Cytotoxic Effect of *Rosmarinus officinalis* L. Leaf Extracts on Tumor Cell Line

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Abstract

This study was conducted to evaluate antitumor effects of roesmarinus (*Rosmarinus officinalis)* extracts (aqueous and methanol) on Rhabdomyosarcoma; RD cell line and a normal cell line; mouse embryo fibroblast; MEF). Chemical detections of Rosemary extracts revealed that the aqueous and methanol extracts were positive for flavonoids, alkaloids, phenol, terpenes, saponine, glycosides, steroids and tannins. The percentage growth inhibition (PGI) of five leaf extract concentrations 50, 100, 250, 500 and 1000 (µg/ml) were assessed *in vitro* using RD and MEF. The results revealed that the five concentrations of the plant extracts showed anti-tumor properties in a concentration-dependent manner, and the methanol extract recorded better values of PGI than aqueous extract in RD cell lines, while, less PGI values were recorded in the MEF cell line. This experiment investigated the cytotoxic activity of the methanolic and aqueous extracts of Rosemary at concentrations (50 -100 -250 -500 -1000 µg/mL) on RD cell lines. A dose-dependent reduction was observed in treated cell line after 24 hrs. of treatment, but lower concentrations exhibited lower cytotoxic effects. Maximum inhibition of proliferation was achieved at the highest concentration (1000 µg/mL).

Keywords: *Rosmarinus officinalis* L., tumor cell lines, cytotoxic effect, leaf extracts.

Introduction

Conventional drugs suffer of their low aqueous solubility and physical stability, reduced absorption, rapid metabolism and instability under high acidic conditions. To combat these constraints intense research is focused on various sources to develop novel anti-cancer drugs. Plants have proved valuable natural anti-cancer therapy source. Increasing number of cancer patients currently use complem - entary and alternative medicines in conjunction with conventional chemotherapeutic treatments [1]. Scientist listed 117 plants having anti-cancer properties [2].

Many studies show that different plants extracts have a potential anticancer activity preventing reverse and/or inhibit certain carcinogenesis processes before the development of invasive cancer [3]. This may be attributed to certain substances present in the plant raw material. Scientific studies are currently under development to prove that these substances possess specific functional activities.

Rosemary (*Rosmarinus officinalis* L.) extracts have been reported to have several and important biological properties, such as hepatoprotective, antidiabetic, antioxidant, antiproliferative, antiviral, antimicrobial, antinociceptive and antidepressant, among others [4]. Some of these activities point to a promising beneficial effect of rosemary in controlling cancer development. Accordingly, it has been previously reported that rosemary extracts and their components show inhibitory effects on the growth of breast, liver, prostate, lung and leukemia cancer cells and represses the initiation and promotion of tumorigenesis of melanoma and glioma in animal models [5].

One of the most appreciated properties of rosemary extract is its antioxidant capacity, which is related to the presence of antioxidant phenolic substances, such as carnosol, rosmanol, carnosic acid, methyl carnosate, rosmarinic and caffeic acids [6].

Materials and Methods

Plant extracts

Plant extracts were obtained by two ways, water and methanolic extracts as follows.

Water extract

A quantity of 50 g of the leaves powder was mixed with 250 ml distilled water. The mixture was left in a shaker incubator at 37°C for 24 hrs., and then filtered through a filter paper (Whatman no. 1). The filtrate was

concentrated using rotary evaporator at 40-60 °C until dryness and the extract residue was weighted and kept until use [7].

Methanolic extract

A quantity of 50 g of leaves powder was extracted with 250 ml of 75%methanol in flask for 6 hr. at 40-60 °C. The solution then evaporated to dryness using a rotary evaporator at 40°C, and the extract residue was weighted and kept until used [8].

Detection of some active compounds of rosemary water and methanolic leaf extracts

Detection of flavonoids was carried out according to [8], alkaloids and saponins [9], phenols [10], glycosides and tannins [11], terpens and steroids [12].

Cell culture

The *in vitro* anti-tumor activity of aqueous and methanol extracts was carried out at the Iraqi Center for Cancer and Medical Genetic Research (Al-Mustansiryah University). In this experiment, the cytotoxic activity of the two plant extracts was evaluated against two tumor cell lines (human rhabdomyosarcoma; RD and a normal cell line (mouse embryonic fibroblasts; MEF). Cells were grown at 37 ºC in humidified atmosphere containing 5% CO_2 in RPMI- 1640 medium supplemented with 10% fetal calf serum (FCS), Glutamine (2Mm), penicillin (100IU/ml), and streptomycin (100µg/ml) [10].

Cytotoxic assay

Cytotoxic assay was carried out according to Freshney [13]. Cell suspension was prepared for each type of cell lines and1×10^5 exponentially growing cells seeded in a 96 well tissue culture plates as (200 µL) in each well and incubated at 37 ºC for 24 hrs. After incubation, the wells examined for the formation of cell monolayers and 200 µl/ well from each concentration (50, 100, 250, 500 and 1000 µg/ml) were added to the wells as three replicates. Three replicates were made for control which contained only the cells with growth medium. After 48 hrs, 50 µl/ well of neutral red dye was added and incubated again for 2 hr. After incubation, the contents of the plate were removed by washing the cells with PBS to remove the excess dye followed by the addition of 20 µl/well of extraction dye solution that draw out the dye from the viable cells that had stained. Results were read using ELISA reader at wave length 492 nm. The percentage of growth inhibition (%GI) was calculated according to the following equation [14].

$$\text{Growth inhibition } (\%) = \left(\frac{\text{Control Absorbance - Treated Absorbance}}{\text{Control Absorbance}} \right) \times 100$$

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer program SPSS version 20.The difference was considered significant when the probability value was equal or less than 0.05.

Results

Detection of some active compounds in rosemary leaves

Chemical detections of *rosemary* aqueous and methanol extracts revealed that the plant was positive for several secondary metabolites, flavonoids, alkaloids, phenols, saponins, glycosides, tannins terpenes and steroids were detected in both extracts (Table (1)).

Table (1)
Detection of some active compounds in rosemary leaves of water and methanolic extracts.

secondary metabolite	*Result of detection*	
	Aqueous extract	*Methanol extract*
Alkaloids	+ve	+ve
Flavonoids	+ve	+ve
Phenols	+ve	+ve
Saponins	+ve	+ve
Glycosides	+ve	+ve
Terpenes	+ve	+ve
Steroids	+ve	+ve
Tannins	+ve	+ve

+ve indicates the presence of secondary metabolites.

Growth inhibition effects of *Rosemary* aqueous and methanol extract on RD cell lines.

Results recorded a significant increase in PGI was recorded in the five tested concentrations for both cell lines, especially the 5th concentration in which the PGI was 82 and 84% for aqueous and methanol extract respectively. Results revealed that methanol extract had a significant $P \leq 0.05$ cytotoxic effect in comparison with aqueous Table (2).

Table (2)
Growth inhibition effects of Rosemary (aqueous and methanol) extract on RD cell lines.

Extract Concentration (µg/ml)	*Percentage of growth inhibition (mean ± S.E.)**	
	Aqueous extract	*Methanol extract*
50	23.00 ± 1.15^{c}	26.0 ± 0.57^{c}
100	26.0 ± 0.57^{c}	32.6 ± 1.45^{b}
250	32.0 ± 0.57^{b}	33.3 ± 0.88^{b}
500	34.6 ± 1.78^{b}	35.3 ± 1.45^{b}
1000	82.3 ± 1.45^{a}	84.00 ± 1.15^{a}

****Different letters: Significant difference (P ≤ 0.5) between means of the columns.***

Growth inhibition effects of *Rosemary* aqueous and methanol extracts on MEF cell line.

The aqueous and methanol extract recorded an approximated range of PGI against the MEF cell line (aqueous extract: 10-14%; methanol extract: 10-15%) with the exception of the concentrations 1000 µg/ml, in which the methanol extract demonstrated no significant increase in methanol PGI as compared with the PGI of the corresponding aqueous extract concentration Table (3).

Table (3)
Growth inhibition effects of Rosemary extracts (aqueous and methanol) on MEF cell line.

Extract concentration (µg/ml)	*Percentage of growth inhibition (Mean ± S.E.)**	
	Aqueous extract	*Methanol extract*
50	10.6 ± 0.33^{b}	10.33 ± 0.88^{b}
100	11.0 ± 0.57^{b}	10.66 ± 0.33^{b}
250	$12.0 \pm 1.15a^{b}$	12.70 ± 1.15^{b}
500	$13.00 \pm 1.0a^{b}$	14.66 ± 0.88^{a}
1000	14.00 ± 0.57^{a}	15.0 ± 0.57^{a}

**** Different letters: Significant difference (P ≤ 0.5) between means of the columns.***

Discussion

Cancer-related research is conducted worldwide every day, since cancer is a leading cause of death. These studies often involve the investigation of the effects of biologically active substances on cancer cells, and they frequently originate from plants. There is a great need to examine reliable and inexhaustible sources of natural substances. In addition, it is important to understand the mechanisms of anticancer agents for future application in cancer therapy [15]. Carcinogenesis is one of the most important phenomena that could be attributed to free radicals/reactive oxygen species. The cytotoxic activity in the present study may due to presence of compounds that were detected such as alkaloids, flavonoids, phenols, saponins, glycosides, terpene and tannins especially the most active antioxidative constituents of rosemary are phenolic diterpenes (carnosic, carnosol, rosmanol, rosmadial, 12-methoxycarnosic acid, epi-, and iso-rosmanol) and phenolic acids (rosmarinic and caffeic) [16]. Compound in rosemary have shown a variety of pharmacological activities for cancer chemoprevention and therapy in *in vitro* and, *in vivo* models [17]. Study indicated that ethanoilc extract of rosemary given to rats showed hepatoprotective and antimutagenic effects due to high content of phenolic compounds with high antioxidant activity [18].

Cheung *et al* [19] showed that the crude ethanolic rosemary extract had anti-proliferative effect on human leukemia and breast carcinoma cells. Addition of rosemary extract delayed the oxidation of lipid fraction of minced meatballs during storage in the freezer. The antioxidative effect was related to the concentration of the active compounds present in the extract [20, 21]. Studies showed that there are biologically active compounds in rosemary essential oil exhibiting cytotoxic, antioxidant, anti-carcinogenic and cognition-enhancing properties [4].

Conclusions

Different active compounds were detected in the aqueous and methanol extracts of rosemary including flavonoids, phenols, terpenes, saponin, steroid, tannins, alkaloids andglycosides**.** Both extracts were *in vitro* effective as anti-tumor agents, and the effect was dependent on the cell line (tumor or normal cell line) that was investigated.

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الخلاصة

اجريت هذه الدراسة لتقييم التأثير المضاد للاورام لمستخلصي نبات اكليل الجبل المائي والكحولي على كل من خط الخلايا العضلية المخططة المصابة بالسرطان للانسان وخط الخلايا الليفية لجنين الفئران الطبيعية. اظهر الكشف الكيميائي للمستخلصات الميثانولية والمائية لاكليل الجبل نتائج إيجابية لكل من الفلافينات والقلويدات والفينولات والتربينات والصابونيات والكلايكوسايدات والسترويدات والتانين. اظهرت النتائج ان نسبة قتل الخلايا للتراكيز الخمس 50 و 100 و 250 و ٥٠٠ و ١٠٠٠ مايكووغرام/ مل التي باستعمال كل من خط الخلايا العضلية المخططة المصابة بالسرطان للانسان وخط الخلايا الليفية لجنين الفئران الطبيعية ان تأثير المضاد للاورام للورم يعتمد على التركيز واظهرت النسبة المئوية لقتل الخلايا للمستخلص الميثانولي اعلى من المستخلص المائي في الخلايا السرطانية بينما سجلت قيم اقل للخلايا الطبيعية.