ORIGINAL ARTICLE

Cytotoxic and genotoxic studies of essential oil from Rosa damascene Mill., Kashan, Iran

Mohammad Shokrzadeh[1], Emran Habibi[2], Mona Modanloo[3]

[1]Department of Toxicology-Pharmacology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, [2]Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences; Sari, Iran , [3]Pharmaceutical Sciences Research Centre, Student research committe Mazandaran University of Medical Sciences, Sari, Iran

ABSTRACT

Aim *Rosa damascene* Mill. belongs to the family of *Roseaceae* and its essential oil is produced in large amounts in Iran. The wide application of rose oil has raised questions about potential adverse health effects. We have investigated cytotoxic activity and genotoxic effects of Rosa oil from Kashan, Iran.

Methods The cytotoxic effect and IC50 of the essential oil on the cell lines was studied followed by MTT assay. In this assay mitochondrial oxidoreductase enzymes with reducing the tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reflect the number of viable cells. Genotoxic effect of the oil was evaluated by micronucleus assay by evaluating produced micronuclei due to cytogenetic damage in binucleated lymphocytes.

Results The results showed that essential oil significantly had cytotoxic and genotoxic effects at doses over 10μg/mL ($p<0.05$). Also, essential oil of Rose showed lower IC50 in cancer cell line (A549) in comparison with the normal cell line (NIH3T3).

Conclusion Cytotoxic and genotoxic properties of essential oil of Rose in Kashan, Iran, are safe at a dose of 10μg/mL. Also, a good cytotoxic effect was shown and could be introduced as an anticancer compound. Further studies are needed with regard to anti-cancer effects of Rose essential oil.

Key words: micronucleus assay, MTT, Rose oil

Corresponding author:
Mona Modanloo
Pharmaceutical Sciences Research Center, Student research committee, Mazandaran University of Medical Sciences,
Khazarabad Road, 48175-861 Sari, Iran;
Phone: +98 911 1520 410;
Fax: +981133543084;
Email: dr_modanloo@yahoo.com
Mohammad Shokrzadeh ORCID ID: http://www.orcid.org/0000-0002-0071-6530

Original submission:
28 February 2017;
Revised submission:
15 March 2017;
Accepted:
09 May 2017.
doi: 10.17392/901-17

Med Glas (Zenica) 2017; 14(2): 152-157

INTRODUCTION

Iran has a long history in cultivation and consumption of *Rosa damascena*, and it is known as an important producer of rose oil in the world (1). *Rosa damascena* Mill. belongs to the family of *Roseaceae* (2). This ornamental is not only known as one of the most valuable sources of flavours and fragrances in the world, but also it has some applications in medicine and food industry (3).

Some evidence showed that Rose oil has some beneficial effects in the treatment of various diseases like premenstrual breast tenderness, inflammatory reactions, gall care and spasms (4).

It also seems to have antidepressant and relaxing effects. It is helpful for long-lasting cough, wound healing, allergies and severe headache (3).

Some evidence reported various potential adverse effects of *Rosa damascene* (5-7). On the other hand, a new study showed that while it is cytotoxic in high doses, it did not show genotoxic effects (8).

The MTT test is an appropriate assay that has often been used to investigate cytotoxicity caused by medical plants (6). It is a rapid, low-cost method based on the reduction of yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), to dark blue formazan in mitochondria of active cells (9). Thus, the amount of produced formazan has a direct relation with viable cells (10).

The micronucleus assay in binucleate human lymphocytes is an effective tool to measure cytogenetic damage of agents with different mechanisms of genotoxicity in vitro (11). Recent studies have shown that genomic instability is an early event that occurs in some malignancies and it can be detected by examination of the peripheral blood lymphocytes as a sample of precursor cells (12).

Different studies reported some differences in the composition of Rose oil in various regions (8, 13, 14).

Due to high application of Rose essential oil and with regard to the fact that Kashan is one of the biggest producers of Rose oil in Iran, this study aimed at assessing cytotoxicity of Rosa damascene Mill.'s essential oil on both normal and cancer cell lines using MTT assay and also evaluating its genotoxicity on human blood lymphocytes using micronuclei assay.

MATERIALS AND METHODS

Essential oil distillation

Flowers were picked by hand before sunrise in May 2015 in the city of Kashan (Vidorj region), Iran. Botanical identification was confirmed by morphologic characteristics at the Department of Pharmacognosy, Sari Faculty of Pharmacy. The flowers were subjected to steam distillation within the same day (400gr fresh flowers in 2.0 L water). After 3h hydrodistillation the obtained oil was dried using anhydrous sodium sulphate. Pure essential oil was stored at 4°C in a dark place (1).

Dimethyl sulphate (DMSO) was used to dissolve essential oil (the final concentration of DMSO was not over 1%) (14).

Cell culture

Experiments were carried out with cell lines NIH3T3 (non-tumour fibroblast) and A549 (human NSCLC cell line) (Pasteur Institute of Iran, Tehran, Iran).

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Germany) with 10% fetal bovine serum (Gibco-BRL, Germany) and 100µg/mL streptomycin (Gibco-BRL, Germany) and 100IU/mL penicillin (Gibco-BRL, Germany). Cell cultures were adjusted to allow for exponential growth.

MTT assay

The protocol was adapted from the method described by Shokrzadeh et al. (15).

Cells (10^4 cells) were cultured with 200 µL DMEM/F12 medium containing bovine serum in 96 wells plate and incubated in 37 °C for 24 hours.

Stock solutions of Rose oil and cisplatin (a platinum coordination complex with potent anti-neoplastic activity induces apoptosis in cancer cells, possibly via caspase-3 activation) (16) were prepared in DMSO (1%) and phosphate buffered saline (PBS), respectively.

After 48 hours of cell incubation with different doses of essential oil (1, 10, 50, 100, 150 and 200 µl), 20µL MTT solution (5 mg/mL) was added to each well. After 4 hours incubation at 37°C, the formazan was dissolved in DMSO. Finally the optical density (OD) of wells was measured on a micro plate ELISA reader at 570 nm. All expe-

riments were performed twice and each experiment was run in triplicate, and mean values were recorded.

A linear relation between cell viability and OD of each well is an exact determination of cell proliferation (17). The percentage of cell viability was calculated using the equation (18): [Mean (OD) of treated cells/mean OD of control cells (1% DMSO)]×100

Micronucleus assay

Fresh blood was collected from 10 healthy, no smoking, no alcoholic, male donors aged between 25-35 years by venepuncture in heparinized falcons; 0.5 mL of whole blood was added to 4.5mL of Roswell Park Memorial Institute (RPMI) culture medium 1640 supplemented with fetal bovine serum containing L-glutamin, antibiotics and phytohemagglutinin (PHA), and different doses of Rose oil (1, 10, 50, 100, 150 and 200 µL) were added. Cytochalasin B (Cyt-B) (Sigma, MO, USA) at the final concentration of 6µg/mL was added at 44h post PHA stimulation.

Cyt B prevents complete cytokinesis in mitosis, thus causing an appearance of multi-nuclear cells (19).

The binucleated lymphocytes were harvested 28 hour after adding Cyt-B, they were treated by hypotonic KCl (0.075M) to red blood cell (RBC) lysis. Then fixative solution (methanol: acetic acid= 6:1) was added to the cells prior to slide preparation and staining. For slide preparation 2-3 drops of cell suspension were thrown on a clean slide. The slides were stained with Giemsa solution (4%) for 7-10 mins. They were observed at 40× and 100× magnifications using a light microscope to estimate mitotic index (the cells with 2 or more nuclei per 1000 observed cells) and micronuclei frequency (the number of micronuclei in at least 1000 binucleated cells) (8,20) binucleated cells are lymphocytes that were once divided by mitosis (21). The experiment was performed twice. Mitotic Index has a direct relation with cells' proliferative activity (8,22).

Statistical analysis

One way analysis of variance and tukey's honestly significant differences (HSD) test were used for multiple comparisons of data. A p value less than 0.05 was considered as significant. The IC50 (half maximal inhibitory concentration) values were calculated by PRISM software using nonlinear regression. Standard deviations represent average results of double experiments. The IC50 values were compared using the student's T-test measuring the effectiveness of a substance to cause cell death or inhibit cell growth. So the lower amount of IC50 represents a higher toxicity of a compound, which leads to death or inhibition of cell growth (23).

RESULTS

During this study 0.2 mL essential oil of 1200 gr Rose flowers was produced. Comparing the results in both MTT and micronucleus tests, data showed no significant difference between DMSO control group and the control without DMSO (Figures 1-4).

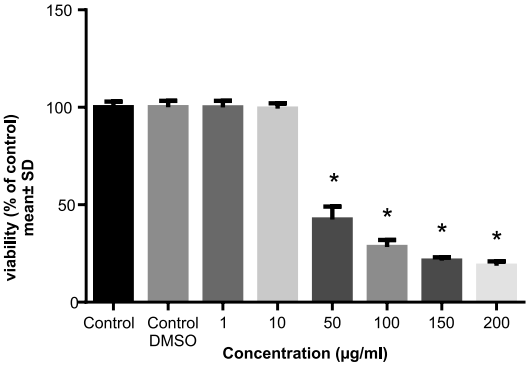

Figure 1. Effect of *Rosa damascene* essential oil on A549 cell viability *significant difference compared to the control group ($p<0.05$)

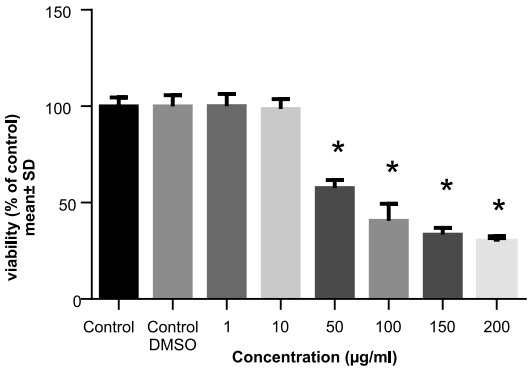

Figure 2. Effect of *Rosa damascene* essential oil on NIH3T3 cell viability *significant difference compared to the control group ($p<0.05$)

The MTT test with increasing doses of oil showed a decrease in viability in both normal and cancer cells, A549 and NIH3T3, respectively (Figures 1, 2). It seems that the dose of 1 and 10 µg/mL in both

Table 1. Micronuclei frequency in different doses of rose essential oil, normal control and colchicine treated cultures

Micronuclei frequency	Control	Control (1% DMSO)	Concentration (µg/mL) 1	10	50	100	150	200	Colchicine 0.1µM
Mean±SD (% of control)	0.70±0.80	0.60±0.69	0.80±0.62	1.00±0.79	5.30±1.22	5.80±1.15	6.40±1.31	8.20±1.80	10.30±2.25

cell lines did not observe toxic effects (the absence of a significant difference with the DMSO control group). However, at higher doses there was a significant difference between the group affected by rose oil and the control group ($p<0.05$) (Figures 1, 2).

The IC50 values were significantly different in the A549 and NIH3T3 cell lines between rose oil (36.43±3.373 and 42.93±0.502, respectively) and cisplatin (8.068±2.670 and 16.67±2.212, respectively) groups ($p=0.0010$ and $p=0.0014$, respectively).

The effect of different doses of rose oil on the frequency of micronuclei in binucleated lymphocytes is shown in Table 1. While the frequency of micronuclei in concentrations of 1 and 10 µg/mL was not much different from the control group, the amount in higher concentrations, of 50-200 µg/mL, was significantly more increased than in the control group ($p<0.05$) (Figure 3).

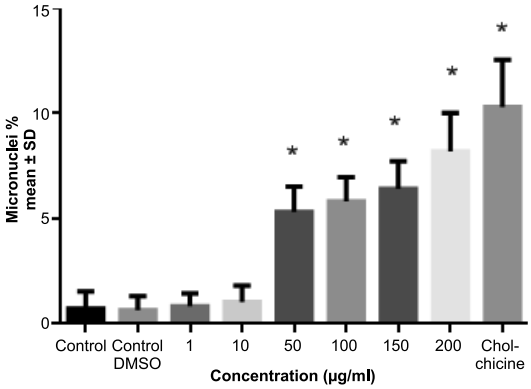

Figure 3. Micronuclei frequency in different doses of rose essential oil, normal control and cholchicin treated cultures
*significant difference compared to the control group ($p<0.05$)

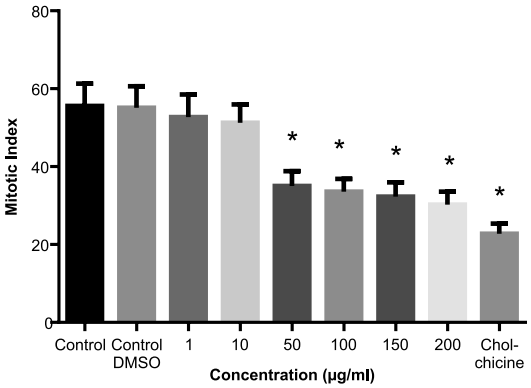

Figure 4. Mitotic index of peripheral blood lymphocytes in different doses of rose essential oil, normal control and colchicine treated cultures *significant difference compared to the control group ($p<0.05$)

The mitotic activity in the cells affected by the rose oil represents an obvious toxic effect at concentrations higher than 10 µg/mL ($p<0.05$ compared to control DMSO group) (Figure 4).

DISCUSSION

The results of this study indicate that the Rose essential oil of Kashan, Iran, at doses higher than10 µg/mL had obvious cytotoxic and genotoxic effects. The material in both normal and cancerous cell lines caused damage and cell death. Also, our findings showed that DMSO in the concentration of 1% had no significant effect on the cells. These findings are similar to previous studies (14).

Based on the results of the MTT test, the sensitivity of cancer cells to rose oil was significantly higher than that of normal cells. This may be due to cancerous cells malfunction, impairment disorders in immune cells process or increased permeability and absorption by them due to the high proliferation rate (24). In a recent study conducted in 2014 by Heba et al., phenyl ethanol blend in Rose essence is reported to have an anticancer activity (8). Rose oil is a matter of gross and includes various pharmaceutical compounds, with each of them having distinct effects (24).

Recent surveys showed that the presence of terpenes in essential oils is able to change the nature of the cell membrane (25). This disturbs the equilibrium concentration of intracellular electrolytes and ultimately causes cell death (26).

In the study of Loghmani-Khouzani et al. (14) carried out on Rose in Kashan, using gas chromatography/mass spectroscopy (GC/MS) of flower essential oil, more than 95 different compounds were identified; the most frequently identified components were β-citronellol (32.49%), nonadecane (23.99%), geraniol (18.12%) and henicosane (9.64%), followed by eicosane (1.29%), linalool (0.29%), methyl eugenol (0.55%) and many other compounds.

It seems that phenolic compounds in this oil through one of two mechanisms of interaction with energy-generating enzymes or protein denaturation leading to cell death (27). Geraniol is the main

ingredient in Rose and is a monoterpene alcohol which causes an increase in cell sensitivity to certain toxic substances by reducing the amount of thymidilatesyntase (TS) and thymidine kinase (TK) enzymes in colon cancer cells (8). Previous research has shown the essential oils by internal and external changes in mitochondrial membrane fluidity thus increasing their permeability; induced cell death by both apoptosis and necrosis (24). Also the inhibitory effects of methylated eugenol on some cancer cells have been reported (28).

The other results of our study included the ability of Rose essence in micronuclei creation induction in peripheral blood lymphocytes. Micronuclei induction is generally recognized as a factor for chromosome damage (19, 29).

In previous studies, the sensitivity of lymphocytes isolated from peripheral blood has been reported into chemicals more than the lymphocytes of the blood as whole blood. This is due to the presence of some protective factors in the blood and also other targets rather than lymphocytes are known for the chemical (19). Since all of these targets and protective factors are in the human body's peripheral blood, it was decided in this study to use whole blood cultures for further similarity of test conditions with the human body (19).

High rate of hydrocarbons and the presence of a minor amount of monoterpenes (linalool 0-0.29%) can prevent the activity of DNA gyrase enzyme thereby causing the genetic damage (30).

Gene toxicity is a very important factor in the safety of a substance because tumours can be caused by mutagenic substances (31). Genetic instability is not the only factor in many malignancies and genetic syndromes including Fanconi anemia, ataxia-telangiectasia and Warner syndrome (31).

In conclusion, since roses have very abroad application in the cosmetics, perfume, food and pharmaceutical industries from the past and considering the fact that this is the first time the cytogenetic safety of rose oil of Kashan as one of the most important producers of oil in the world was evaluated, based on the results of this study, a dose of 10 μg/mL and less is considered as the dose of confidence. On the other hand, despite the Rose oil's IC50 in cancer cell line was higher than the cisplatin's IC50, its value is considerably low because the lower IC50 the more power of sample in killing cell or inhibiting their growth (24). Accordingly, we can suggest that the Rose essential oil can be used as a complementary therapy in cancer.

ACKNOWLEDGMENT

The authors thank the Vice Chancellor for Research of the Mazandaran University of Medical Sciences for his cooperation and also the volunteers who participated in this study.

FUNDING

No specific funding was received for this study.

REFERENCES

1. Jalali-Heravi M, Parastar H, Sereshti H. Development of a method for analysis of Iranian damask rose oil: Combination of gas chromatography–mass spectrometry with Chemometric techniques. Anal Chim Acta 2008; 623:11-21.
2. Pellati F, Orlandini G, Leeuwen KA, Anesin G, Bertelli D, Paolini M, Benvenuti S, Camin F. Gas chromatography combined with mass spectrometry, flame ionization detection and elemental analyzer/isotope ratio mass spectrometry for characterizing and detecting the authenticity of commercial essential oils of Rosa damascena Mill. Rapid Commun Mass Spectrom 2013; 27:591-602.
3. Shafei MN, Saberi Z, Amini S. Pharmacological effects of Rosa damascena. Iran J Basic Med Sci 2011; 14:295-307.
4. Kaul VK, Singh V, Singh B. Damask rose and marigold: prospective industrial crops. Int J Med Arom Plants 2000; 313-8.
5. Rezaie-Tavirani M, Fayazfar S, Heydari-Keshel S, Rezaee MB, Zamanian-Azodi M, Rezaei-Tavirani M, Khodarahmi R. Effect of essential oil of Rosa Damascena on human colon cancer cell line SW742. Gastroenterol Hepatol Bed Bench 2013; 6.
6. Talib WH, Mahasneh AM. Antimicrobial, cytotoxicity and phytochemical screening of Jordanian plants used in traditional medicine. Molecules 2010; 15:1811-24.
7. Khatib H, Rezaei-Tavirani M, Keshel SH, Azodi MZ, Omidi R, Biglarian M, Sobhi S. Flow cytometry analysis of Rosa damascena effects on gastric cancer cell line (MKN45). Iran J Cancer Prev 2013; 6:30-6.
8. Hagag HA, Bazaid SA, Abdel-Hameed E-SS, Salman M. Cytogenetic, cytotoxic and GC–MS studies on concrete and absolute oils from Taif rose, Saudi Arabia. Cytotechnology 2014; 66:913-23.
9. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55-63.

10. Kiani M, Zamani Z, Khalighi A, Fatahi R, Byrne DH. A unique germplasm of Damask roses in Iran. Proceedings of the Vth International Symposium on Rose Research and Cultivation, Gifu/Japan, May 24-28 2009. Acta Hort (ISHS) 2010; 870:131-6.
11. Kirsch-Volders M, Decordier I, Elhajouji A, Plas G, Aardema MJ, Fenech M. In vitro genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models. Mutagenesis 2011; 26:177-84.
12. Maffei F, Moraga JM, Angelini S, Zenesini C, Musti M, Festi D, Cantelli-Forti G, Hrelia P. Micronucleus frequency in human peripheral blood lymphocytes as a biomarker for the early detection of colorectal cancer risk. Mutagenesis 2014; 29:221-5.
13. Yassa N, Masoomi F, Rankouhi SR, Hadjiakhoondi A. Chemical composition and antioxidant activity of the extract and essential oil of Rosa damascena from Iran, population of Guilan. DARU 2015; 17:175-80.
14. Loghmani-Khouzani H, Sabzi Fini O, Safari J. Essential oil composition of Rosa damascena mill cultivated in central Iran. Scientia Iranica 2007; 14:316-9.
15. Shokrzadeh M, Saravi SS, Mirzayi M. Cytotoxic effects of ethyl acetate extract of Sambucus ebulus compared with etoposide on normal and cancer cell lines. Pharmacogn Mag 2009; 5:316.
16. Fuertes MA, Alonso C, Pérez JM. Biochemical modulation of cisplatin mechanisms of action: enhancement of antitumor activity and circumvention of drug resistance. Chem Rev 2003; 103:645-62.
17. Greim H, Bury D, Klimisch H-J, Oeben-Negele M, Ziegler-Skylakakis K. Toxicity of aliphatic amines: Structure-activity relationship. Chemosphere 1998; 36:271-95.
18. Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. Carol Stream, IL: Allured publishing corporation, 2007.
19. Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. Mutat Res 1985; 147:29-36.
20. Shokrzadeh M, Ahangar N, Abdollahi M, Shadboorestan A, Omidi M, Payam SH. Potential chemoprotective effects of selenium on diazinon-induced DNA damage in rat peripheral blood lymphocyte. Hum Exp Toxicol 2013; 32:759-65.
21. Burt S. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol 2004; 94:223-53.
22. Shokrzadeh M, Chabra A, Naghshvar F, Ahmadi A. The mitigating effect of Citrullus colocynthis (L.) fruit extract against genotoxicity induced by cyclophosphamide in mice bone marrow cells. Scientific World J 2013; 2013.
23. Saravi SS, Shokrzadeh M, Shirazi FH. Cytotoxicity of Sambucus ebulus on cancer cell lines and protective effects of vitamins C and E against its cytotoxicity on normal cell lines. Afr J Biotechnol 2013; 12.
24. Hussain AI. Characterization and biological activities of essential oils of some species of Lamiaceae. University of Agriculture, Faisalabad, 2009; Ph. D. Thesis.
25. Oussalah M, Caillet S, Lacroix M. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of Escherichia coli O157: H7 and Listeria monocytogenes. J Food Prot 2006; 69:1046-55.
26. Marrufo T, Nazzaro F, Mancini E, Fratianni F, Coppola R, De Martino L, Agostinho AB, De Feo V. Chemical composition and biological activity of the essential oil from leaves of Moringa oleifera Lam. cultivated in Mozambique. Molecules 2013; 18:10989-1000.
27. Tiwari BK, Valdramidis VP, O'Donnell CP, Muthukumarappan K, Bourke P, Cullen P. Application of natural antimicrobials for food preservation. J Agric Food Chem 2009; 57:5987-6000.
28. Pisano M, Pagnan G, Loi M, Mura ME, Tilocca MG, Palmieri G, Fabbri D, Dettori MA, Delogu G, Ponzoni M, Rozzo C. Antiproliferative and pro-apoptotic activity of eugenol-related biphenyls on malignant melanoma cells. Mol Cancer 2007; 6:1.
29. Titenko-Holland N, Windham G, Kolachana P, Reinisch F, Parvatham S, Osorio AM, Smith MT. Genotoxicity of malathion in human lymphocytes assessed using the micronucleus assay in vitro and in vivo: a study of malathion-exposed workers. Mutat Res 1997; 388:85-95.
30. Cushnie TT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005; 26:343-56.
31. Bonassi S, El-Zein R, Bolognesi C, Fenech M. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 2011; 26:93-100.