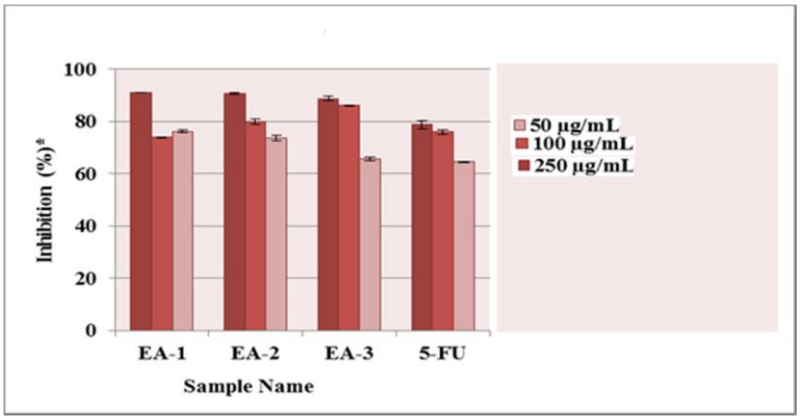

Figure 1: Antiproliferative activities of ethyl acetate extracts against C6 cells.

* Each extract was tested twice in triplicate against cell lines. The data show the average of two individual experiments [p <0.01]

3.3.2. Hella cells

The antiproliferative effect of the extracts was studied on HeLa cell line at the concentrations of 50, 100 and 250 µg/mL by using real time cell analyzer xCELLigence technique. The system quantifies the impedance variation in order to sort out the Cell Index values at time points whose periods can be set by the operator. These impedance variations and thus the CI values depend on the cell activity at the base of the wells [28]. CI is a dimensionless parameter resulting 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. Our records showed that the extracts displayed antiproliferative effect against HeLa cell line mainly at 250 µg/mL (Figure 2).

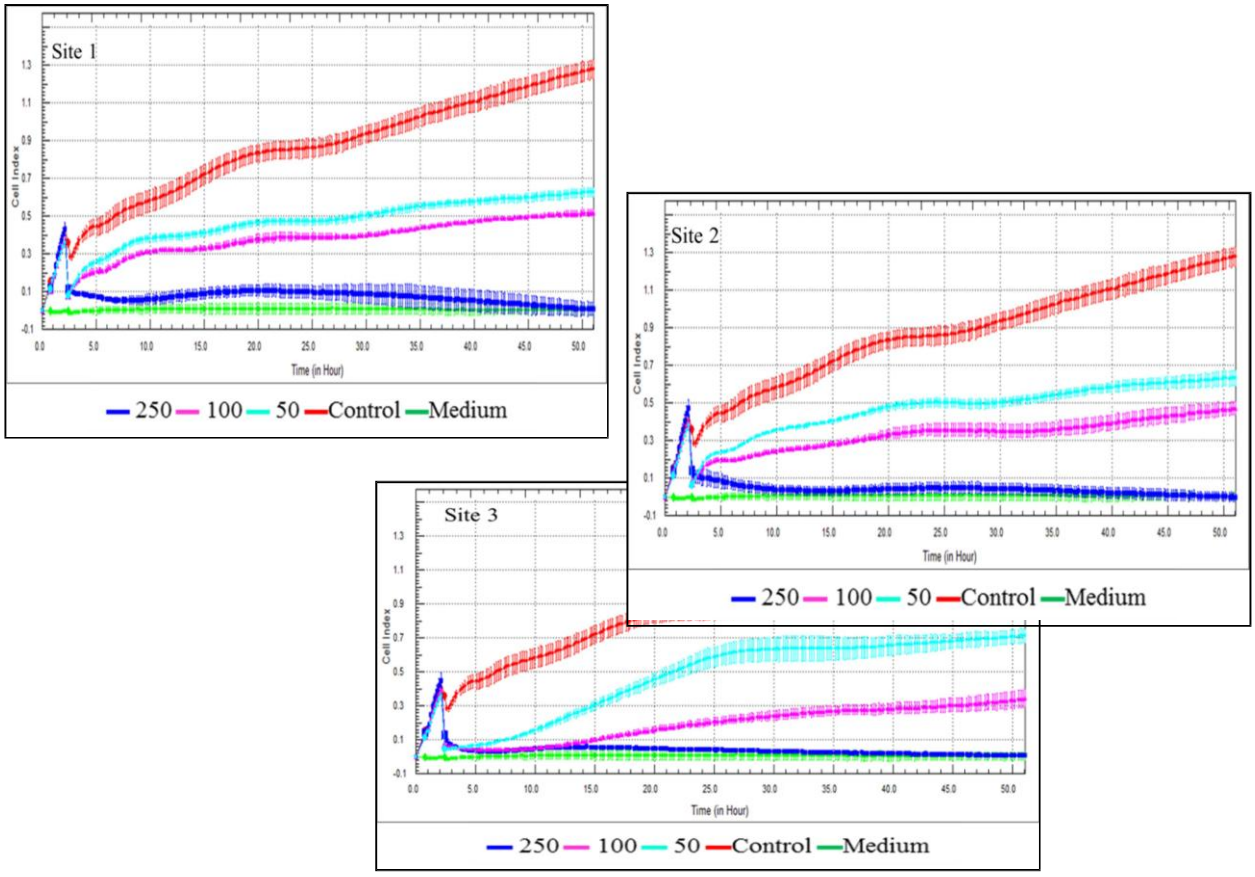

Figure 2: antiproliferative activity of the ethyl acetate extract in *R. officinalis* using xCELLigence RTCA instrument

For the ethyl acetate extract of *R. officinalis*, there is a slight effect on the cancer cells at a high dose (250 µg/mL) for the three sites.

4. Conclusions

The amount of total phenolic compounds investigated in plant extracts is more or less correlated with their antiproliferative activity. It was shown through two tests used to evaluate the antiproliferative activity that ethyl acetate extract at 250 µg/mL showed a good action upon evaluation by BrdU ELISA and xCELLigence. The geographical locations and hence abiotic factors seem to have significant role in affecting either the polyphenolic content or the cytotoxic potency of the crude extracts. Based on these results the plant can be a potential source of natural cytotoxic agents and *in vivo* studies are needed to promote the advantageous quality 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 such activities.

Conflict of Interest

The authors declare no conflict of interest

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