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Cell cycle analysis and cytotoxic potential of *Ruta graveolens* against human tumor cell lines

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There are reports on the presence of various compounds exerting different biological activities in *Ruta graveolens*, a plant of Rutaceae family. The aim of the present study was to evaluate in vitro cytotoxicity of the total extract of *R. graveolens* against tumor cell lines of different origin. Aerial parts of the plant was extracted with 70% ethanol by sonication method and cytotoxic activity was examined on RAJI, RAMOS, RPMI8866, U937, Jurkat, MDA-MB-453, MCF-7, LNCap-FGC-10, 5637, HeLa, SK-OV-3, A549, Mehr-80 and also peripheral blood mononuclear cells (PBMC) by the use of WST-1 assay. Results were expressed as IC_{50} values. *R. graveolens* extract showed high cytotoxic activity against RAJI and RAMOS, two Burkitt's lymphoma cell lines, with an IC_{50} equal to 24.3μg/ml and 35.2μg/ml respectively and LNCap-FGC-10, a prostate adenocarcinoma cell line with an IC_{50} equal to 27.6μg/ml as well as Mehr-80, a newly established Large Cell Lung Carcinoma (IC_{50}=46.2 μg/ml). No significant anti-proliferative activity was observed on other cell lines including MCF-7, MDA-MB-453, SK-OV-3, HeLa, 5637, JURKAT and RPMI8866. Adverse cytotoxic effect of *R. graveolens* was investigated against PBMCs and a significantly lower effect of this extract (IC_{50}=104 μg/ml) was seen on normal cells compared with RAJI and RAMOS, two haematopoietic cell lines ($p<0.05$). Flow cytometry analysis of *R. graveolens* extract for determining cell cycle distribution in RAJI cells demonstrated that its anti-proliferative effects is associated with a significant increase in sub-G1 population. These results indicate the presence of naturally occurring cytotoxic compound(s) in *R. graveolens* with probable apoptosis inducing capacity with low cytotoxicity on normal mononuclear cells.

Keywords: *Ruta graveolens*, Rutaceae, cell line, cytotoxicity, cell cycle analysis

Cancer is one of the most severe health problems worldwide and the development of new anticancer drugs and more effective treatment strategies are fields of utmost importance in drug discovery and clinical therapy [1]. The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants with medicinal activity have been assayed. A significant percentage of drugs are from natural resources and nearly 50% of drugs used in medicine are of plant origin [2]. This percentage becomes even higher for anticancer compounds, over 60% [3].

Ruta graveolens, a plant of Rutaceae, is native to Europe and cultivated in Northern parts of Iran. Constituents of the plant include volatile oils, coumarin, yellow glucoside, alkaloids, and Rutin [4]. *R. graveolens* was reported to have spasmolytic activity to the isolated rabbit ileum [5]. Rutin (C27H30O16·3H2O), the main active compound, and its glycone, first isolated from the leaves of *R. graveolens*, are well known protectors against nuclear exposures and capillary bleedings [6]. An extract from *R. graveolens* has also shown mutagenic activity when tested in *Salmonella* [7]. Medicine in ancient Greece and Rome also employed it as an abortifacient. Laboratory studies in adult albino mice have shown that Ruta provides protection against the clastogenic effects induced by X-radiation. Ruta 6, which is a diluted potency of the mother tincture (Ruta Q), a plant extract homeopathic drug, has also been effective in the treatment of cysticercosis. In addition Ruta 6, in combination with calcium phosphate [(Ca3PO4)2], has shown potent antitumor activity in patients with brain cancer [8]. There are also reports on the anti-inflammatory effect of *R. graveolens* in murine macrophages [9] as well as cytotoxicity of two naturally occurring furacridone alkaloids which are isolated from *R. graveolens* too [10].

Despite several surveys on the biological activities of *R. graveolens* and its components, there are only few reports on the antiproliferative and cytotoxic effects of this plant against tumor cell lines and normal cells. Therefore, in the current study we investigated the cytotoxic activites of *R. graveolens* growing in Iran which has not been studied previously against selected tumor cell lines and normal Peripheral Blood Mononuclear cells (PBMCs). Additionally, in order to obtain some preliminary

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insights on the mechanism of action, *R. graveolens* extract was examined for its effects on RAJI cell cycle distribution by flow cytometry analysis.

Materials and methods:

Plant material. The aerial parts of cultivated plants were collected from Fars province of Iran, in July 2003 and identified by Dr. A. R. Khosravi. Voucher specimen has been deposited in the Herbarium of Shiraz University, Department of Biology, Faculty of Science.

Extraction of the plant material. Dried and powdered plant material (1.6 g) was suspended in 16 ml of absolute ethanol and sonicated two times for 1 min at room temperature. Extraction was continued by the addition of 144 ml of ethanol 67% and sonicated at 70˚C for 10 min. The extract was concentrated under reduced pressure by rotary evaporation below 45˚C and then lyophilized to yield dried powder. The yield of dried extract as percentage weight of starting dried plant material was 24.187%.

Preparation of human peripheral blood mononuclear cells and cell lines. Mononuclear cells were isolated from healthy volunteers by ficoll gradient centrifugation. Mononuclear cells were washed 3 times with RPMI 1640 and then resuspended in the same medium supplemented with 10% heat inactivated fetal calf serum at concentration of 1 x 10^6 cells/ml.

Cells were activated with 10µg/ml phytohemagglutinin (PHA) and cultured in a 96 well micro plate. Adherent and suspension cell lines including *Solid tumor cell lines*: MDA-MB-453(Breast Adenocarcinoma, ER-), MCF-7 (Breast Adenocarcinoma, ER+), SK-OV-3 (Ovary Adenocarcinoma), HeLa (Cervix Carcinoma), LNCap-FGC-10 (Prostate Adenocarcinoma), 5637(Bladder Carcinoma), A549 (Lung Adenocarcinoma), Mehr-80(Large Cell Lungcarcinoma) and *Hematopoietic Cell lines*: Jurkat (Acute Lymphoblastic Leukemia), RPMI 8866 (EBV Transformed B Cell Lymphoma), RAMOS (Burkitt's Lymphoma), U937 (Histiocytic lymphoma), were seeded in 50 µl plastic culture flasks in RPMI 1640 or DMEM media supplemented with 10% heat inactivated fetal bovine serum, 100µg/ml streptomycin, 100 IU/ml penicillin and incubated at 37˚C in a 5% CO_2 incubator.

WST-1 cytotoxicity assay. The assay was based on the cleavage of the tetrazolium salt WST-1 (Roche, Germany) producing a soluble formazan salt. 96 well tissue culture micro plates (Nunc, Denmark) were seeded with 90 µl medium containing cells in suspension. Different concentrations of the extracts, positive controls and solvent controls (PBS/Ethanol) were added to the cells in 10 µl volumes in triplicates. The final concentration of extracts in each well was reached to 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, 15.625µg/ml and 7.813µg/ml. After 48 hrs of incubation in a CO2 incubator (37˚C), 10 µl of WST-1 was added to the test wells and the plate was further incubated in 37˚C for 4 hrs. The absorbance was then determined in 450 nm and a reference wavelength of 630 nm by an ELISA spectrophotometer (Anthos 2020, Austria). Viability percent was calculated from the following equation, and then IC_{50} values were mathematically interpolated:

$$\frac{\text{Mean OD of test wells} \times 100}{\text{Mean OD of control (-) wells}}$$

Flow cytometry analysis for cell cycle distribution. RAJI cells were seeded in a 24-well plate and treated with 200µg/ml, 100µg/ml and 50µg/ml of *R. graveolens* extract for 48hr. After washing with cold PBS, approximately 1x10^6 cells were suspended in 500 µl cold Hypotonic solution containing 50µg/ml PI (Sigma, USA), 0.1% Triton X-100 (Merck, Germany), 100µg/ml RNase(Sigma, USA) and 0.1% sodium citrate solution. Tubes were placed in dark at 4˚C. Analysis was made between 20min and 2hr of incubation by Flow cytometry (FACSCalibure, Becton Dickinson, USA). Results were analyzed by Cell Quest program.

Statistical analysis. The statistical significance of the data was determined by One-way ANOVA test using SPSS software version 10. P-values less than 0.05 were taken significant.

Results

The inhibitory effects of *R. graveolens* extract was determined by exposure of human cancer cell lines to increasing concentrations of the extract for 48 hr. Results were expressed

Table 1: IC_{50} values for *R. graveolens* against tumor cell lines

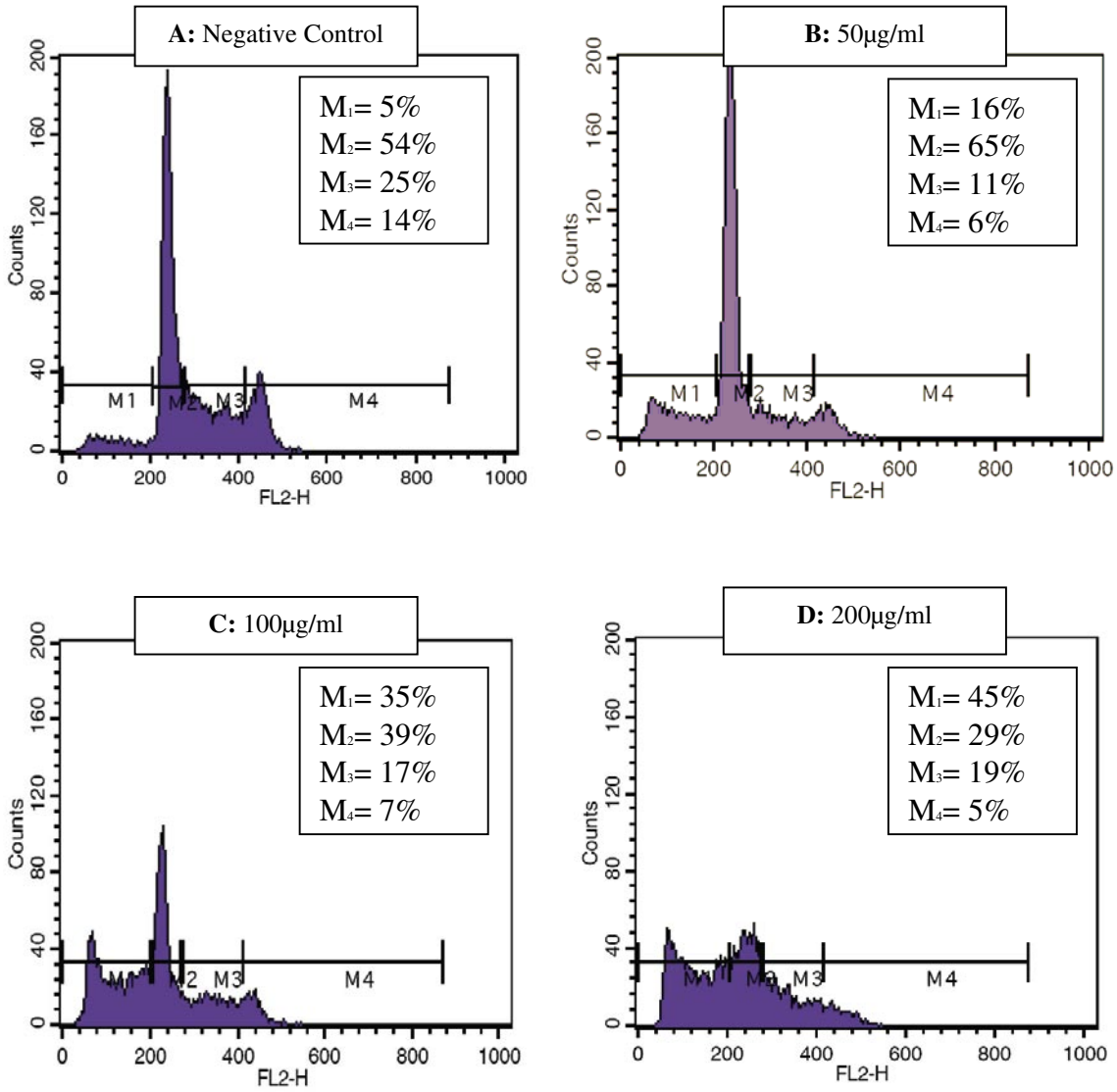
Cell Line	IC_{50} values(µg/ml)	
	R.graveolens	**C+**[a]
Jurkat	127.0+22.0	<7.813
RPMI8866	107.6+13.6	<7.813
RAMOS*	35.1+4.2	<7.813
RAJI*	24.3+3.8	<7.813
U937	146.0+ 29.1	<7.813
MDA-MB-453	403.2+55.4	<7.813
MCF-7	>500	<7.813
HeLa	225.8+48.5	<7.813
SKOV-3	>500	<7.813
LNCap-FGC-10*	27.5+1.1	<7.813
5637	429.5+34.7	<7.813
A549	397.8+18.2	<7.813
Mehr-80*	46.2+6.7	19.6
PBMC[b]	104.1+15.9	-

a: Positive controls including doxorubicin (for Jurkat, RPMI8866,RAJI, RAMOS, U937, LNCap-FGC-10, SKOV-3) , vinblastine (for MDA-MB-453, MCF-7, HeLa, 5637) and etoposide (for A549)

b: Peripheral Blood Mononuclear Cells

* Statistical significance compared with Negative control (p<0.05). *R. graveolens* extract was significantly cytotoxic against RAMOS, RAJI, LNCap-FGC-10 and the newly established cell line, Mehr-80.

Fig. 1 Effect of *R. graveolens* extract on DNA distribution pattern of RAJI cells**.** Propidium iodide (PI) fluorescent intensity was measured using flow cytometry. Histograms *A-D* show cell cycle pattern of cells treated with 0, 50, 100 and 200µg/ml of the extract for 48h respectively. The proportion of cells with sub-G1 DNA content (M1) increased significantly up to 9 fold at 200µg/ml compared with negative control.



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as $IC_{50,}$ the concentration of the extract that inhibited cell proliferation up to 50% of the negative control [Table 1].

The highest antiproliferative effect of *R. graveolens* extract was observed on RAJI and RAMOS cell line with an IC_{50} equal to 24.3µg/ml and 35.1µg/ml which are significantly lower than the resulted IC_{50} for Jurkat, RPMI8866 and U937 equal to 127µg/ml, 107µg/ml and 146µg/ml respectively ($p<0.05$).

The treatment of LNcap-FGC10 and Mehr-80 cell lines with the hydro-alcohol extract resulted in a significant reduction in cell number and IC_{50}s equal to 27.5µg/ml and 46.2µg/ml obtained respectively. No significant inhibition of proliferation occurred on other solid tumor cell lines.

Normal peripheral blood mononuclear cells were used as controls to be compared with haematopoietic tumor cell lines. The IC_{50} obtained for PBMCs was 104.1µg/ml while this value was equal to 24.3µg/ml and 35.1µg/ml for the most affected haematopoietic cell lines, RAJI and RAMOS.

R. graveolens extract was examined for cell cycle changes using PI staining. Treatment of RAJI cells with 200µg/ml, 100µg/ml and 50µg/ml of the extract increased the proportion

of sub-G1 cells compared with the negative control, which was equal to 54.7%, 24.7% and 9.1% respectively (Fig 1).

Discussion

There are reports on various biological activities of *R. graveolens* in literature. Rutin which is the main active compound isolated from *R. graveolens* has been studied for different in vivo and in vitro effects such as protection against nuclear exposure and capillary bleedings [6]. Extract of *R. graveolens* has exerted antimicrobial effects against *salmonella* sp. and a diluted tincture called Ruta 6 has show anti-tumor activity in patients with brain cancer [8]. Cytotoxic and apoptosis inducing effects of two furanoacridone alkaloids isolated from *R. graveolens* have also been studied against HeLa, MCF-7 and A431 [10]. In contrast to the high cytotoxic potency of the two isolated alkaloids reported against HeLa and MCF-7, in this study the crude sonicated extract of this plant had roughly no cytotoxicity against these two cell lines. This might have been due to the low concentration of furanoacridone alkaloids in this plant. An extract from *R. graveolens* has been reported to have significant anti-inflamatory effect in murine macrophage cells [9]. In this study we investigated the cytotoxic properties of the hydro-alcohol extract of *R. graveolens* on normal peripheral blood mononuclear cells (PBMCs) in order to compare this effect with the cytotoxicity of the plant extract against the haematopoietic cell lines, RAJI and RAMOS. Interestingly, a significantly lower effect was observed against PBMCs in comparison with these two cell lines ($p<0.05$).

In accordance with resulting IC_{50} values for *R. graveolens* extract against different tumor cell lines, a significant anti-proliferative effect of the extract observed on RAJI and RAMOS which are Burkitt's lymphoma cell line among haematopoietic cell lines and LNCap-FGC-10, a prostate adenocarcinoma cell line as well as Mehr-80 which is a newly established Large Cell Lung Carcinoma among solid tumors [11] ($p<0.05$), while roughly no inhibitory effect was seen on the other lung cancer cell line, A549, which is originally an adenocarcinoma cell line. There has been no previous report on the cytotoxic and anti-proliferative effects of *R. graveolens* against above tumor cell lines. In general, the inhibitory effect of the extract was higher against haematopoietic cell lines compared with solid tumor cells except for LNCap-FGC-10.

According to the results of cell cycle analysis, *R. graveolens* extract caused profound increase in the number of cells with the sub-G1 DNA content. This could be suggestive of the fact that cytotoxic activity of this plant has been due to its apoptosis inducing properties; however, further investigations are required to confirm and determine the exact mechanism of the inhibitory effect of this plant.

As a conclusion, these data suggest that there is/are active constituent(s) in *R. graveolens* with anti-proliferative effect against a Burkitt's Lymphoma, a prostate adenocarcinoma and a LCLC cell line, While our results shed light on the significantly lower cytotoxicity of this plant extract against normal blood cells.

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