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Original article

Milk thistle may induce apoptosis in development of carbontetrachloride-induced liver DNA damage in rats

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Summary. The leaf and flower extracts of *Slybum marianum* [*Milk thistle* (MT)] have been used for liver, gall bladder and spleen disorders for centuries. 28 male Wistar albino (n=28, 8 weeks old) rats used in the study. The rats divided into 4 groups according to their live weights. The groups were: (i) Negative Control (NC): Normal water consuming group to which no CCl_4 and MT is administered; (ii) Positive Control (PC): Normal water consuming group to which no CCl_4 is administered but MT is administered; (iii) CCl_4 Group: Normal water consuming and group to which CCl_4 is administered (2 ml/kg live weight, ip); CCl_4 + MT group: CCl_4 and MT administered group (2 ml/kg live weight, ip). Tissue apoptotic index was examined via TUNEL method. MDA determination in plasma tissue was carried out using a spectrophotometer. As a Results, MDA amount decreased in the CCl_4 + MT group (3,65 ± 0,15 nmol/ml) in comparison to CCl_4 group (4,25 ± 0,21 nmol/ml) whereas it was observed in the CCl_4+MT group (12,62 ± 0,91 %) that the apoptotic index (TUNEL results) decreases in comparison with the CCl4 group (22,62 ± 0,91 %) thus approaching normal values. These results show that MT plant has a protective effect on liver.

Keywords: CCl_4, DNA damage, liver damage, *Milk thistle*, TUNEL

Introduction

CCl_4 is frequently preferred and used in experimental studies due to its similarities with the development stage of cirrhosis in humans (1, 2) despite the fact that it can cause liver damage together with many factors. CCl_4 is a transparent, dense and non-flammable liquid that can evaporate easily. It has a high stability. It can be found in nature and can also be generated as a result of many chemical reactions (3). It is known that medical plants are used widely for the treatment of diseases since ancient ages until today. The leaf and flower extracts of *Slybum marianum* [*Milk thistle* (MT)] have been used for liver, gall bladder and spleen disorders for centuries. In the 1960s, active biological principles of seed and fruit extracts were examined and light was thrown on their chemical structures (4-6). Recent studies have focused on the possible effects of MT in preventing cancer. MT extract consist chemically of isomer flavonolignans known as silymarin, silybin, isosilybin, silychristin and dehydrosilybin (7-9). The liver is a multifunctional organ that can carry out endocrine as well as exocrine functions (10). Liver has important storage functions. The food components absorbed from the digestive tract are carried to the liver where they are stored. Glucose is stored as glycogen. When blood glucose exceeds a certain level, insulin is secreted from the endocrine section of the pancreas

that enables the transport of glucose to the interior of the hepatocytes thereby transforming glucose into glycogen at the hepatocytes. Some vitamins are stored in the liver for future use. When the storage function is considered, vitamins soluble in fat (A, D, E, K), B_{12} and B_{19} vitamins can be given as examples. (10, 11). In case of DNA damage, the cell where the damage takes place is forced to apoptosis and is removed. Thus, the potential of the cells that have harmful DNA mutations to become cancerous is eliminated. The ineffective T lymphocytes which are important for the immune system as well as those that have the potential to cause reactions that are harmful to the tissues of the organism itself are removed via apoptosis prior to entering circulation (12). In this study carbontetrachloride was administered to rats and it was examined whether MT has protective effects against the damage that occurs in the liver or not.

Material and Method

Animal material and research groups

The animal experimentation work of our study was carried out at the F.U. Experimental Animals Research Institute (FUDAM) with the consent numbered 132 of the Firat University Animal Experiments Ethics Council during the 27.11.2013 dated meeting with a meeting number of 2013-11. 28 male Wistar albino (n=28, 8 weeks) rats were used in the study. Light and dark periods of 12 hours were applied to rats. The rats were distributed into 4 groups according to their live weights. The groups were: (i) Negative Control (NC): Normal water consuming group to which no CCl_4 and MT is administered; (ii) Positive Control (PC): Normal water consuming group to which no CCl_4 is administered but MT is administered; (iii) CCl_4 Group: Normal water consuming and group to which CCl_4 is administered (2 ml/kg live weight, ip); CCl_4 + MT group: CCl_4 and MT administered group (2 ml/kg live weight, ip). The extract of boiled MT seeds was mixed into the drinking water of animals to be used as MT source (10% w/v). The initial live weights of the animals were arranged to be equal. Live weights were recorded 3 times weekly throughout the study.

CCl_4 application

CCl_4 application was carried out twice per week for four weeks intraperitoneally at 2 ml/kg live weight injected together with olive oil at 1: 3 ratio (13).

Chemical substances and Milk thistle

All chemicals used in the experimental work were acquired from Sigma-Aldrich (Germany), Bio-Rad (USA), BioShop (Canada) and Merck (USA). Dust form MT seeds to be boiled and mixed in the daily drinking waters of rats were acquired from Oz Gida Organik and Yoresel Urunler Ltd. Sti. (Elazığ).

Preparation of Milk thistle extract

The extract prepared by mixing the ground MT seeds in water and boiling them was added to the drinking water of rats at a ratio of 10%. The water consumption of rats was monitored and recorded regularly.

Tissue homogenization

Liver tissue samples were divided into small parts and broken down inside lizis buffer (0,5 M Tris; pH: 8, EDTA, ß-Mercaptoethanol, Phenyl methyl sulphonyl fluoride [PMSF]) in mechanical homogenizator. These broken down tissue samples were centrifuged at 15000 rpm for 45 minutes. Supernatant was taken and was stored at -80^{0}C until usage time (13).

Analysis of DNA damage

The DNA samples acquired from tissues were dissolved in Tris-EDTA solution and analyzed in 1 % gel via DNA agarose gel electrophoresis (12, 14). Images of the DNA bands were taken afterwards and similarities as well as differences were determined.

Determination of apoptosis levels via TUNEL method

Apoptosis in paraffin blocks, frozen cross sections, cells grown on cultured "plates" in the form of solutions or in lamellae can be determined using this method (15, 16). In our study, the damage that occurs

Table 1. MDA level between groups in the liver tissue.

Groups	Negative control	Positive control	CCl_4	CCl_4 + milk thistle
Plasma MDA (nmol/ml)	2.72 ± 0.18[c]	1.59 ± 0.25[d]	4.25 ± 0.21[a]	3.65 ± 0.15[b]

a-d: Differences between groups in the same column with different letters are statistically significant (p<0.05); One-Way ANOVA Post Hoc Tukey Test

in the DNA of liver tissue was determined via the TUNEL method.

Plasma MDA measurements

MDA determination of the plasma samples was carried out in accordance with the method developed by Ohkawa et al. (1979) (17).

Statistical analyses

All data were evaluated via SPSS 20 package software using variance analysis. One-Way ANOVA *post hoc* Tukey test was applied to determine the differences within the groups. The measurements were repeated at least 3 times to ensure the reliability of the statistical analyses after which they were recorded in the SPSS 20 package software and evaluation process was started.

Results

MDA measurement results

The values in Table 1 and Figure 1A show that MDA level decreases in the plasma tissue in groups to which MT is administered and that the highest MDA levels are observed in CCl_4 groups (p<0.05).

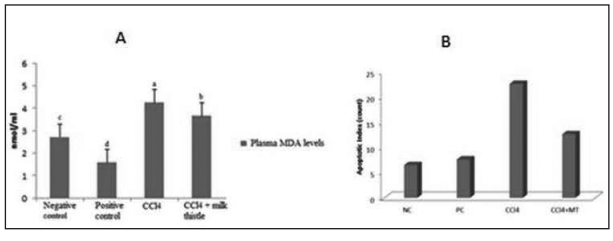

Figure 1. A) Comparison of the MDA levels in plasma between groups. B) Apoptotic index in liver tissue. a-d: Differences between groups in the same column with different letters are statistically significant (p<0.05). One-Way ANOVA Post Hoc Tukey Test

DNA damage result

When the DNA agarose gel image in Figure 2 is examined, it is observed that there are more fragmentations (ladder pattern) in the carbon tetrachloride group than in the control groups. Whereas the DNA fragmentations in the CCl_4 + MT group is comparatively less than those of the CCl_4 group. According to these results, the following interpretations can be made: the DNA damage ratio of the liver damage in the MT administered group with liver damage is less in comparison with that in the group with liver damage to which no MT is administered and accordingly,

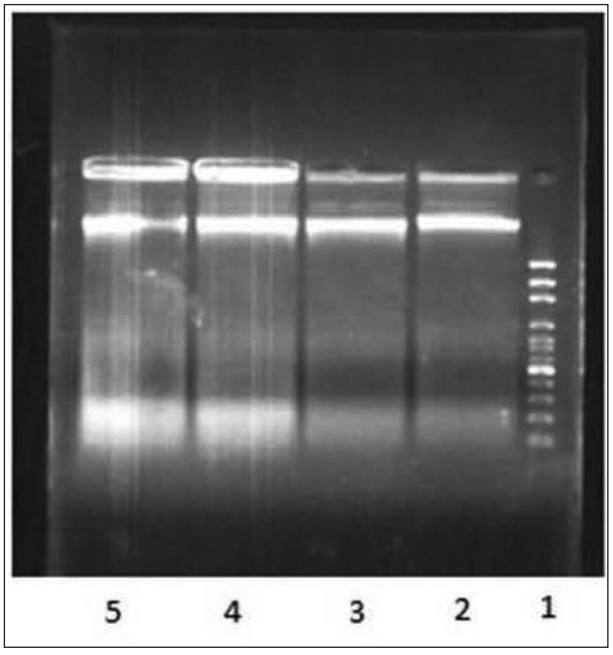

Figure 2. Cellular DNA damage in the control group and experimental group rats. Lines (right to left): (1) Marker; (2) NC; (3) PC; (4) CCl4; (5) CCl4 + MT

it can be stated that MT can decrease the DNA damage with regard to liver damage due to its antioxidant effect (7, 18).

Histological results determined via TUNEL method

After completing the study, apoptosis levels were determined via TUNEL method. Damages in the liver tissue have been determined using the acquired images. A statistically significant increase was observed in the CCl_4 (Fig. 3D) group ($p<0.05$) as a result of the comparison of TUNEL positivity in the liver tissue with the NC (Figure 3A) group following the examination of the TUNEL dyeing under the light microscope. When compared with the CCl_4, no statistically significant difference was determined only in the PC group (Fig. 3C) to which only MT was administered. Whereas it was observed in the CCl_4+MT (Fig. 3B) group that the apoptotic index decreases in comparison with the CCl4 group (Fig. 3D) and approaches normal values.

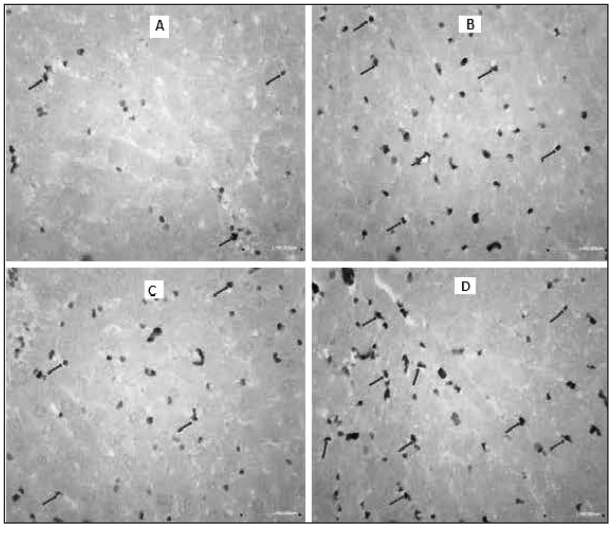

Figure 3. A) TUNEL positive cells (→) in the Negative Control group liver tissue. Scale bar: 50 μm.
B) TUNEL positive cells (→) in the CCl4 + Milk thistle group liver tissue. Scale bar: 50 μm.
C) TUNEL positive cells (→) in the Positive Control group liver tissue. Scale bar: 50 μm.
D) TUNEL positive cells (→) in the CCl4 group liver tissue. Scale bar: 50 μm.

Discussion

We hope that the results obtained in this study will contribute greatly to scientific studies when the current relevant literature is examined. Shaker et al. (2010) put forth that MDA which is the final product of lipid peroxidation plays a remarkable free radical role in liver damage. It has been observed in the study carried out by Shaker et al. (2010) that the MDA level in the plasma of rats who have a normal diet and are not treated in any manner is significantly lower in comparison with the group that is treated with CCl_4. ($p<0.05$) (19). Senturk et al. (2010) observed in their study carried out to examine the role of MT in the oxidative damage that occurs in the rat kidneys that MDA level in the ischemia reperfusion group is high and that it decreases in the 50 mg/kg MT group, whereas they also observed that the MDA level is high in the 100 mg/kg MT group after which they put forth that MT extract decreases MDA level at a certain ratio but that it might have adverse effects at high doses (7). When the plasma MDA levels are compared (Table 1, Fig. 1A) no statistically significant difference was observed among the groups ($p<0.05$). The highest MDA level was observed in the CCl_4 group. Whereas the MDA level of the CCl_4+MT group was determined to be lower in comparison with that of the CCl_4 group and a statistically significant difference was observed. It is striking to observe that MDA level is low only in the PC group which was treated with MT. Zhao et al. (2012) carried out a study examining the negative effects of acetoaminophenin on liver and when the TUNEL method results are examined, apoptotic index ratio of groups damaged with this substance was observed to be statistically significantly higher in comparison with other groups ($p<0.05$) (20). Tas et al. (2012) put forth that toluene mostly causes apoptotic neurodegeneration in the brain tissue but that it also affects tissues such as liver, lungs, testicles and put forth as a result of TUNEL method examinations that toluene causes caspase-3 activity increase (21). Yu et al. (2014) carried out a study examining the effects of *Dioscorea nipponica* (Dioscorea) on CCl_4 caused liver damage and when the TUNEL method results are examined, it was observed that the apoptotic index values in the CCl_4 administered group are statistically significantly

Table 2. Apoptotic Index

Groups	Negative control	Positive control	CCl_4	CCl_4 + milk thistle
Apoptotic index	6.5 ± 0.53[c]	7.62 ± 0.51[c]	22.62 ± 0.91[a]	12.62 ± 0.91[b]

a-c: Differences between groups in the same column with different letters are statistically significant (p<0.05); One-Way ANOVA Post Hoc Tukey Test

higher in comparison with other groups (p<0.05) (22). And also Aslan and Can (2015a, 2015b) and Aslan (2015) indicated that different fruit juice extract has positive effect on microorganism growth (23-25). According to our results, when the DNA agarose gel electrophoresis images are examined (Fig. 2) for liver damage caused in rats after CCl_4 was administered it was observed that there was a significant difference in terms of DNA fragmentation between the damaged group administered with MT and the damaged group to which no MT was administered. DNA fragmentation is less in the MT administered damaged group in comparison with the damaged group to which no MT was administered. According to Aslan and Can (2014) MT has protective effect on CCl4 induced liver damage in rats (13). When the TUNEL method results in our study are examined, no statistically significant difference was determined between the NC and PC (Fig. 1B, Table 2) groups; however an increase was observed in the CCl_4 group (Fig. 3D) that caused a statistically significant difference when compared with the NC group (p<0.05). Whereas it was observed that the apoptotic index of the CCl_4+MT group approaches normal values in comparison with the CCl_4 group (Fig. 3B, Fig. 3D). These differences led us to think that MT extract can relatively prevent DNA damage in damaged cells and that it protects the liver against possible damage in rats.

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References

1. Jeon T, Hwang SG, Park NG, Jung YR, Shin SI, Choi SD, et al. Antioxidative effects of chitosan on chronic carbon tetrachloride induced hepatic ınjury in rats. Toxicology 2003; 187: 67-73.
2. Singhal KG, Gupta GD. Hepatoprotective and antioxidant activity of methanolic extract of flowers of nerium oleander against ccl4-ınduced liver ınjury in rats. Asian Pac J Trop Med 2012; 5(9): 677-685.
3. Stehbens EW. Oxidative stress, toxic hepatitis, and antioxidant with particular emphasis on zinc. Exp Mol Pathol 2003; 75: 265-276.
4. Sonnenbichler J, Scalera F, Sonnenbichler I, Weyhenmeyer R. Stimulatory effects of silibinin and silicristin from the Milk thistle silybum marianum on kidney cells. JPET 1999; 290: 1375-1383.
5. Aslan A, Boydak D, Can MI, Kuloglu T, Baspinar S. Black cumin may be a potential drug for development of carbon-tetrachloride-induced lung damage in rats. Progress in Nutrition 2016; 18(1): 56-62.
6. Aslan A, Can MI, Boydak D. Anti-oxidant effects of pomegranate juice on saccharomyces cerevisiae cell growth. African Journal of Traditional Complementary and Alternative Medicines 2014; 11(4): 14-18.
7. Senturk H, Kolankaya D, Sahin Y. The effect of silymarin on oxidative stress during renal ischemia-reperfusion ınjury in rat kidney crashed. Çankaya University J of Science and Eng 2010; 1: 59-74.
8. Aghazadeh S, Amini R, Yazdanparast R, Ghaffari SH. Anti-apoptotic and Anti-inflammatory effects of Silybum marianum in treatment of experimental steatohepatitis. Exp Toxicol Pathol 2011; 63: 569-574.
9. Aslan A, Boydak D, Can MI, Kuloglu T. Nigella sativa improves the carbon tetrachloride-induced lung damage in rats through repression of erk/akt pathway. Bangladesh Journal of Pharmacology 2015, 10(3): 654-659.
10. Campbell NA, Reece JB. 2006; Biology, San Francisco.
11. Aktumsek A. Human Physiology, Nobel Broadcast Distribution, 2007; Ankara, Turkey
12. Aslan A. The effect of lycopene on the cyclooxygenase (cox-2), caspase-3, caspase-9, bax, bcl-2, p53 Protein expression and DNA damage in rats with azoxhymethane-induced colorectal cancer, Phd Thesis, 2011; Fırat University Institute of Natural and Applied Sciences, Elazığ Turkey.
13. Aslan A and Can MI. Milk thistle impedes the develop-

ment of carbontetrachloride-induced liver damage in rats through suppression of bcl-2 and regulating caspase pathway. Life Sciences 2014; 117: 13-18.
14. Wang JY, Shum AY, Ho YJ, Wang JY. Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and no on apoptosis ınvolving activation of p38 mitogen-activated protein kinase and caspase-3. J Neurosci Res 2003; 72: 508-519.
15. Yilmaz I. The Apoptosis levels rise in adult rats after experimental varicocele testicular germ cel and the assessment of the increased apoptosis after varicocelectomy throwback level and time for viatunnel method, expertise thesis, 2005; Taksim Training and Research Hospital, Department of Urology, Istanbul.
16. Qiu J, Liu Z, Li Y, Xuan H, Lin Q, Li F, et al. Overexpression of the gene for transmembrane 4 superfamily member 4 accelerates liver damage in rats treated with CCl4, J Hepatol 2007; 46: 266-275.
17. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analyses of Biochemichal 1979; 95: 351-358.
18. Ustundag B, Bahcecioglu I, Sahin K, Gulcu F, Duzgun S, Ozercan IH, et al. Effects of soy ısoflavones on carbon tetrachloride (ccl4)-ınduced liver damage and on the level of plasma paraoxonase with arylesterase activities. Firat University Medical J of Health 2005; 19: 263-271.
19. Shaker E, Mahmoud H, Mnaa S. Silymarin, the antioxidant component, and silybum marianum extracts prevent liver damage. Food Chem Toxicol, 2010; 48: 803-806.
20. Zhao X, Cong X, Zheng L, Xu L, Yin L, Peng J. Dioscin, a natural steroid saponin, shows remarkable protective effect against acetaminophen-ınduced liver damage in vitro and in vivo, Toxicol Lett 2012; 214: 69-80.
21. Tas U, Ogeturk M, Sapmaz H, Karaca ZI, Ozyurt B, Sogut E, et al. Investigation of protective effect of melatonin against toluene-induced apoptosis in rat prefrontal cortex. Firat University Medical J of Health 2012; 26: 1-6.
22. Yu H, Zheng L, Yin L, Xu L, Qi Y, Han X, et al. Protective effects of the total saponins from *Dioscorea nipponica* makino against carbon tetrachloride-induced liver injury in mice through suppression of apoptosis and ınflammation. Int Immunopharmacol. 2014; 19: 233-244.
23. Aslan A, Can MI. The inhbition of chromium effect in *Saccharomyces cerevisiae* thrive from grapefruit. Progress in Nutrition 2015a; 17(4): 339-343.
24. Aslan A, Can MI. The effect of orange juice against to H_2O_2 stress in *Saccharomyces cerevisiae*. Progress in Nutrition 2015b; 17(3): 250-254.
25. Aslan A. The effects of different essential fruit juice and their combination on *Saccharomyces cerevisiae* cell growth. Progress in Nutrition 2015; 17(1): 36-40.

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