ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2022; 27(1): 3292-3301
doi: 10.25083/rbl/27.1/3292-3301

Received for publication, December, 03, 2021
Accepted, January, 16, 2022

Original paper

Evaluation of the genotoxic activity of wastewater obtained after steam distillation of essential oil of Bulgarian Rosa alba L. – in vivo study

TSVETELINA GERASIMOVA[1], MARGARITA TOPASHKA-ANCHEVA[1], ANA DOBREVA[2], ALMIRA GEORGIEVA[3,4], MILKA MILEVA[3]

[1] Department of Ecosystem Research, Environmental Risk Assessment and Conservation Biology, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Str., Bulgarian Academy of Sciences, Sofia 1113, Bulgaria
[2] Institute for Roses and Aromatic Plants, Agricultural Academy, Kazanlak 6100, 49 Osvobojdenie Blvd, Bulgaria
[3] Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 23, Acad. G. Bonchev, str., 1113 Sofia, Bulgaria
[4] Institute of Neurobiology, Bulgarian Academy of Sciences, 23, Acad. G. Bonchev, str., 1113 Sofia, Bulgaria

Abstract

The process of essential oil water-steam distillation leaves a water fraction as a rest material of the technological process. This residual fraction represents a serious environmental pollutant. The aim of the present study was to investigate the clastogenic and cytotoxic effects of *R. alba* L. distillation wastewater in laboratory animal's test model *in vivo*. The ICR mice received a single dose (0.01 mL/b. w.) of 20% (v/v) or 11% (v/v) wastewater solution by intraperitoneal administration. The chromosomal aberrations frequency, mitotic index and micronuclei formation in peripheral blood were scored. The results suggested that the distillation wastewater extracts of white oil-bearing rose *R. alba* L. did not induce a considerable amount of chromosome aberrations, but a cell proliferation inhibition in mice bone marrow cells, compared to the negative control group ($p<0.001$). The rodent erythrocyte MN assay showed a slightly increased frequency of micronucleated polychromatic erythrocytes under the present experimental conditions. *Rosa alba* L. wastewater solution applied showed a negligible genotoxic effect, but a slight antiproliferative effect.

Keywords *Rosa alba* L., wastewater products, genotoxicity, *in vivo* mammalian erythrocyte micronucleus test.

To cite this article: GERASIMOVA T, TOPASHKA-ANCHEVA M, DOBREVA A, GEORGIEVA A, MILEVA M. Evaluation of the genotoxic activity of wastewater obtained after steam distillation of essential oil of Bulgarian *Rosa alba* L. – *in vivo* study. *Rom Biotechnol Lett*. 2022; 27(1): 3292-3301. DOI: 10.25083/rbl/27.1/3292-3301.

*Corresponding author: TSVETELINA GERASIMOVA, Department of Ecosystem Research, Environmental Risk Assessment and Conservation Biology, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Str., Bulgarian Academy of Sciences, Sofia 1113, Bulgaria
E-mail: cvetij@yahoo.com

Introduction

Plants in the genus Rosa of the family Rosaceae are among the most valuable oil-bearing plants. From ancient times the rose has been declared the Queen of Flowers, due to its wonderful aroma and beautiful inflorescences. The application of rose essential oils, aqueous and alcoholic extracts from flowers for the treatment and prevention of various diseases dates back to the beginning of human civilization due to their therapeutic efficacy - antidepressant effects, psychological relaxation, improvement of sexual dysfunction, antioxidant, antimicrobial, antifungal, probiotic and antipyretic effects, smooth muscle relaxation, lipid-lowering, antiulcerogenic effects, etc. (Gochev et al., 2010; Shohayeb et al., 2014; Abdel-Hameed et al., 2015; Georgieva et al., 2019). Rose flowers are a rich phytocomplex of ingredients that determine their biological activity (Bakkali et al., 2008).

In Bulgaria, the most popular method for the production of rose oil is a classical method of water-steam distillation, which leaves a water fraction as a rest material of the technological process. They are data existing that only in Bulgaria the essential oil of *Rosa alba* L. is distilled separately from *Rosa damascena* Mill. (Kovacheva et al., 2010; Dobreva et al., 2011). The low yield of essential oil of *Rosa alba* L. leads to the release of bulky waste - (i) spent rose petals as a solid residue, as well as (ii) wastewater - liquid residue (Slavov et al., 2017).

So, the wastewater after rose oil distillation represents a serious environmental problem as pollutants because they are discarded into the drainage system and the rivers (Wedler et al., 2016).

Recently, there is great attention on the valorization of plant materials and reusing the potentially bioactive ingredients of these materials. Many new novel approaches are studied, including the extraction of polysaccharides from the biomass and the introduction of integrated methods for the more complete valorization of the rose waste by-products. Due to insufficient data on their composition and biological activities, most of the methods for rose waste valorization still remain on a laboratory scale (Slavov et al., 2017).

As a by-product of hydrodistillation, wastewater is rich in water-soluble compounds as polyphenols, glycosides, tannins, etc., so they could be used as a good natural inexpensive source of biologically active compounds after more phytochemical, *in vitro*, and *in vivo* studies. These compounds are the subject of a rather scarce but growing number of studies in terms of their genotoxic potential. Sabahi et al. (2020) applied a cytotoxicity assay on HepG2 cells (human liver cancer cell line) exposed to different concentrations of *R. damascena* Mill. polyphenol-enriched fraction of wastewater for 24 and 48 h. A significant toxicity at a concentration of 100 µg/mL and higher was reported in this cell line, but on human lymphocytes a range of concentrations (25-100 µg/mL) did not show any genotoxicity and could be considered as nongenotoxic concentrations. However, this type the results need to be validated in *in vivo* settings (Rothfuss et al., 2011). Different components of *Rosa alba* L. have been subjected to various studies. Naikwade et al. (2009) reported a learning and memory-enhancing activity of *Rosa alba* L. Authors indicated that aqueous extract of *Rosa alba* L. calyces might be useful memory restorative agent when treating cognitive disorders such as Alzheimer's disease. Yon et al. (2018) demonstrated that petal extract of white rose (*Rosa hybrida* L.) containing polyphenols and flavonoids exerted neuroprotective effects via antioxidative and anti-inflammatory activities in glutamate-treated HB1.F3 human neural stem cells and kainic acid-challenged male ICR mice. Therefore, authors suggested that petal extract of *Rosa hybrida* could be a good candidate as an anti-epileptic or neuroprotective agent for clinical trials to attenuate seizure-related brain injury.

In the scientific literature are described various methods and technology for rose waste utilization are sought as well as to reuse these wastewaters and the compounds contained therein with potential beneficial effects. One such study provides evidence that phenolic compounds in water by-product obtained after hydro-distillation of Taif rose (*Rosa damascena Miller var. trigintipetala Dieck*) showed antioxidant activity (Abdel-Hameed et al., 2015). Taif rose water by-product extract did not produce pathological abnormality and had an excellent safety profile in acute, sub-chronic, and chronic toxicity studies in adult male Swiss albino mice. Polyphenol-containing rose oil distillation wastewater from *Rosa damascena* Mill. was also proved to have dose-dependent antiproliferative activity on immortalized human keratinocytes (Wedler et al., 2016).

Since rose petals are known to contain compounds with potential antiproliferative activity (Wedler et al., 2016), it was therefore of interest to investigate *Rosa alba* L. distillation wastewater extracts and their contribution to possible genotoxic and antiproliferative effect. Based on this data consideration, we formulated the objective of the present work - to investigate the clastogenic and cytotoxic effects of *Rosa alba* L. distillation wastewaters through classical cytogenetic methods on a laboratory animals test model.

Materials and methods

Preparation of wastewaters

The wastewaters were collected after the distillation of rose flowers, in the semi-industrial installation of the Institute for Roses and Aromatic Plants, Kazanlak. The process parameters were as follows: raw material 8 - 10 kg; hydro module 1:4; flow rate 16-20 ml/min; duration 150 min. The wastewaters were collected in cool conditions for the next stage of the investigation.

Chemicals

The standard experimental mutagen N-methyl-N'-nitro-N-nitrosoguanidine MNNG (50 µg/mL) (CAS-Nr.: 70-25-7, from Fluka – AG, Switzerland) was used as a positive control. A parallel experiment with 0.9% NaCl solution was used to a negative control.

Total flavonoid and phenolic content assay

The Folin-Ciocalteu method with some modifications was used for the measurement of the total phenolic content. 150 µL of the tested waters were added to 1.25 mL of Folin-Ciocalteu reagent (diluted 1:10) and 1 mL of Na_2CO_3 solution (7.5%). After incubation of 30 min at room temperature, the absorbance was measured at 765 nm against blank. Gallic acid was used as a reference standard for plotting the calibration curve for polyphenols, and quercetin – for flavonoid content respectively. The final results were calculated as gallic acid equivalents per 1 mL wastewater (GAE/1 ml water) and QueE/ml respectively, on the basis of a standard curves.

Animals and treatment

Eight-week old male and female ICR strain albino mice (20.0±1.5g b.w.), were delivered from Slivnitza animal breeding house, Bulgarian Academy of Sciences, Sofia. Animals were transported to the Animal house facility of Institute of biodiversity and ecosystem research and were kept for several days at standard laboratory conditions - temperature 20-22^{0}C, photoperiod 7 am to 7 pm, free access to standard for laboratory animal's food and water.

The experiments were performed in accordance with Bulgaria's Directorate of Health Prevention and Humane Behavior toward Animals. Bulgarian Food Safety Agency (BFSA) published a Certificate number 125 and standpoint 45/2015 for 5 years period for use of animals in experiments for the Stephan Angeloff Institute. The Ethical Committee of The Stephan Angeloff Institute approved the experimental design and protocols of the work with decision from 4.10.2020.

The ICR mice were randomly assigned to four experimental groups (eight male/eight female animals each) and kept in standard cages isolating the control and the treatment groups to avoid cross contamination. All tested substances were given as a single treatment by intraperitoneal (i.p.) injection.

The following experimental groups (n=8, 4♂4♀ each) were defined: Group 1. Animals, injected with 20 % wastewater solution (0.01 mg/mL); Group 2. Animals, injected with 11 % - wastewater solution (0.01 mg/mL). Group 3: MNNG 50 µg/mL (0.01 mg/mL). Group 4: Untreated control group, injected only with 0.9% NaCl (0.01 mg/mL). Throughout the experiment, all animals were observed twice daily after initial i.p. treatment for any clinical signs of toxicity.

Cytogenetic assay

The cytogenetic assay protocol (Preston et al., 1987) was applied for each experimental group starting at the 24th (4♂4♀) or 48th (4♂4♀) after intraperitoneally treatment with the respective solution. To obtain metaphase chromosomes suitable for cytogenetic analysis, one hour before bone marrow cell isolation a mitotic inhibitor colchicine – 0.04 mg /g b.w. was intraperitoneally (i.p.) injected. For scoring of micronuclei, blood smears were prepared prior colchicine treatment. Animals were euthanized by diethyl ether anesthesia, bone marrow cells were flushed from femur and hypotonized in a 0.075 M KCl at 37^{0}C for 15 min. The cell fixation procedure includes a solution of cold methanol: glacial acetic acid (3:1), followed by resuspendation and dripping on precleaned cold wet glass slides and air dried. The slides were stained in 5 % Giemsa solution (Sigma Diagnostic). Up to 50 well-scattered metaphase plates were analyzed from each animal using light microscopy (Olympus CX 31) x 1000. The structural changes in the chromosomes - chromosomal aberrations (CA) were used as an endpoint for genotoxicity. The main types of aberrations – breaks and fragments, were separately scored for each animal in all eight experimental groups. Mitotic indices (MI) were determined by counting the number of dividing cells among 1500 cells per animal (Darzynkiewicz et al., 1987). The frequencies of abnormalities and the mitotic index were determined for each animal and then the mean ± standard error of mean for each group was calculated.

The in vivo mammalian erythrocyte micronucleus test (MN test)

The MN test was performed in accordance with the OECD test guideline No. 474 for the testing of chemicals (OECD, 2016). Samples of peripheral blood were taken once 48 hours after initial treatment from all dose groups to facilitate integration with the other toxicity tests. For each of the eight mice in the group 5µL peripheral blood was collected through the tail vein. The blood was diluted with 45 µL Sörensen's phosphate buffer (pH 6,8) (Mitkovska et al., 2012) and a drop of this solution was smeared on a microscopic slide. Slides were air dried and fixed for 10 min with absolute methanol. Dry slides were independently coded prior to scoring by a single investigator. The smeared preparations were stained with Acridine orange (AO) (Merck) according to Hayashi et al. (1983) with some modifications. The A.O. stock solution was prepared as a 0.1% aqueous solution, available for several weeks at 4°C. A.O., 0.24 mM in 1/15 M Sörensen's phosphate buffer (pH 6.8) which is 1/15 M Na_2HPO_4 and 1/15 M KH_2PO_4, prepared separately and mixed together in a ratio to have pH 6.8 (2 parts of stock solution and 30 parts of the buffer), was used as a working solution. AO (50 µL of a 1mg/mL solution) was dropped on dry blood sample slides and spread by immediately covering the slide with coverslip glass (24x32mm). The cells were allowed to settle for few minutes and the analysis was then performed with a fluorescence microscope at 400x magnification. Observations were made using Axio Scope A1 – Carl Zeiss Fluorescent Microscope with 40X EC Plan-NEOPLUAR objective equipped with FITC 495 nm excitation filter.

The criteria used for the identification of micronuclei (MNi) and to distinguish them from artifacts in the cytoplasm, were described by Schmid (1975). MNi were generally round or oval in shape, with sharp borders and exhibited a strong yellow-green fluorescence. MNi were easily distinguishable in PCE, which emit red fluorescence (Hayashi et al., 1983).

From each slide, at least 2000 polychromatic erythrocytes (PCE) or at least 4000 per animal were screened for the incidence of micronucleated immature

erythrocytes. The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 1000 normochromatic erythrocytes (NCE) per slide or 2000 NCE per animal. Only monolayers without overlapping cells were targeted for each slide.

Statistical analysis

Results are expressed as mean value ± standard deviation (SD) for each group and the data were statistically evaluated for their significance by analysis of variance using two-tailed, two-sample Student's t-test. Statistical significance is expressed as $p<0.001$; $p<0.01$; $p<0.05$; $p>0.05$ - (not significant).

Results and discussion

The wastewater of *Rosa alba* L. was examined for its total phenolic composition and total flavonoid content. Our results showed that it contained 7.6 μg GAE/ml polyphenols and 5.2 μg QueE/mL liquid. The low level of polyphenols is probably due to their low water solubility, or higher levels of polyphenolic glycosides that are difficult to detecting.

Polyphenols regulate many cellular, biochemical, and immunological events involved in the initiation and progress of genotoxic processes (Chahar et al., 2011). Dietary polyphenols, including flavonoids, have protective effects against DNA damage induced by different genotoxic agents. They are data existing that they quite often realize their genoprotection action by the mechanisms include (i) reducing oxidative stress; (ii) metal ion chelating; (iii) modulation of enzymes responsible for bioactivation of genotoxic agents and detoxification of their reactive metabolites. The quercetin-like structures containing in the wastewater of *Rosa alba* L. have the same chemical analogs as were reported by Luca et al. (2016), so they are able to exert their genoprotective effect by a similar mechanism.

The paper presents the effect of oil-bearing Bulgarian rose *Rosa alba* L. distillation wastewater introduced intraperitoneally over a well-known *in vivo* test model - ICR mice.

The changes in chromosomal aberrations frequency (CAF), mitotic index (MI) and micronuclei formation (MN) in peripheral blood were studied. Micronuclei are small satellite structures, representing chromosomal fragments lacking centromeres. The frequency of micronuclei is also commonly used as a cytogenetic biomarker. The *in vivo* MN test in peripheral blood erythrocytes is widely used as a short-term assay for the detection of agents able to induce chromosomal aberrations in somatic cells (Hayashi et al., 1990). For short-term studies, polychromatic erythrocytes are taken into account (OECD, 2016). An increase in the frequency of micronuclear polychromatic erythrocytes is an indication of induced chromosomal damage.

In this study, we present data on the presumptive genotoxic and cytotoxic effects of *Rosa alba* L. wastewater in two concentrations – 20% and 11% solution applying the bone marrow test system scheme by Preston et al. (1987) and Darzynkiewicz et al. (1987), as well as the *in vivo* mammalian erythrocyte micronucleus test (OECD, 2016).

Table 1. Clastogenic effect and antiproliferative activity of bone marrow cells in ICR line laboratory mice after *i.p.* supplementation with *R. alba L.* wastewater

			Type of chromosomal aberrations				
Compound and dose	*Time after treatment*	*Number of metaphases scored*	*Breaks*	*fragments*	*Rearrangements* **c/c**	*% of cells with aberrations (mean±SD)*	*Mitotic index (‰) (mean±SD)*
Rosa alba L. wastewaters *20 %*	24 h	*400*	**4**	**4**	**18**	**2.0 ± 1.51**	**16,83 ± 3.18**
	48 h	*400*	**1**	**3**	**19**	**1.0 ± 1.07**	**10.49 ± 1.79**
Rosa alba L. wastewaters *11 %*	24 h	*400*	**2**	**1**	**17**	**0.75 ± 1.03**	**8.91 ± 3.07**
	48 h	*400*	**1**	**2**	**17**	**0.75 ± 1.49**	**13.87 ± 4.10**
MNNG (50μg/mL)	24 h	*400*	**18**	**17**	**4**	**10.75 ± 4.27**	**11.25 ± 3.59**
	48 h	*400*	**21**	**19**	**14**	**13.00 ± 3.38**	**11.21 ± 2.68**
Control 0.9%NaCl	24 h	*350*	**0**	**5**	**11**	**1.14 ± 1.57**	**17.73 ± 2.99**
	48 h	*500*	**0**	**0**	**3**	**0.6 ± 0.3**	**16.88 ± 0.56**

c/c – centromere/centromeric fusion

Chromosomal aberration assay

The data about the percentage of chromosomal aberration frequencies (breaks and fragments) and mitotic indices are presented in Table 1.

Cytogenetic analysis revealed breaks, fragments, translocations, and other chromosomal rearrangements in the bone marrow metaphase plates. Centromere-centromeric fusions predominate. These chromosomal rearrangements

lead to the appearance of biarmed chromosomes without altering the amount of genetic material. Breaks and fragments were presented almost equally in all experimental groups. Chromatid type of aberrations (breaks and fragments) in metaphases may occur in response to single-chain ruptures induced in the early S phase, as well as a result of incomplete or unsuccessful reparation (Pfeiffer et al., 2000).

The percentage of aberrant mitoses in the experimental mice group, treated with 20% solution of *Rosa alba* L. wastewaters, calculated at the 24th hour from the beginning of the experiment was 2.0% ± 1.51 and slightly decreased at 48th h (1.0% ± 1.07), but the means are not significantly different (p>0.05).

The results about the percentage of cells with chromosomal aberrations showed a slight reduction in the percentage of mitoses with aberrations (0.75% ± 1.03 and 0.75% ± 1.49) in the bone marrow mice cells, treated with 11% wastewater at the 24th and 48th h compared to the data in 20% wastewater treated groups, but the difference is not statistically significant (p>0.001). The data of experimental groups, treated with wastewater in both concentrations (20% and 11%), are statistically undistinguishable from those calculated in the 0.9% NaCl control groups (p>0.001) (t_{st}=1.0755 and t_{st}=0.579, respectively) (Fig. 1).

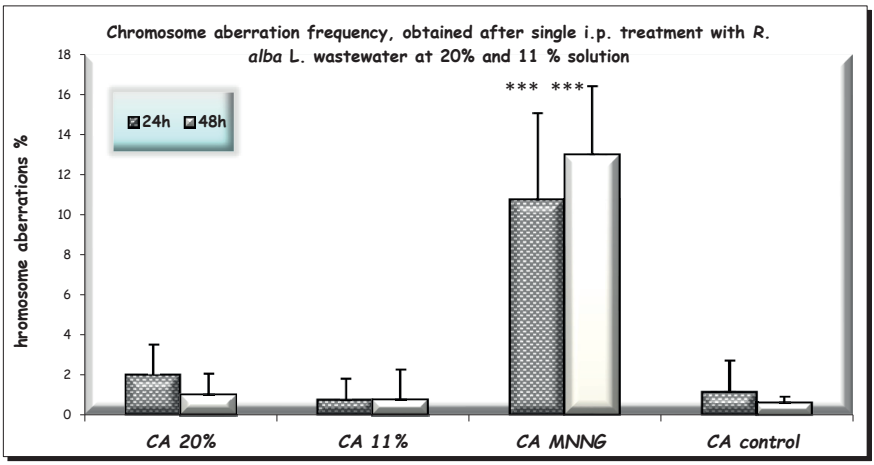

Figure 1. Frequency of chromosomal aberrations (CA) observed after *Rosa alba* L. wastewater treatment (20% and 11 % wastewater solution) in bone marrow of ICR mice. Data are expressed as mean ± SD; ***p<0.001 compared with the positive control MNNG.

Obviously, the number of aberrant metaphases in all experimental groups is dramatically lower in comparison with the relevant values obtained for the positive control group (MNNG 50μg/mL) (10.75% ± 4.27 and 13.00% ± 3.38) (t=5.2915 and t=7.202, respectively) (p<0.001).

The results of our experiments clearly showed that the tested wastewater concentrations from *Rosa alba*'s water-steam distillation does not cause a real clastogenic effect in the bone marrow cells of the experimental mouse line.

These results are consistent with the data of *in vitro* cytotoxicity assay (Sabahi et al., 2020). Authors tested a range of concentrations of *R. damascena* polyphenol-enriched fraction (25–100 μg/mL) and the results did not show any genotoxicity, so these concentrations could be considered as nongenotoxic. The comet assay analysis in the same paper showed that this *R. damascena* polyphenol-enriched wastewater fraction (25–100 μg/mL) protects human lymphocytes against H_2O_2-induced DNA damages significantly. This, according to the authors, is attributable to that the phenolic compounds reduce free radicals' side effects before they induce any DNA damages.

Mitotic index

The mitotic index as a measure of the proliferation status in treated with *Rosa alba* L. wastewater bone marrow cell populations displays a different picture from that described in the experimental chromosomal aberration test assessment (Fig. 2). The results showed a significant decrement in mitotic activity of bone marrow cells at the 48th hour compared to the 24th hour experimental group in 20% wastewater concentrations tested (p<0.001).

On the contrary, MI significantly increases at the 48thh in the lower concentration tested – 11% (p<0.05). This contradictory result is also repeated when we compare the MI data in bone marrow cells of 20% and 11% wastewater treated animal groups at the 24th h from the beginning of the experiment (p<0.001). The means were not statistically distinguishable in 48th h mice group (p>0.05) - at the 48th hour the mitotic activity was not suppressed in the lower *R. alba* L. concentration tested (11% wastewater solution). A longer period of treatment (48 hours) does not provide clear evidence of cytostatic effect, i.e. does not significantly affect the mitotic cycle preparation and progression in stem bone marrow cells population.

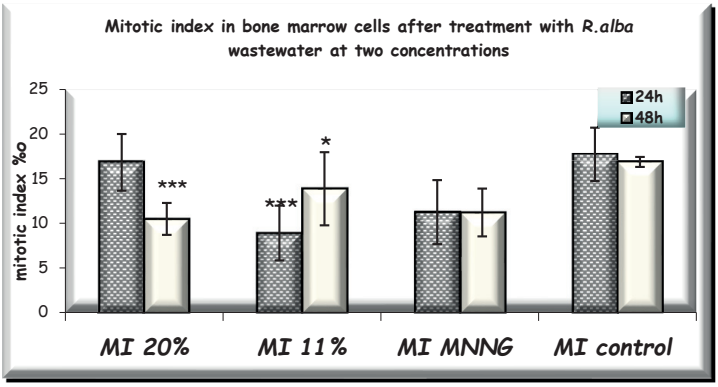

Figure 2. Value of mitotic activity (MI) observed after *Rosa alba* L. wastewater treatment in bone marrow cells. Data are expressed as mean ± SD. *p<0.05; ***p<0.001 compared with the negative control group.

The values of MI as an indication of the degree of cytotoxicity in the treated bone marrow cell population were compared with those in the negative control groups. These data show that acute treatment with *R. alba* L. wastewater for a short period of time (24 hours) leads to a suppression of dividing activity in bone marrow cells only in the experimental group, treated with the lower dose (11% solution of *R. alba* wastewater) (p<0.001).

With regard to the values for the mitotic index in all experimental variants there is no significant difference compared to the values for MI obtained in the positive control (MNNG) (p>0.05), with one exception - 20%/24h *R. alba* L. wastewater (t_{st}=3.523) (p<0.01).

This significant reduction in mitotic activity of bone marrow cells in almost all tested concentrations points that *R. alba* L. wastewater in the concentrations applied significantly influences the replicative capacity of the cells.

Such dose-dependent antiproliferative activity in immortalized human keratinocytes with half maximal inhibitory concentration (IC50) of 9.78 μg/mL was reported by Wedler et al. (2016). Authors pointed that the polyphenol-enriched wastewater fraction from *Rosa damascena* Mill. could be developed as a supportive therapy against hyperproliferation-involved skin diseases. Antiproliferative activity of geraniol in cancer cells has been also reported by Carnesecchi et al. (2002; 2004) using various assays. Sabahi et al. (2020) pointed out that polyphenol-enriched *R. damascena* fraction exerted significant cytotoxicity *in vitro* at a concentration of 100 μg/ml and higher.

Consistent with the above mentioned, this flavonoid content is apparently the reason for the observed reduction in mitotic activity in bone marrow cells. In the wastewater sample of *Rosa alba* L., used in our study, the phenolic composition was 7.6 mg GAE and 5.2 μg QueE/mL total flavonoid content.

Our results obtained for the antiproliferative effects of wastewater are logical due to the certain flavonoids contain, in particular quercetin, which is known for its inhibitory effect on cell division (Delgado et al., 2014; Klimaszewska-Wiśniewska et al., 2017). Klimaszewska-Wiśniewska et al. (2017) found that quercetin induced G_2/M arrest and polyploidy, and also exerted a dose-dependent cytotoxic effect on the tested cells with an IC_{50} value of 74 μM. Authors suggested that "the possible mechanism underlying quercetin-induced mitotic catastrophe involves the perturbation of mitotic microtubules leading to monopolar spindle formation, and, consequently, to the failure of cytokinesis".

Table 2. *In vivo* micronucleus test, performed in ICR line laboratory mice after single *i.p*. supplementation with *R. alba* L. wastewater. The frequencies of micronuclei in peripheral blood erythrocytes are presented as mean ± standard deviation per group.

Compound and dose	*Time after treatment*	*Number of animals*	*Number of PCE scored*	*Total MNPCE*	*MNPCE/4000 PCE (%) Mean ± SD %*	*PCE/(PCE+NCE) (%) Mean ± SD %*
Rosa alba L. wastewater *20%*	48 h	**4♂ 4♀**	**32561**	**48**	**0.15 ± 0.02**	**8.57 ± 0.84**
Rosa alba L. wastewater *11%*	48 h	**4♂ 4♀**	**32373**	**30**	**0.09 ± 0.03**	**9.25 ± 1.66**
MNNG (50μg/mL)	48 h	**4♂ 4♀**	**32681**	**226**	**0.69 ± 0.03**	**8.67 ± 1.50**
Control 0.9%NaCl	48 h	**4♂ 4♀**	**32600**	**20**	**0.06 ± 0.01**	**21.27 ± 2.77**

Total MNPCE – total number of observed MN in at least 4000 PCE per animal; MNPCE – micronucleated polychromatic erythrocytes; PCE - polychromatic erythrocyte; NCE – normochromatic erythrocyte;

Since polyphenols are the major component of wastewaters (Wedler et al., 2016; Sabahi et al., 2020), such investigations are important in the context of further efforts to create appropriate technology for rose wastewater utilization to reduce the disposal of the product with a large volume and valuable composition.

Micronucleus assay

Throughout the test period, no signs of toxicity were detected in any of the animals, treated with the listed substances.

The rodent erythrocyte MN assay, according to the regulatory requirements (The National Toxicology Program, OECD TG, Rothfuss et al., 2011), is usually the first choice among *in vivo* assays for subsequent testing when *in vitro* gentotoxicity tests proved to be positive. In the experimental scheme presented in this paper, a single-dose regimen was applied, thus only MNPCEs were taken into account for determining a positive result. The number of MNPCE was given separately for each treatment group. The results of the micronucleus assay with peripheral blood erythrocytes are summarized in Table 2.

Table 2. *In vivo* micronucleus test, performed in ICR line laboratory mice after single *i.p*. supplementation with *R. alba* L. wastewater. The frequencies of micronuclei in peripheral blood erythrocytes are presented as mean ± standard deviation per group.

Compound and dose	*Time after treatment*	*Number of animals*	*Number of PCE scored*	*Total MNPCE*	*MNPCE/4000 PCE (%) Mean ± SD %*	*PCE/(PCE+NCE) (%) Mean ± SD %*
Rosa alba L. wastewater *20%*	48 h	**4♂ 4♀**	**32561**	**48**	**0.15 ± 0.02**	**8.57 ± 0.84**
Rosa alba L. wastewater *11%*	48 h	**4♂ 4♀**	**32373**	**30**	**0.09 ± 0.03**	**9.25 ± 1.66**
MNNG (50μg/mL)	48 h	**4♂ 4♀**	**32681**	**226**	**0.69 ± 0.03**	**8.67 ± 1.50**
Control 0.9%NaCl	48 h	**4♂ 4♀**	**32600**	**20**	**0.06 ± 0.01**	**21.27 ± 2.77**

Total MNPCE – total number of observed MN in at least 4000 PCE per animal; MNPCE – micronucleated polychromatic erythrocytes; PCE - polychromatic erythrocyte; NCE – normochromatic erythrocyte;

Initially, the ratio of PCE to total erythrocytes (PCE+NCE) was determined for each animal and used as an index of cytotoxicity. These ratios showed significant differences only among the *R. alba* treatment groups (20% and 11%) and the negative control group ($p<0.01$ and $p<0.001$, respectively) (Table 2). In presence of *R. alba* wastewater, the percentage PCE decreased significantly, which may indicate a suppression of bone marrow proliferation probably due to mitotic arrest. The lack of statistical significance between the positive control group (treated with the model alkylating agent MNNG 50μg/mL) and the two wastewater concentrations ($p>0.001$) supports this assumption since MNNG is well known mutagen and is cytotoxic to mammalian cells (O'Brien & Brown, 2006).

The MNPCE frequencies were statistically significant and did yield dose-dependent pattern among the two wastewater treatment groups ($p<0.001$). *R. alba* wastewater at concentration of 20% significantly increased the frequency on MNPCE from 0.06% ± 0.01 (mean value for the positive control group) to 0.15% ± 0.02 ($p<0.001$). A statistically significant increase in the incidence of MNPCE over the control value was also observed following treatment with 11 % *R. alba* wastewater (0.09% MNPCE/4000 PCE, $p<0.05$) (Fig. 3).

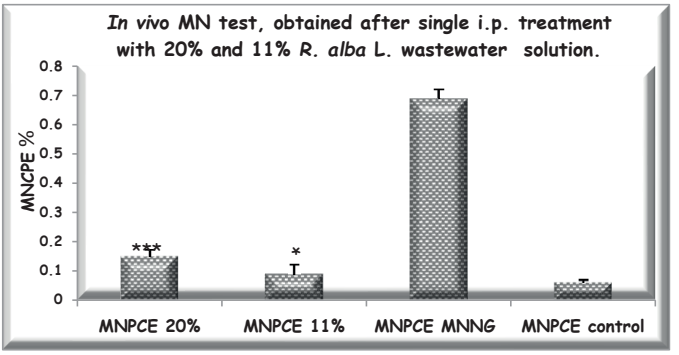

Figure 3. Induction of MN observed after *Rosa alba* L. wastewater treatment in peripheral polychromatic erythrocytes. Data are expressed as mean ± SD. *$p<0.01$ and ***$p<0.001$ compared with the negative control.

These results are consistent with the data of Gateva et al. (2021), who reported that *in vitro* treatment with Rosa alba L. oil in a range of concentrations from 50 to 500 μg/mL enhanced the frequency of micronuclei in dose-related manner. The formation of micronuclei increased above two-fold compared to the negative control in human lymphocyte cultures.

Regarding wastewater, the original results obtained by us are difficult to compare with literature data. This is especially true for the scarce experimental data on genotoxic activity. There are some data for the individual components of wastewater - flavonoids and polyphenols, as well as for the main product of water-steam distillation of R. alba L. - rose oil.

Geraniol is one of monoterpenes, present in high percentage in *Rosa alba* L. waste biomass (Slavov et al., 2017), in hydrosol (Georgieva et al., 2019) as well as in *Rosa alba* L. essential oil (Gateva et al., 2021). It was found to possess well-expressed antioxidant properties. Geraniol was tested for cytotoxicity and genotoxicity by Doppalapudi et al. (2007). The authors observed that geraniol did not induce a significant increase in bone marrow micronucleus of mice treated with 375, 750, and 1500 mg/kg. No statistically significant increases in the frequency of micronucleated PCE were seen at any dose level in either male or female mice at the 24 or 48 h time point, and there was no dose-related increase in the frequency of micronuclei.

Our results demonstrated a slight increase in the frequency of MNPCE in treated with *Rosa alba* L. wastewater animals, which is an indication of induced chromosome damage, which ends up as MN.

Conclusion

Under the conditions employed in this study, our results suggested that the white oil-bearing rose *Rosa alba* L. distillation wastewater extracts at 20% and 11% doses did not induce a considerable amount of CAs. The reported low percentage of breaks and fragments in bone marrow cells of experimental ICR mice can be considered as a sure indicator, predicting the absence of clastogenic wastewater effect (lack of significant chromosome-damaging action). This result was accompanied by cell proliferation inhibition in ICR mice bone marrow following intraperitoneally administration of both wastewater concentrations, compared to the negative control group ($p<0.001$).

As an additional standard battery for genotoxicity - rodent erythrocyte micronucleus assay in peripheral blood was applied *in vivo*. From the results is evident that *Rosa alba* L. wastewater was determined to induce a slightly increased frequency of micronuclei in peripheral blood erythrocytes of male and female ICR mice under the present experimental conditions.

Rosa alba L. wastewater solution in both concentrations applied showed a negligible genotoxic effect, but a slight antiproliferative effect.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

The team expresses heartfelt thanks to the financial support of Project of Bulgarian National Sciences Fund КП-06 H36/17 (granted to Assoc. prof. M. Mileva, PhD)

References

1. ABDEL-HAMEED E, BAZAID S, SABRA A. Total phenolic, *in vitro* antioxidant activity and safety assessment (acute, sub-chronic and chronic toxicity) of industrial taif rose water by-product in mice, *Pharmaceutical Letter*, 2015; 7(2): 251–259.
2. ALMONTE-FLORES DC, PANIAGUA-CASTRO N, ESCALONA-CARDOSO G, ROSALES-CASTRO M. Pharmacological and Genotoxic Properties of Polyphenolic Extracts of *Cedrela odorata* L. and *Juglans regia* L. Barks in Rodents. Evidence-based complementary and alternative medicine. *eCAM*. 2015; 187346. doi.org/10.1155/2015/187346
3. BAKKALI F, AVERBECK S, AVERBECK D, IDAOMAR M. Biological effects of essential oils - a review. *Food and Chem Toxicol*. 2008; 46: 446-475. doi: 10.1016/j.fct.2007.09.106
4. CARNESECCHI S, BRAS-GONCALVES R, BRADAIA A, ZEISEL M, GOSSÉ F, POUPON M-F, RAUL F. Geraniol, a component of plant essential oils, modulates DNA synthesis and potentiates 5-fluorouracil efficacy on human colon tumor xenografts. *Cancer Lett*. 2004; 215(1): 53-59. doi: 10.1016/j.canlet.2004.06.019
5. CARNESECCHI S, LANGLEY K, EXINGER F, GOSSE F, RAUL F. Geraniol, a component of plant essential oils, sensitizes human colonic cancer cells to 5-Fluorouracil treatment, *J. Pharmacol. Exp. Ther*. 2002; 301(2):625-630. doi: 10.1124/jpet.301.2.625
6. CHAHAR MK, SHARMA N, DOBHAL MP, JOSHI YC. Flavonoids: A versatile source of anticancer drugs. *Pharmacogn Rev*.2011; 5(9): 1–12. doi: 10.4103/0973-7847.79093
7. DARZYNKIEWICZ Z. Cytochemical probes of cycling and quiescent cells applicable to flow cytometry. In: *Techniques in Cell Cycle Analysis (Biological Methods) 1987th Edition*, Kindle Edition by Joe W. Gray (Author), Zbigniew Darzynkiewicz, 1987; 272 – 290. ISBN-13: 978-0896030978
8. DELGADO A, FERNANDES I, GONZÁLEZ-MANZANO S, DE FREITAS V, MATEUS N, SANTOS-BUELGA C. Anti-proliferative effects of quercetin and catechin metabolites *Food Funct*. 2014; 5(4): 797-803. doi: 10.1039/c3fo60441a
9. DOBREVA A, KOVATCHEVA N, ASTATKIE T, ZHELJAZKOV VD. Improvement of essential oil yield of oil-bearing (*Rosa damascena* Mill.) due to

surfactant and maceration. *Ind Crop Prod*. 2011; 34:1649–1651. doi:10.1016/j.indcrop.2011.04.017
10. DOPPALAPUDI RS, RICCIO ES, RAUSCH LL, SHIMON JA, LEE P, MORTELMANS K, KAPETANOVIC I, Crowell J, MIRSALIS J. Evaluation of chemopreventive agents for genotoxic activity. *Mutat. Res*. 2007; 629: 148-160. https://doi.org/10.1016/j.mrgentox.2007.02.004
11. GATEVA S, JOVTCHEV G, CHANEV C, GEORGIEVA A, STANKOV A, DOBREVA A, MILEVA M. Assessment of anti-cytotoxic, anti-genotoxic and antioxidant potential of Bulgarian *Rosa alba* L. essential oil. *Caryologia*. 2020; 73(3): 71-88. doi: 10.13128/caryologia-260.
12. GEORGIEVA A, DOBREVA A, TZVETANOVA E, ALEXANDROVA A, MILEVA M. Comparative study of phytochemical profiles and antioxidant properties of hydrosols from Bulgarian *Rosa alba* L. and *Rosa damascena* Mill., *Journal of Essential Oil Bearing Plants*, 2019; 22(5): 1362-1371, DOI: 10.1080/0972060X.2019.1699867
13. GOCHEV V, DOBREVA A, GIROVA T, STOYANOVA A. Antimicrobial activity of essential Oil from *Rosa alba*. *Biotechnology & Biotechnological Equipment*, 2010; 24(1): 512-515. DOI: 10.1080/13102818.2010.10817892
14. HAYASHI M, MORITA T, KODAMA Y, SOFUNI T, ISHIDATE JR.M. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutat. Res. Letters*. 1990; 245(4): 245-249. https://doi.org/10.1016/0165-7992(90)90153-B
15. HAYASHI M, SOFUNI T, ISHIDATE M. An application of Acridine Orange fluorescent staining to the micronucleus test. *Mutation Research Letters*. 1983; 120(4): 241-247. doi: 10.1016/0165-7992(83)90096-9
16. KLIMASZEWSKA-WIŚNIEWSKA A, HAŁAS-WIŚNIEWSKA M, IZDEBSKA M, GAGAT, M, GRZANKA A, GRZANKA D. Antiproliferative and antimetastatic action of quercetin on A549 non-small cell lung cancer cells through its effect on the cytoskeleton. *Acta Histochemica.* 2017; 119(2): 99-112. https://doi.org/10.1016/j.acthis.2016.11.003
17. KOVACHEVA N, RUSANOV K, ATANASSOV I. Industrial cultivation of oil bearing rose and rose oil production in Bulgaria during 21st century, directions and challenges. *Biotechnol Biotechnol Eq.* 2010; 24:1793–1798. doi:10.2478/ V10133-010-0032-4
18. LUCA VS, MIRON A, APROTOSOAIE AC. The antigenotoxic potential of dietary flavonoids. *Phytochem Rev*. 2016; 15: 591–625. https://doi.org/10.1007/s11101-016-9457-1
19. MILEVA M, KUSOVSKI V, KRASTEV D, DOBREVA A, GALABOV A. Chemical composition, *in vitro* antiradical and antimicrobial activities of Bulgarian *Rosa alba* L. essential oil against some oral pathogens. *Int. J. Curr. Microbiol. App. Sci*. 2014; 3(7): 11-20. ISSN: 2319-7706
20. MITKOVSKA V, CHASSOVNIKAROVA TS, ATANASOV N, DIMITROV H. Environmental genotoxicity evaluation using a micronucleus test and frequency of chromosome aberration in free-living small rodents. *J. BioSci. Biotech*. 2012; 1(1): 67-71. ISSN: 1314-6246
21. NAIKWADE NS, MULE SN, ADNAIK RS, MAGDUM CS. Memory-enhancing activity of *Rose alba* in mice. *International Journal of Green Pharmacy*. 2009; 3(3): 239-242. ISSN: 0973-8258
22. O'BRIEN V, BROWN R. Signalling cell cycle arrest and cell death through the MMR System, *Carcinogenesis*. 2006; 27(4): 682–692, https://doi.org/10.1093/carcin/bgi298
23. OECD, 2016. OECD test guideline No. 474 for the testing of chemicals https://www.oecd-ilibrary.org/docserver/9789264264762en.pdf?expires=1616068940&id=id&accname=guest&checksum=80F54DA4886040B71182A492D85C19FE
24. PRESTON R, DEAN B, GALLOWAY S, HOLDEN H, MCFEE A, SHELDY M. Mammalian *in vivo* cytogenetic assay analysis of chromosome aberrations in bone marrow cells. *Mutation Research/Genetic Toxicology.* 1987; 189(2): 157–165. https://doi.org/10.1016/0165-1218(87)90021-8
25. PFEIFFER P, GOEDECKE W, OBE G. Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis. 2000 Jul;15(4):289-302. doi: 10.1093/mutage/15.4.289. PMID: 10887207.
26. ROTHFUSS A, HONMA M, CZICH A, AARDEMA M, BURLINSON B, GALLOWAY S, HAMADA S, KIRKLAND D, HEFLICH R, HOWE J, NAKAJIMA M, O'DONOVAN M, PLAPPERT-HELBIG U, PRIESTLEY C, RECIO L, SCHULER M, UNO Y, MARTUS H-J. Improvement of *in vivo* genotoxicity assessment: Combination of acute tests and integration into standard toxicity testing. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2011; 723(2): 108-120. https://doi.org/10.1016/j.mrgentox.2010.12.005
27. SABAHI Z, FARMANI F, MOUSAVINOOR E, MOEIN M. Valorization of waste water of *Rosa damascena* oil distillation process by Ion exchange chromatography. *Sci World J*. 2020; 5409493. doi: 10.1155/2020/5409493. eCollection 2020.
28. SCHMID W. The micronucleus test. *Mutation Research*. 1975; 31: 9–15. https://doi.org/10.1016/0165-1161(75)90058-8
29. SHOHAYEB M, ABDEL-HAMEED S, BAZAID SA, MAGHRABI I. Antibacterial and antifungal activity of *Rosa damascena* Mill. essential oil, different extracts of rose petals. *Global Journal of Pharmacology*. 2014; 8(1): 1–7. DOI: 10.5829/idosi.gjp.2014.8.1.81275
30. SLAVOV A, VASILEVA I, STEFANOV L, STOYANOVA A. Valorization of wastes from the rose oil industry. *Reviews in Environmental Science and Bio/Technology*. 2017; 16(2): 309–325. https://doi.org/10.1007/s11157-017-9430-5

31. WEDLER J, RUSANOV K, ATANASSOV I, BUTTERWECK VA. Polyphenol-enriched fraction of rose oil distillation wastewater inhibits cell proliferation, migration and TNF-α-induced VEGF secretion in human immortalized keratinocytes. *Planta Med.* 2016; 82(11-12): 1000-1008. doi: 10.1055/s-0042-105158

32. YON JM, KIM YB, PARK D. The ethanol fraction of white rose petal extract abrogates excitotoxicity-induced neuronal damage *in vivo* and *in vitro* through inhibition of oxidative stress and proinflammation. *Nutrients.* 2018; 10(10): 1375. doi: 10.3390/ nu10101375.