Original Article

Flow Cytometry Analysis of Rosa Damascena Effects on Gastric Cancer Cell Line (MKN45)

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Abstract

Background: Gastric cancer is a life threatening disease. Recent studies have shown that Rosa Damascene possesses noticeable biological effects on the human body. This study, investigates the anticancer effect of Rosa Damascena on gastric cancer cell line MKN45.

Methods: Microscopic studies and MTT assay were used to examine morphological alteration, and cell survival of the cancer cells while exposing to differentvolumes of the essential oilrespectively. In addition, flow cytometry was applied to determine the cell death mechanism of the gastric cancer treated with the essential oil.

Results: The findings indicate that Rosa Damascena essential oil affects gastric cancer cells in two distinctive ways: the soluble phase increases cell viability, while the vapor phase decreases cellsurvival. Moreover, flow cytometry analysis revealed that apoptosis is the main mechanism accompanied with cell death.

Conclusion: Consequently, Rosa Damascena essential oil can be used as a potent anticancer agent in the future. However,more evaluation of this essential oil is still needed to elicit its effectivebiological activities.

Keywords: Cell line; Essential oils; Rosa Damascene; Anticancer properties; Flow cytometry

Please cite this article as: Khatib H, Rezaei-Tavirani M, Heidari Keshel S, Zamanian Azodi M, Omidi R, Biglarian M, Sobhi S. Flow Cytometry Analysis of Rosa Damascena Effects on Gastric Cancer Cell Line (MKN45). Iran J Cancer Prev. 2013; 6(Suppl):30-6.

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Received: 8 Nov. 2012
Accepted: 25 Dec. 2012
Iran J Cancer Prev 2013; Suppl: 30-6

Introduction

Gastric cancer is the second most common type of cancer around the world. The prevalence of gastric cancer in developing countries is very high [1]. In Iran it is the most prevalent cancer among Iranian males and more fatal cancer in both sexes [2].While the rate of gastric cancer is slipping, it is still remained a major health problem, and a widespread cause of cancer mortality globally [3, 4].In addition, it is also reported that the rate of this malignancy is higher in men than women.Gastric adenocarcinoma is a malignant epithelial tumor which is derived from glandular epithelium of the gastric mucosa; and it comprisesaround 90% of all gastric cancers. Development of gastric cancer is assumed to be a slow process with primary etiological determinants for gastric cancer being exposed to various etiological factors [5] such as chemical carcinogens and/or infection with Helicobacter pylori;this bacterium has been known as the major risk for the development of gastric cancer for inducing chronic gastritis [6, 7]. However, this malignancy is a multistep process, andthe molecular mechanism of itsincident and process has been remained elusive. There are different types of treatment available for this cancer therapy. Chemotherapy or radiation can improve symptoms and may lengthen survival, but they are notcurative. Additionally, surgery is another option which is the only best one, but it works just for patients with small and early cancer lesions[8, 9]. Unfortunately, most patients are diagnosed at an advanced stage; consequently, have a very low five-year survival rate (less than 10%)[7,10].As there are high side effects accompanied with common therapies available for gastric cancer, other eligible sources

are needed to be evaluated. Rosa damascena as a one of the recent studied plant has shown promising biological activities [11-14]. It is one of the most important species of Rosaceae family. Presently, over 200 rose species and more than 18000cultivars form of the plant have been identified[15].It has been reported that essential oil of this plant has different properties such as antimicrobial, antidiabetic, anti-HIV and anticancer activities [15-17]. Recent studies have reported that this potent plant has effective anticancer properties against colon cancer cell line.

Since there is not a solid cure for malignant cancers such as gastric cancer, the aim of this study was to decipher the probable anticancer activity of Rosa Damascene, and its mechanism on gastric cancer via flow cytometry.

Materials and Methods

Essential Oil Distillation

A natural drying method was applied for drying the plant. It was dried at room temperature for 48 hours. Distillation is one of the most common methodsfor purification. It means to separate thematerial into one, two or more different materials.These compounds have different evaporation time.The essential oil was extracted by a different apparatus - a steam distillation method (designed by Jaimand- Rezaee)[18].

Cell Culture

The cell culture medium (RPMI), Fetal Bovine Serum (FBS), penicillin and streptomycin were provided by Gibco BRL (Life Technologies, Paisley, Scotland). MKN45 cell line was obtained from cell bank (Pastuer Institute, Tehran, Iran). Moreover, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT), Annexin V-FLOUS staining kit (Cat. No. 11 988 549 001) were provided from Roche Diagnostics GmbH (Germany). The adenocarcinoma gastric cell line (MKN45) was cultured in the RPMI medium that had been treated with FBS (10%, v/v), streptomycin (100 µ g/mL), and penicillin (100 U/mL). Cultures were maintained at 37°C in 5% CO2 and 95% air, and the medium changed two times for each week.

Microscopic Study

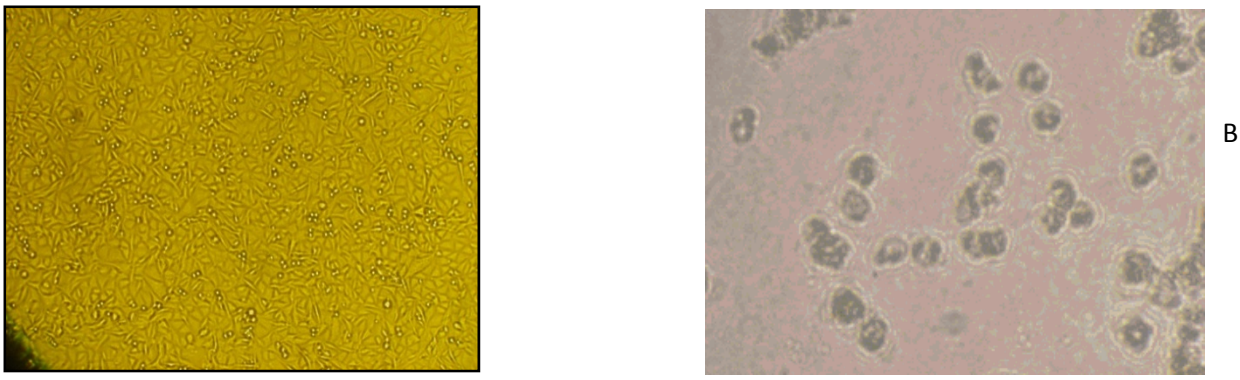
In order to compare the cell morphology and pattern of cell distribution in the absence (without essential oil) and presence of the essential oil, an inverted microscope (Celti) was used.

MTT Assay

The cells were seeded in the 96 well plates and incubated at 37 °C under 5% CO2 atmosphere for 48 hours. After that, the various concentrations of essential oil (1, 5, 10, 20, 40, and60µl) and 0µl as control pattern were induced into cells for 48 hours for MTT assay proposes. The inhibition of cellular proliferation was determined by the modified MTT 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay based on the ability of live cells to convert thiazolyl blue to dark blue formazan[19]. After treatmentof MKN45 cells with essential oil of Rosa Damascene for 48 hours, 20 ml MTT (5 g/l) was added into the wells and incubation continued at 37°C for 4 hours, and 100 ml DMSO was pipetted to solubilize the formazan product for 30 min at room temperature. The absorbency at 570 nm was measured using ELISA reader. Percent of Cytotoxicity = (1 - mean absorbance of toxicant - treated cells) × 100 Mean absorbance of negative control Percent Viability = 100 – percent of Cytotoxicity

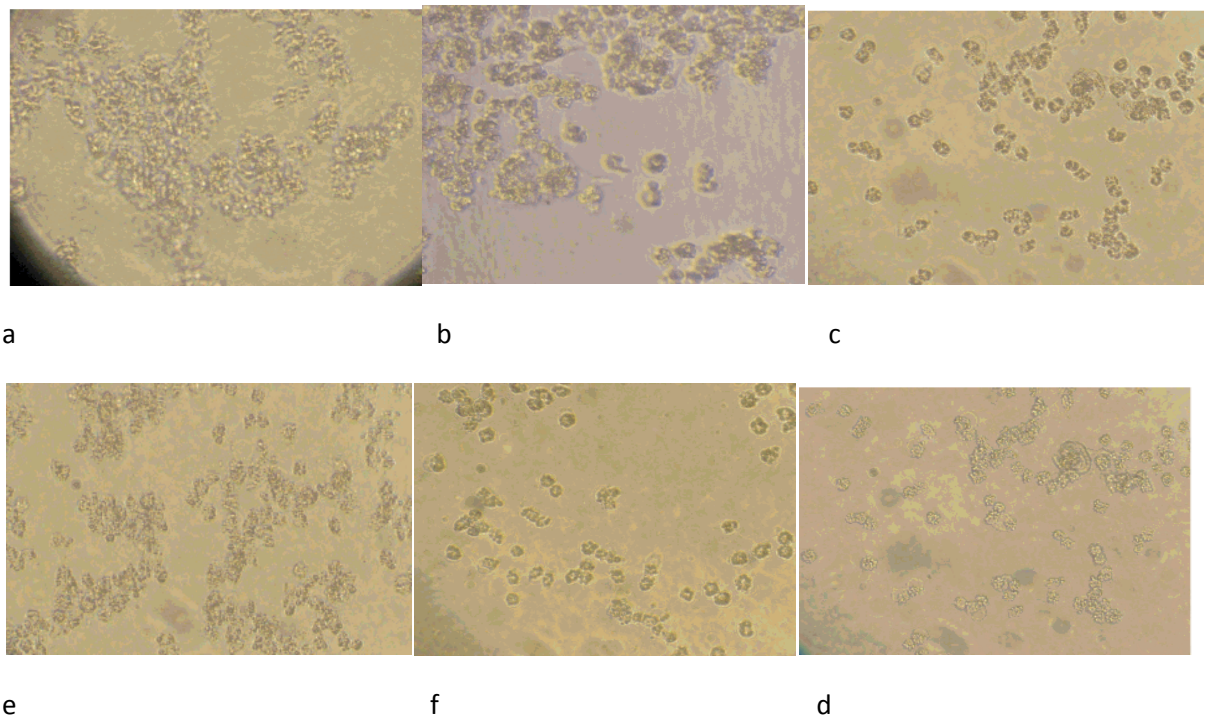
Flow Cytometry Analysis

Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection andprotein engineering via suspending cells in a stream of fluid and passing them by an electronic detection apparatus [20]. It deals out simultaneous multi-parametric analysis of the physical and/or chemical distinctiveness of up to thousands of particles for each second. For flow cytometry analysis, MKN45 cells were cultured into 6-well plates at a density of 1 × 106 cells in the presence and absence of the cytotoxic agents for 48 hours. All floated and adherent cells were harvested and centrifuged at 200 ×g for 10 min. Cell pellet was washed with 1X phosphate buffer saline solution and centrifuged at 200 ×g for 10 min. The cell pellet was then re-suspended in 100 □L of Annexin V/FLUOS labeling solution (predilute 20□LAnnexin V/FLUOS labeling reagent in 1 mL incubation buffer and add 20 □L propidium iodide solution), and incubated at 15-25 °C for 10-15 min. It was then employed to analyze the cell population evaluated by Flow Cytometer (Bio-Rad, USA). In this experiment, the cells were aspirated by PBS, and then 1×106, MKN45 cells were used. The samples were read in a FACS flow cytometer (USA) using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter > 600 nm for propidium iodide revealing. Analyses were done by Cell Quest software supplied in the instrument.



A B

Figure 1. MKN45 cell line morphologyA) in the absence of essential oil as outer control (the cells are cultured in the another plate) and B) in the absence of essential oil as Inner control (the cells are cultured in the plate included treated cells)



a b c

e f d

Figure 2. MKN45 cell line treated with different dosages of essential oil a) 1,b) 5, c) 10,d) 20, e) 40 and f)60 µl after 48 hours incubation

Results

Microscopic studies have been used to examine morphological changes in MKN45 cell line treated with the essential oil (Figure 1 and 2).

Viability evalution of MKN45 in the peresece of various dosages of Rosa Damascenehas been done by MTT assay (Figure 3).

As it is depicted in Figure 3, the vapor phase of essential oil affects inner control, so viability is decreased to less than 10%. Here, viability is calculated by considering inner control as 100% and the data are presented in Figure 4.

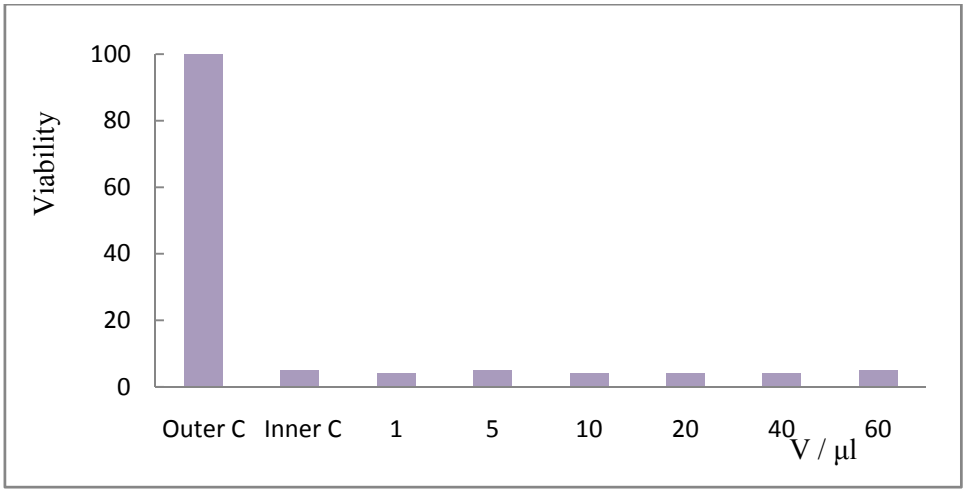


Figure 3. Viability test of various volumes of essential oilon MKN45 cell line. It shows that as the volume of the essential oil increases from 1 to 60 □l and also for inner control the viability of the cells follows steady trend in the manner of reduction (P value ≤ 0.001).

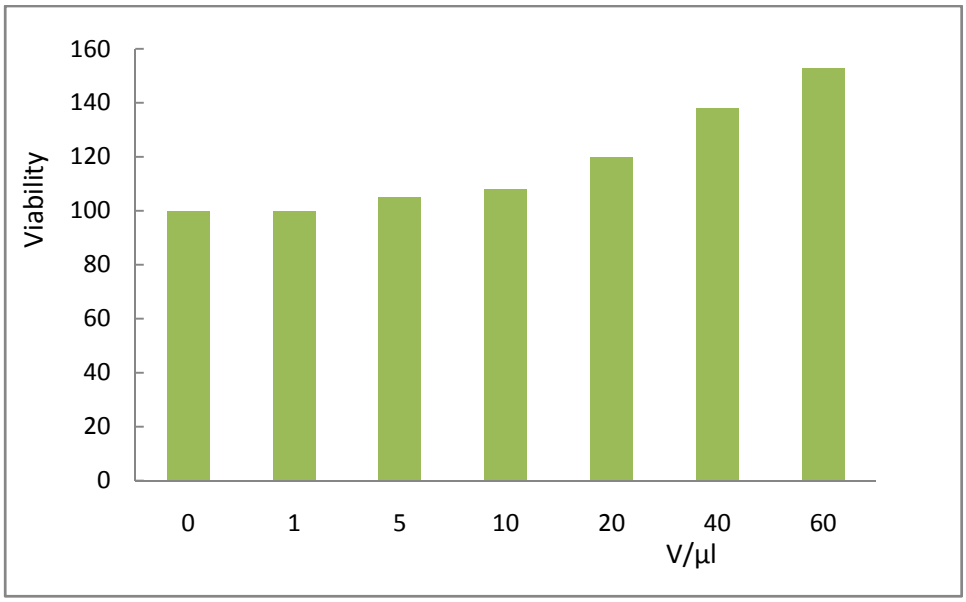
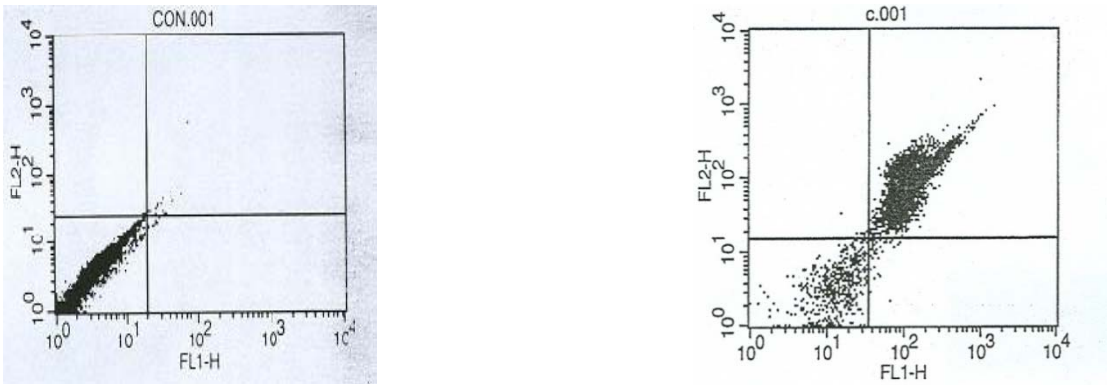


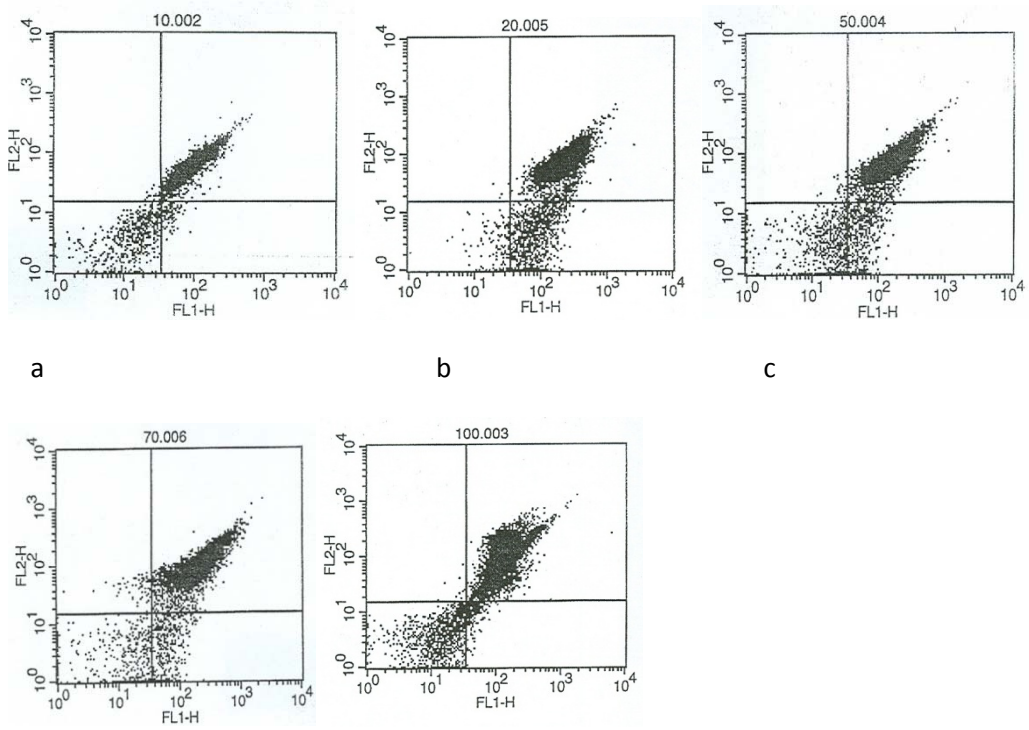
Figure 4. Effect of different doses (0, 1, 5, 10, 20, 40, 60 □l) of Rosa Damascene essential oil on MKN45 cell line (viability is calculated by considering inner control as 100%). Viability in the presence of 20, 40 and 60 µl is increased correspond to Pvalues ≤ 0.01, ≤ 0.001 and ≤ 0.001 respectively.

Flow cytometry method was used for cell death mechanism evaluations. Alive cells were observed in the Lower Left part [LL]. Cells that are Annexin V-FITC (+)/PI (-) [LR] are apoptotic and are seen in the lower right. Annexin V-FITC (-)/ PI (+) [UL] may be bare nuclei, cells in late necrosis, or cellular debris (Upper Left).The cell population with Annexin V-FITC (+)/PI (+) [UR] has been indicated as necrotic or advanced apoptotic (Upper Right)(Figure 5 and 6).



A B

Figure 5. Flow cytometry analysis of MKN45 cell line in the absence of essential oil of Rosa Damascene A: Outer control, B: Inner control



a b c

d e

Figure 6. Flow cytometry analysis of MKN45 cell line in the presence of different volumes of Rosa Damascene essential oil on MKN45 cells: a) 5, b) 10, c) 20, d) 40, and e) 60 µ l after 48 hours incubation

Discussion

Gastric cancer is one of the most prevalent malignancies after lung cancer worldwide, and its incidence in developing countries has been raised recently [8]. Owing to high prevalence, and less efficiency of common treatments, the study of this life threatening disease is crucialas its underling natural source is accounted for fewer side effects. Rosa Damascenas as one of the most recent studied plant is possessing different biological properties [11,12,21,22]; therefore, it could be an eligible candidate for more studies.This study focuses on Rosa Damascenas essential oil activities on gastric cancer. Morphological studies are important to understand visualized changes of the cells treated with the essential oil.Outer and inner controls are

depicted in Figure 1. In Figure A, outer control with high population of cells was cultivated in a separated plate, whereas in Figure B viability of inner control was influenced by strong cytotoxic effects of the vapor phase of the essential oil. As can be inferred from Figure 2, different dosages of none-soluble phase of the essential oil (vapor phase) have cytotoxic activities. As the volume increases, the viability of the gastric cells decreases dramatically. MTT assay is a technique to evaluate cytotoxic properties of the drug [23]. Here, observed in Figure 3, MTT assay was applied to determine the effect of the herbal essential oil against gastric cancercell proliferation and death. Different volumes of this essential oil have significant cytotoxic properties which are merely related to its vapor part. For better resolution,viability was calculated by considering inner control as 100% (Figure 4). Since the vapor phase has similar effect on all cells, (Figure 4) water-soluble part of Rosa Damascena oil increases cell proliferation of MKN45 cell line in high dosages in which the influence of vapor part is omitted. It can be concluded that there are at least two different reagents with opposite effect in the essential oil, the soluble factor that evokes proliferation and the other that inhibits cell proliferation. It is recognized that the dynamic balance between cell proliferation and apoptosis is very important to maintain the homeostasis in the human body which can be influenced by cancer development [24]. Flow cytometry is a reliable technique for assessing cell death mechanism [20]. Figure 5illustrates that how vapor part of the essential oil decreases cell viability of gastric cancer cell line that are not treated with the essential oil (the inner control) versus the outer control. Moreover, apoptosis is the main mechanism of the cell death in inner control. Furthermore, as it is shown in Figure 6, we have observed that various dosages of the essential oil have positive effects on MKN45 cell line, and the cytotoxicity of the essential oil on MN45 cells occurs through apoptosis.

Conclusion

In sum, Rosa Damascena can be considered as ananticancer agent. However, more investigation is needed for underlining the exact cytotoxic effects of this plant. Furthermore, the soluble part of essential oil included a cell growth factor that can be investigated separately. It is suggested that vivo studies would be done to assure the probable anticancer properties of Rosa Damascena essential oil.

Acknowledgment

This research has been supported by Proteomics Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Conflict of Interest

The authors have no conflict of interest in this article.

Authors' Contribution

Sara Sobhi and Mostafa Rezaei-Tavirani designed the study, Hanieh khatib analyzed the data, Saeed Heidari Keshel contributed to study design and analysis, Mona Zamanian Azodi contributed to the data entry, and wrote the paper, Roghiyeh Omidi and Mohsen Biglarian contributed to literature review and writing-up process. All the authors read and approved the final manuscript.

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