GENOTOXIC EFFECTS OF *RHAMNUS CATHARTICUS* IN HUMAN BLOOD LYMPHOCYTE

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KEYWORDS: *Rhamnus catharticus*, genotoxicity, human peripheral blood lymphocytes, micronucleus.

ABSTRACT

Rhamnus catharticus L., has an antioxidant and antimicrobial activity whereas the cytotoxic effect of the plant may be responsible for the emergence of these properties.

The aim of this study was to evaluate the genotoxic and cytotoxic potential of the water and methanolic extract obtained from the *Rhamnus catharticus* leaf in peripheral lymphocyte culture by micronucleus assay for 24- and 48-hours treatment period. Three doses of *Rhamnus catharticus* extract (1, 2, 4 mg/ml) were selected as treatment group; Mitomycin C (MMC) as positive and water is used as negative control.

We found that all extracts had greater toxicity compared to the negative control and MMC toxicity was higher than other concentrations. This might be caused by the various ingredients from the plant extract that are obtained due to the polarity of solvents.

REZUMAT: Efectul genotoxic al *Rhamnus catharticus* asupra limfocitelor din sângele uman.

Rhamnus catharticus L., are o activitate antioxidantă și antimicrobiană, în timp ce efectul citotoxic al plantei poate fi responsabil de apariția acestor proprietăți.

Scopul acestui studiu a fost de a evalua potențialul genotoxic și citotoxic al extractului apă metanol obținut din frunza de *Rhamnus catharticus* în cultura limfocitelor periferice prin analiza micronucleului pentru perioada de tratament de 24 și 48 de ore. Trei doze de extract de *Rhamnus catharticus* (1, 2, 4 mg/ml) au fost selectate ca grup de tratament; Mitomicina C (MMC) drept control pozitiv și apă drept control negativ.

Am constatat că toate extractele au fost mai toxice comparativ cu controlul negativ, iar toxicitatea MMC a fost mai mare decât alte concentrații. Acest lucru ar putea fi datorită extractului de plante care are diverse ingrediente din cauza polarității solvenților.

RÉSUMÉ: Effets génotoxiques de *Rhamnus catharticus* sur les lymphocytes du sang humain.

Rhamnus catharticus L., a une activité antioxydante et antimicrobienne alors que l'effet cytotoxique de la plante peut être responsable de l'apparition de ces propriétés.

Le but de cette étude était d'évaluer le potentiel génotoxique et cytotoxique de l'eau et de l'extrait méthanolique obtenus à partir de la feuille *Rhamnus catharticus* en culture de lymphocytes périphériques par dosage du micronoyau pour une période de traitement de 24 et 48 heures. Trois doses d'extrait *Rhamnus catharticus* (1, 2, 4 mg/ml) ont été sélectionnées comme groupe de traitement; La mitomycine C (MMC) est positive et l'eau est utilisée comme contrôle négatif.

Nous avons constaté que tous les extraits étaient plus toxiques que le contrôle négatif et que la toxicité du MMC était supérieure à celle des autres concentrations. Cela pourrait être dû au fait que l'extraction des plantes contient divers ingrédients en raison de la polarité des solvants.

INTRODUCTION

The family *Rhamnaceae*, which has 59 genera and 900 species, consists of small trees and bushes. In the flora of Turkey, there are 21 *Rhamnus* species, seven of which are native (Davis, 1966). *Rhamnus catharticus* L. has been used as a laxative, diuretic, depurative, and cathartic. Aside, *Rhamnus catharticus* is used to treat gout, rheumatism, chronic skin diseases, and liver disorders (Qaderi et al., 2009).

Compounds in the bark, leaves, and fruits of *Rhamnus catharticus* are well known to have purgative effects when it is eaten (Soper et al., 1982), and the fruit has been often used for constipation (Mozingo, 1987). Up to date research shows *Rhamnus catharticus* has reduced toothache (*, 1890), and it also has antioxidant and antimicrobial properties. The cytotoxic effect of the plant may be responsible for the emergence of the antimicrobial properties (Coskun, 1992).

There is information about the rich purgative quinones which were identified earlier as antioxidant molecules in many studies that are found at high concentrations in *Rhamnus catharticus* (Yokozozowa, 1998; Coskun, 1992; Vaya, 2003; Park, 2004; Ammar, 2005).

The carcinogenic and mutagenic potentials of the synthetic and natural substances whose biological effects are not yet known should be tested. In this study, we aimed to investigate the genotoxic effects of methanol and water extracts of *Rhamnus catharticus*, which is widely used by the public for its laxative effects, on lymphocyte cells isolated from human blood.

MATERIAL AND METHODS

Rhamnus catharticus seed was purchased from the local market as stock start-up. The weeds were dried at room temperature in dark, and powdered in order to prepare extracts.

The plant extraction (20 g) was prepared with 200 ml of methanol (which may develop toxicity) or water (which has no toxic effect) an ultrasonic water bath for 24 hours. After the extraction steps, the extracts were filtered and then the solvent (methanol/water) was evaporated to prepare aqueous stock solution (Ha'znagy-Radnai et al., 2006). The obtained extract (100 mg) was dissolved in 20 ml of water. The most commonly used doses are 1, 2, and 4 mg/ml, thus we examined the genotoxicity of these concentrations on human lymphocytes.

Patients

The study population consisted of 10 healthy individuals (20-30 years of age) with no exposure to genotoxic agents. Participants did not use cigarettes, alcohol or drugs. All participants were enrolled in the study after they were informed about it and consent was obtained. The study was performed in accordance with the ethical standards of Çanakkale Onsekiz Mart University.

200 µl blood samples were added to 2.5 ml Chromosome Medium B (Merck). Afterwards lymphocytes were cultured at 37°C for 72 h. The cells were treated with 1, 2, 4 mg/ml concentrations of methanol or aqueous extract of *Rhamnus catharticus* for 24 and 48 hours. The negative control group was treated with dH_2O and positive control group was treated with 0.2 mg/ml of mitomycin C (MMC); (Kyowa Hakko Co.). Slides were stained by standard method for CA assay (Speit and Haupter, 1985).

Cytochalasin B (Sigma-Aldrich), was added to the final concentration of six mg/ml and verified after 44 h. Cells were then harvested to perform micronucleus (MN) test (Fenech, 2000; Kirsch-Volders and Wakata, 2003). 1000 cells for each donor were scored to determine frequency of cells with one to four nuclei and then the mitotic index (MI) was calculated (Fenech, 2000).

Statistical Analysis

The data was analysed with SPSS 20.0. Data processing was carried out with count, mean value, standard deviation, median value, minimum/maximum values. As a result of Normal Distribution Suitability Test, non-parametric test Mann-Whitney U was used. $P < 0.05$ was accepted for statistical significance.

RESULTS

The goal of this study was to evaluate the *in vitro* cytotoxic activity of aqueous and methanol extracts of *Rhamnus catharticus* in human lymphocyte culture. The same three concentrations of methanol and aqueous extracts of *Rhamnus catharticus* were applied to human lymphocyte cultures for 24 and 48 hours periods (Tabs. 1 and 2).

It was observed that both methanol and aqueous extracts of *Rhamnus catharticus* increase the MI when compared to the negative control (Tabs. 1 and 2). In addition, there was no difference in MI between 24 hours and 48 hours application groups; despite the increasing concentration of methanol extracts of *Rhamnus catharticus* with 1 mg/ml being the exception (p values are 0.112, 0.821, and 0.049 respectively). Similar results were observed in aqueous extracts of *Rhamnus catharticus* when comparing the MI of groups which depend on time of exposure for three different concentrations (p values are 0.821, 0.199, and 0.496).

A dose-dependent effect was observed in both extracts of *Rhamnus catharticus* in micronucleus, binucleus and tetranucleus formation (Tabs. 1 and 2). The peak level of binucleate formation was achieved at 24 hours of incubation in both extracts for both two and four mg/ml doses and then the binuclear formation decreases at the following cell cycles. Binucleus formation is significantly induced in aqueous extracts in the 24th hours for the four mg/ml dose, more than methanol extract ($p < 0.001$). However, statistical evaluation of data has been shown there is no difference in binucleate formation between methanol and aqueous extracts of plants at the end of the 48th hours in three doses.

The comparison of tetranucleate formation between methanol/aqueous extracts of plant with negative control shown that the plant extract triggered tetra nucleate formation (0.004 and 0.007 respectively). When the tetra nucleus were counted mathematically by light microscopy after application in one, two, and four mg/ml doses, it was observed that the number of nuclei at 24 and 48 hours of methanol extract were higher than the water extract, in contrast, the results were not statistically significant. It was also observed the amount of tetranuclear formation decreased significantly by time in the administration of two mg/ml group both in methanol and aqueous extracts.

The micronucleus formation was triggered by methanol and aqueous extracts of *Rhamnus catharticus* and both extracts showed increase micronucleus formation when compared with the negative control. The amount of micronucleus formation decreased significantly in comparison with the positive control (Tabs. 1 and 2).

When the positive and the negative controls of all methanol doses were performed, statistically significant changes were observed. Compared to negative control, all parameters were higher and compared to positive control all parameters were lower (Tabs. 1 and 2).

Table 1: Comparison of micronucleus (MN), binucleate (BN), tetranucleate (TN), total cell counts (TCCs) and mitotic index (MI).

Treatment							
Period (h) n = 10	Conc. (mg/ml)		MN (Micronucleus)	BN (Binucleate)	TN (Tetranucleata)	Total cell counts (TCCs)	Mitotic Index (MI)
Methanol 48 h	4 mg	Mean Median (Min-Max)	9.90 ± 5.40 7.00 (6.00-23.00)	20.00 ± 6.27 19.50 (9.00-30.00)	7.20 ± 5.22 5,00 (3.00-18.00)	1042.00 ± 27.08 1046.50 (1007-1072)	4.94 ± 1.73 4.50 (2.60-8.10)
Methanol 48 h	2 mg	Mean Median (Min-Max)	7.90 ± 4.58 6.00 (4.00-19.00)	17.70 ± 8.94 19.50 (3.00-36.00)	4.40 ± 5.04 3.00 (1.00-8.00)	1051.60 ± 22.98 1054 (1008-1089)	4.33 ± 2.11 4.50 (1.30-9.10)
Methanol 48 h	1 mg	Mean Median (Min-Max)	5.40 ± 1.65 5.50 (3.00-9.00)	17.40 ± 3.75 17.50 (10.00-3.00)	3.20 ± 2.15 2.50 (1.00-8.00)	1060.00 ± 17.93 1051.50 (1037-1094)	4.02 ± 0.73 4.10 (2.60-5.10)
Methanol 24 h	4 mg	Mean Median (Min-Max)	7.30 ± 1.16 7.00 (6.00-9.00)	25.10 ± 3.72 24.00 (19.00-3.00)	6.90 ± 1.37 7.00 (5.00-9.00)	1048.80 ± 25.14 1050 (1008-1088)	5.75 ± 0.70 5.65 (4.70-7.30)
Methanol 24 h	2 mg	Mean Median (Min-Max)	8.00 ± 5.45 6.00 (4.00-23.00)	19.10 ± 2.02 19.00 (17.00-23.00)	5.90 ± 4.01 5.00 (3.00-17.00)	1053.60 ± 33.02 1054.50 (1014-1098)	4.62 ± 0.88 4.30 (3.90-6.91)
Methanol 24 h	1 mg	Mean Median (Min-Max)	5.70 ± 2.06 6.00 (2.00-9.00)	14.20 ± 1.81 14.50 (11.00-7.00)	3.80 ± 2.66 3.00 (2.00-0.00)	1060.60 ± 24.44 1061.50 (1023-1104)	3.41 ± 0.50 3.45 (2.40-4.10)
Positive control	MMC	Mean Median (Min-Max)	9.90 ± 2.47 9.00 (7.00-14.00)	24.20 ± 2.62 24.50 (19.00-8.00)	11.10 ± 4.12 10.00 (8.00-22.00)	1066.6 ± 50.80 1061.00 (1017-1196)	5.83 ± 0.56 6.00 (4.60-6.50)
Negative control	dH_2O	Mean Median (Min-Max)	3.20 ± 1.40 3.50 (1.00-5.00)	15.40 ± 2.63 16.00 (11.00-19.00)	2.50 ± 1.84 2.00 (1.00-7.00)	1074.20 ± 52.03 1055.50 (1025-1188)	3.40 ± 0.53 3.50 (2.60-4.30)

Table 2: Comparison of micronucleus (MN), binucleate (BN), tetranucleate (TN), total cell counts (TCCs) and mitotic index (MI) of aquaus extract time and dose depended.

Treatment							
Period (h) n = 10	Conc. (mg/ml)		MN (Micronucleus)	BN (Binucleate)	TN (Tetranucleate)	Total cell counts (TCCs)	Mitotic Index (MI)
Water 48 h	4 mg	Mean Median (Min-Max)	8.00 ± 2.58 7.00 (6.00-13.00)	19.60 ± 5.50 19.00 (9.00-29.00)	5.70 ± 2.54 5.50 (3.00-9.00)	1038.16 ± 28.4 1039.00 (1007-1069)	4.85 ± 1.07 4.55 (3.40-7.00)
Water 48 h	2 mg	Mean Median (Min-Max)	5.70 ± 1066.00 (4.00-7.00)	16.90 ± 3.54 18.00 (11.00-21.00)	4.40 ± 5.04 3.00 (1.00-18.00)	1058.00 ± 21.28 1060,00 (10271089)	3.95 ± 0.74 4.10 (2.70-4.90)
Water 48 h	1 mg	Mean Median (Min-Max)	5.40 ± 1.65 5.50 (3.00-9.00)	15.50 ± 4.08 14.00 (10.00-23.00)	2.90 ± 1.52 2.50 (1.00-6.00)	1060.00 ± 17.94 1051.50 (1037-1094)	3.64 ± 0.82 3.45 (2.60-5.10)
Water 24 h	4 mg	Mean Median (Min-Max)	7.30 ± 1.16 7.00 (6.00-9.00)	19.10 ± 2.38 19.00 (15.00-23.00)	5.10 ± 2.28 5.00 (2.00-9.00)	1041.70 ± 25.68 1040.00 (1008-1088)	4.55 ± 0.41 4.45 (3.80-5.20)
Water 24 h	2 mg	Mean Median (Min-Max)	6.60 ± 1.65 6.00 (4.00-9.00)	18.70 ± 1.49 19.00 (17.00-21.00)	5.90 ± 4.01 5.00 (3.00-17.00)	1053.60 ± 33.02 1054.50 (1014-1098)	4.40 ± 0.38 4.30 (3.90-5.10)
Water 24 h	1 mg	Mean Median (Min-Max)	5.70 ± 2.06 6.00 (2.00-9.00)	13.30 ± 2.06 13.50 (11.00-17.00)	3.30 ± 1.64 3.00 (2.00-7.00)	1060 ± 24.44 1061.50 (1023-1104)	3.23 ± 0.41 3.20 (2.40-3.90)
Positive control	MMC	Mean Median (Min-Max)	9.90 ± 2.47 9.00 (7.00-14.00)	24.20 ± 2.62 24.50 (19.00-8.00)	11.10 ± 4.12 10.00 (8.00-22.00)	1066.6 ± 50.80 1061.00 (1017-1196)	5.83 ± 0.56 6.00 (4.60-6.50)
Negative control	dH_2O	Mean Median (Min-Max)	3.20 ± 1.40 3.50 (1.00-5.00)	15.40 ± 2.63 16.00 (11.00-19.00)	2.50 ± 1.84 2.00 (1.00-7.00)	1074.20 ± 52.03 1055.50 (1025-1188)	3.40 ± 0.53 3.50

DISCUSSION

Micronucleus, binucleate and tetranucleate amounts can be significant parameters for some illnesses (Šošić et al., 2017). Micronucleus presence is an indicator of genomic instability and accumulated damages that appeared during the lymphocyte's life cycle and can be detected in vitro (Kopjar et al., 2010). The presence of micronuclei shed light on numerical and structural chromosomal aberrations (Albertini et al., 2000).

When *Rhamnus catharticus* methanol-extract is applied for a longer period, the data has shown that MI has decreased (Tab. 1). In fact the same time depended lower MI was obtained because the compounds that are soluble in methanol extract may inhibit the start of mitosis. In the cell cycle, there are several check points that regulate cell division. DNA damage is always controlled by molecular mechanisms during the cell cycle. These results have shown that the cells which are under pressure might stop mitosis in a longer treatment period. Thus, we could speculate the longer period of exposure time could cause an increase genotoxic activity of the methanol extract.

The results have shown that methanol extract of *Rhamnus catharticus* triggers tetranucleate and binucleate formation in the doses of four mg/ml and two mg/ml respectively (Tab. 1). These results are an indication of the increased toxicity of the doses over time. The significant increase in the amount of binucleate and MI suggests that this dose (one mg/ml) might be reliable (Tab. 1). However, the potential cytotoxic and genotoxic effects of these substances on human cells are not yet well-known. In comparison with the MMC substance, which is known to be toxic in human lymphocyte culture, and methanol exposure doses, it was determined that *Rhamnus catharticus* samples showed lower amounts of micronucleus, binucleate, tetranucleate, and MI levels. However, these micronucleus, binucleate, tetranucleate amounts and MI levels are considered to be toxic values in the literature (Kopjar et al., 2010).

In healthy individuals, micronucleus amount is found in certain ratios. Thus when we compare the results of different concentration of both methanol and aqueous extract of *Rhamnus catharticus* with negative control. And we observed that the number of micronucleus counts in the individuals included in the study was consistent with the data obtained from healthy persons as a result of the literature research.

The significant decrease in the amount of tetranucleate in the two mg/ml water extract group is indicative of the toxicity of this dose. Although the four mg/ml water extract group shows no change in time, for which all parameters were toxic (Tab. 2).

As expected, the water extracts of *Rhamnus catharticus* are more toxic compared to the negative control aside that there was a decrease when compared to MMC, toxicity was observed in all doses.

We could not find any significant difference between methanol and water extract for four mg/ml dose in 48 hours of application. The content of the methanol and aqueous extract of *Rhamnus catharticus* differs to each other, for that reason if higher concentrations of both extracts was evaluated the results may differ significantly. There is a huge information gap in literature on this topic. The level of cytotoxic activity on human cells and the genotoxic potential of non-cytotoxic concentrations should be established for all medicinal plants introduced to routine use for humans.

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