BENEFICIAL EFFECTS OF PROPOLIS ON METHOTREXATE-INDUCED LIVER INJURY IN RATS

Sıçanlarda Metotreksata Bağlı Karaciğer Hasarında Propolisin Olumlu Etkileri

Aysun Çetin, Leylagül Kaynar, Barış Eser, Canan Karadağ, Berkay Saraymen,

Ahmet Öztürk, İsmail Koçyiğit, Sibel Kabukçu Hacıoğlu, Betül Çiçek, Sibel Silici

Running title: Beneficial effects of propolis on liver injury in rats

Aysun Çetin, Assist. Prof., MD, (Department of Biochemistry and Clinical Biochemistry, School of Medicine, Erciyes University, Kayseri, Turkey)

Leylagül Kaynar, MD, (Department of Hematology, School of Medicine, Erciyes University, Kayseri, Turkey)

Canan Karadağ, Research Assistant, MD, (Department of Biochemistry and Clinical Biochemistry, School of Medicine, Erciyes University, Kayseri, Turkey)

Berkay Saraymen, MSc Candidate, Chemist, (School of Pharmacy, Erciyes University, Kayseri, Turkey)

Ahmet Öztürk, Lecturer, PhD, (Department of Biostatistics, School of Medicine, Erciyes University, Kayseri, Turkey)

İsmail Koçyiğit, Specialist, MD, (Department of Hematology, School of Medicine, Erciyes University, Kayseri, Turkey)

Sibel Kabukçu Hacıoğlu, Specialist, MD, (Department of Hematology, School of Medicine, Pamukkale University, Denizli,

Sibel Silici, Assoc. Prof., PhD, (Department of Animal Sciences, Safiye Cikrikcioglu Vocational College, Erciyes University, Kayseri, Turkey)

Betül Çiçek, Assist. Prof., PhD, (Department of Nutrition and Dietetics, Ataturk Health School, Erciyes University, Kayseri, Turkey)

Barış Eser, Specialist, MD (Department of Hematology, School of Medicine, Erciyes University, Kayseri, Turkey).

Corresponding Author:

Aysun Çetin, Assist. Prof., MD, (Department of Biochemistry and Clinical Biochemistry, School of Medicine, Erciyes University, Kayseri, Turkey)

Phone: +90 352 – 437 9348

Fax: +90 352 – 437 9348

e-mail: aysuncetin@yahoo.com

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Background/aim: The efficacy of methotrexate (MTX), a widely used cytotoxic chemotherapeutic agent, is often limited by severe liver injury. Propolis is a natural bee product rich in polyphenolic compounds known for antioxidant activity. We investigated the protective role of propolis on MTX-induced liver injury in rats. **Methods:** *Forty-eight Wistar Albino male rats* were assigned equally into four groups: MTX group received placebo (distilled water) orally (for ten days) and a single dose of 20mg/kg MTX was administered intraperitoneally (on the seventh day). Propolis-MTX group received 100 mg/kg/day propolis extract orally (for ten days), and same dose MTX. Propolis group received 100 mg/kg/day propolis extract (for ten days), and equally volume physiological saline was administered intraperitoneally instead of MTX (on the seventh day). Control group received distilled water (for ten days), and also physiological saline was administered intraperitoneally (on the seventh day). Malondialdehyde (MDA) concentration, and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activity levels were determined in the homogenate of liver. **Results:** MTX increased liver MDA concentration and decreased the SOD, GSH-Px and CAT activity levels ($p<0.001$). The addition of propolis significantly decreased MDA concentrations and increased the GSH-Px levels in liver of the rats receiving MTX ($p<0.001$). There was numeric improvement SOD and CAT activity levels but it did not reach to the statistically significance ($p>0.05$). **Conclusions:** The MTX-induced oxidative stress was decreased by propolis, probably by its antioxidant components.

Key Words: Antioxidant activity, liver injury, methotrexate, oxidative stress, rats, Turkish propolis.

SIÇANLARDA METOTREKSATA BAĞLI KARACIĞER HASARINDA PROPOLISIN OLUMLU ETKILERI

Amaç: Yaygın olarak kullanılan sitotoksik bir ilaç olan metotreksatın kullanımı sıklıkla ciddi karaciğer hasarı nedeniyle kısıtlanmaktadır. Propolis antioksidan aktiviteleri bilinen polifenolik bileşiklerden zengin doğal bir arı ürünüdür. Bu çalışmada, sıçanlarda metotreksata (MTK) bağlı karaciğer hasarı üzerine propolisin koruyucu rolünü araştırdık.

Yöntem: Wistar albino 48 erkek sıçan, eşit olarak dört gruba ayrılmıştır. MTK grubu 10 gün boyunca 100 mg/kg/gün plasebo distile su oral yolla ve tek doz 20 mg/kg MTK'ı 7. günde intraperitoneal almıştır. Propolis-MTK grubu 10 gün boyunca 100 mg/kg/gün propolis ve aynı doz MTK almıştır. Propolis grubu 10 gün boyunca 100 mg/kg/gün propolis ve MTK yerine 7. günde eşit hacim fizyolojik tuzlu su intraperitoneal almışlardır. Kontrol grubu 10 gün boyunca distile su ve 7. günde eşit hacim fizyolojik tuzlu su intraperitoneal almıştır. Karaciğer homojenat örneklerinde malondialdehit (MDA) konsantrasyonu, süperoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px) ve katalaz (KAT) aktivite düzeyleri ölçülmüştür.

Bulgular: MTK karaciğer MDA düzeylerini artırmış ve SOD, GSH-Px ve KAT düzeylerini belirgin olarak azaltmıştır ($p<0.001$). Propolis eklenmesi, MTK alan sıçanların karaciğerinde MDA düzeyini azaltmış ve GSH-Px seviyelerini anlamlı olarak artırmıştır. SOD ve KAT seviyelerinde izlenen rakamsal iyileşme istatistiksel olarak anlamlı seviyelere ulaşmadı. ($p>0.05$). **Sonuç:** MTK'ın neden olduğu oksidatif stres propolis verilmesiyle azalmış ve bu etkinin büyük olasılıkla propolisin yapısındaki antioksidan bileşenlerden kaynaklandığı düşünülmüştür.

Anahtar Kelimeler: Antioksidan aktivite, karaciğer hasarı, metotreksat, oksidatif stres, sıçan, Türk propolisi

INTRODUCTION

Methotrexate (MTX), a structural analogue of folic acid, is widely preferred as a cytotoxic chemotherapeutic agent in the treatment of malignancies and some autoimmune diseases. While the cytotoxic effect of MTX is not selective for cancer cells, it also affects the normal tissues which have a high rate of proliferation, including the hematopoetic cells in the bone marrow and actively dividing cells of the intestinal mucosa (1). Thus, the efficacy of MTX is limited due to its toxic side effects. Studies revealed that the systemic oxidative stress is an important factors background of the MTX induced toxicity (2-5). MTX causes differential toxic effects on lipid peroxidation by significant reduction in glutathione (GSH) levels leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to eactive oxygen species (ROS) (6). MTX may also depress nucleic acid metabolism. Thus, the significant reduction in glutathione levels promoted by MTX, leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to ROS (1).

In biological structures, malondialdehyde (MDA) is considered to be the most significant indicator of membrane lipid peroxidation arising from the interaction of ROS with cellular membranes (6). The important enzymatic antioxidant defense mechanisms in the tissues are dismutation of superoxide ($.O_2^-$) to form hydrogen peroxide (H_2O_2) and O_2 by superoxide dismutase (SOD), as well as the conversion of H_2O_2 to molecular O_2 and H_2O by catalase (CAT) or conversion of H_2O_2 to H_2O by glutathione peroxidase (GSH-Px). CAT and GSH-Px are unique key enzymes scavenging hydroperoxides (6,7).

Propolis (bee glue), a natural product produced by the honeybee (*Apis mellifera*, L.), has been used for thousands of years in folk medicine for several purposes. Its chemical composition is very complex and varies with geographic origin. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other substances, including organic debris (8). The propolis extract contains amino acids, phenolic

acids, phenolic acid esters, flavonoids, cinnamic acid, terpenes and caffeic acid. Many of the physiological actions of these flavonoids have been attributed to their antioxidant properties, via their reducing capacities (catalysis of electron transport, ability to scavenge free radicals) (9-15). In addition, propolis has been determined to reverse the depletion of liver glutathione, and has radical scavenging activity (16). Recent reports suggest that propolis may have an important role in balancing antioxidant systems and has an antiperoxidant effect on several tissues, which may account for its beneficial effect in oxidant induced injury (15, 16).

However, no previous study exists on the declining/preventive effects of propolis on MTX-induced oxidative stress, the role of antioxidant enzymes in the inhibition of this potential. We aimed to investigate the beneficial effect of propolis extract against MTX-induced hepatotoxicity using biochemical approaches, including the assessment of MDA levels, in addition to SOD, GSH-Px and CAT activities.

MATERIALS AND METHODS

Animals and experimental design

Forty-eight male, Wistar Albino rats, weighing 230–310 g, were obtained from The Hakan Cetinsaya Experimental and Clinical Research Animal Laboratory of The Erciyes University. The animals were maintained in a 12:12 h light/dark photoperiod, at a fixed temperature of 22-24°C, and were provided *ad libitum* access to animal feed and drinking water, and were given a rat feed containing 7% crude cellulose, 23% crude protein and 2600 kcal/kg, for at least two weeks prior to and throughout the experiment. All experimental protocols were approved by the Erciyes University, School of Medicine, Animal Care and Use Committee (date: 05/12/2006; number: 01/429). Experiments were performed in in adherence to the National Institutes of Health guidelines on the use of experimental animals.

We randomized 48 rats into 4 groups as follows:

MTX group: The rats received placebo (distilled water) orally for 10 days and a single dose of 20mg/kg MTX, was administered intraperitoneally (ip) on the eighth day.

Propolis-MTX group: The rats were orally administered 100 mg/kg/day body weight propolis extract for 10 days, and a single dose of 20mg/kg MTX, was administered ip on the seventh day.

Propolis group: The rats were orally administered 100 mg/kg/day body weight propolis extract for 10 days, and instead of MTX, physiological saline was administered ip on the eighth day.

Control group: The rats received distilled water for ten days and physiological saline was administered ip on the eighth day.

Liver tissue samples were obtained under deep anesthesia, On the 11th day. The animals were scarified by 100 mg/kg ip ketamine administration. The liver samples were excised immediately and homogenized in 10-fold volume of 50 milimolar (mM) phosphate buffer solution pH 7.4 using a homogenizer (Ultra-Turrax T25, IKA, Werke 24000 r.p.m.j. Germany). The homogenates were centrifuged at 10000x g for about 60 min and the resulting supernatant were stored at -80°C until the time for MDA, SOD, GSH-Px and CAT assays.

The collection and extraction of propolis

Turkish poplar type propolis was used. Propolis sample was collected manually from honey bee colonies of *A. mellifera caucasica,* kept Bunyan, in Kayseri (Central Anatolia, Turkey). The obtained propolis samples were stored in dark conditions prior to processing. The chemical composition of propolis used in this study was determined in a previous study (17). Voucher specimen was kept in Department of Oncology, Faculty of Medicine, University of Erciyes, Kayseri. 30 g of propolis was extracted with 80% ethyl alcohol for three days. The extract was filtered through Whatmann filtrate paper. The process was repeated twice and the extracts were mixed. The alcohol fragment of the extract was evaporated by a vacuum evaporator and pure propolis was obtained.

The chemical composition of ethanol-extracted Turkish (Kayseri) propolis was given in Table 1.

Biochemical analysis

Determination of liver malondialdehyde (MDA) activity

The levels of MDA in liver tissue were assessed according to the method described by Ohkawa *et al.* (18). The assay procedure for MDA level in rat liver was set up as follows: to samples less than 0.2 ml of 10% (w/v) tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS) and 1.5 ml of 20% acetic acid solution were added. pH was adjusted to 3.5 with NaOH and 1.5 ml of 0.8% thiobarbituric acid (TBA). The final volume was brought to 4.0 ml by distilled water and then heated in an water bath at 95°C for 60 min using a glass ball as a condenser. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously. After centrifugation at 4.000 rpm for 10 min, the organic layer was removed and its absorbance was measured with a spectrophotometer (Hitachi, Japan) at 532 nm. MDA levels were expressed in nanomoles MDA per milligram of protein in tissue homogenates (nmol/mg protein). Protein concentrations in tissue homogenates were measured according to Lowry *et al*. (19).

Determination of liver SOD activity

SOD activity was determined according to the method of Sun *et al.* (20). The principle of the method is based on inhibition of Nitro Blue Tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as unit/mg protein (U/mg protein)

Determination of liver GSH-Px activity

Liver GSH-Px activity was determined according to Paglia and Valentine (21), using hydrogen peroxide as substrate. The reaction mixture contained 2.48 ml of a 50mM/l phosphate buffer, pH 7 (Sigma), 0.01 ml 112.5 mM/l sodium azide, and 4.6 U glutathione reductase (Type

III, Sigma). The reaction was initiated by adding 0.1 ml 2.2 mM H_2O_2 to the reaction mixture containing 500-1000 µg protein. The change in the absorbance was read at 340 nm for 4 min. The data was expressed as U/g prot.

Determination of liver CAT activity

CAT activity was determined in the homogenate as described by Aebi (22). Briefly, 100µl of the tissue supernatant was incubated with an equal volume of absolute alcohol for 30 min at 0°C followed by the addition of triton-X-100. A known volume of this tissue reaction mixture was taken in an equal volume of 0.066 M H_2O_2 in phosphate buffer and absorbance was measured at 240 nm for 30 sec in a spectrophotometer. An extinction coefficient of 43.6 mM/cm was used to determine the enzyme activity, which was expressed as U/mg protein.

Statistical analysis

Data were expressed as mean ± standard deviation ($\overline{X} \pm SD$). Comparisons of MDA, SOD, GSH-Px and CAT between the groups were made using the one-way analysis of variance (ANOVA). Post-hoc comparisons were conducted by Tukey's procedure. $p<0.05$ was considered to be statistically significant. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 15.0 for windows (SPSS Inc., Chicago, IL, USA).

RESULTS

In the course of the study, two rats in both the MTX and propolis-MTX groups died 48 to 72 hours after the MTX administration.

MDA levels were significantly increased in MTX group versus propolis-MTX, propolis and control groups in the multiple comparisons (Table 1; $p<0.001$). Propolis significantly decreased MDA levels in MTX-propolis groups compared to MTX group ($p<0.001$), but this improvement was not reach to the levels of propolis and control group ($p<0.001$).

SOD activity levels significantly reduced in MTX group as compared to other groups ($p<0.001$). The addition of the propolis numerically increased SOD activity levels in propolis-MTX group, but this improvement was not statistically important ($p>0.05$). There was no significant difference with respect to SOD activity levels between control and propolis groups ($p>0.05$).

GSH-Px activity measurement revealed that a significant reduction in MTX group than MTX-propolis, propolis and control groups in multiple comparison analysis, ($p<0.001$). The addition of the propolis significantly prevented MTX induced decrement in GSH-Px levels in propolis-MTX group ($p<0.001$). GSH-Px activity numerically increased in propolis group as compared with control group but this increment was not significant ($p>0.05$).

MTX also induced a significantly decreased CAT activity in MTX groups ($p<0.001$). CAT activity numerically was higher propolis-MTX groups than MTX group, but this difference was not significant ($p>0.05$).

DISCUSSION

In studies using different propolis types belonging to the other regions of the world, Christov *et al*. (23), demonstrated Canadian propolis, including phenolic compounds, to exhibit strong radical scavenging property. Similarly, the antioxidant activities of Argentinian, Brazilian, Greek, Cypriot, and Croatian propolis samples were demonstrated by several researchers (24-27). As claimed by other researchers, this beneficial effect of propolis was demonstrated to originate from flavonoids. In accordance with our previous study (17), in the current study, the main components primarily responsible for antioxidant and antiradical activity were determined to be the phenolic compounds existing in the propolis extract used, including caffeic acid phenethyl ester (CAPE), naringenin, 4-vinyl-2-methoxyphenol, fatty and aromatic acids in the propolis extract.

Propolis administration reported to improve the activity of hepatic microsomal drug metabolizing enzymes, significantly inhibited lipid peroxidation and markedly enhanced glutathione in liver and kidney. Propolis treatment also reversed carbon tetrachloride induced severe alterations in histological architecture of liver and kidney (15). Elevated MDA levels revealed that lipid peroxidation mediated by oxygen-free radicals is an important cause of destruction and damage to cell membranes, which was an important contributing factor to the development of MTX-mediated tissue damage. Co-administration of propolis with MTX, lowered MDA formation in the rat liver tissue. This effect may be due to the phenolic components of propolis and their antioxidant activity (15, 16).

Flavonoids may also exert antioxidant abilities through protection or enhancement of endogenous antioxidants. The free radicals activated by chemotherapeutic drugs are scavenged

by SOD and CAT. Numerous flavonoids have been shown to alleviate oxidative stress by increasing the endogenous antioxidant status, protecting cells against free-radical damage by increasing resistance to oxidative stress. In accordance with previous studies, we detected diminished SOD, GSH-Px, and CAT activities in MTX-treated rat liver. These depletions were reversed when the rats were co-administered propolis. These data lead to the conclusion that oxidative stress is one of the mechanisms of MTX cytotoxicity and that propolis may have a protective effect due to this kind of oxidative stress.

Several components of the propolis extract used in this study possess antioxidant properties (17). Gnanasoundari and Pari (28), have reported naringenin found in the propolis sample studied, to exert a positive effect on the increment in lipid peroxide levels and decline in CAT, SOD and GSH-Px activities, resulting from renal and liver oxidative injury in rats exposed to oxytetracycline. Furthermore, Salah *et al.,* (29) have reported β-eudesmol to exhibit antioxidant effects. Another organic compound propenoic acid has been reported to induce GSH-Px and antiradical activity (30). Besides, the antioxidant effects of Turkish propolis against propetamphos and sodium floride exposure in rats, was reported (5, 17).

Okutan *et al.* (31), reported CAPE, an active component of propolis has an ameliorating effect on oxidative stress via its antioxidant property. CAPE induced a decrease in MDA levels, SOD and CAT activities. CAPE exerted a dose-dependent free radical scavenging effect and antilipoperoxidative capacity. Ozyurt *et al.* (32), reported the antioxidant properties of CAPE to be similar to those of vitamin E. Ates *et al.* (33), reported CAPE to regulate antioxidant enzymes, inhibit lipid peroxidation and reduced liver injury in rats exposed to cold stress.

In the current study, the administration of 20 mg/kg MTX in rats was determined to cause oxidative stress. The liver MDA levels of rats administered MTX alone were determined to be increased. On the other hand, analyses of SOD, GSH-Px and CAT activities revealed a decrease. The changes in these variables suggest the antioxidant enzymes activities to be insufficient in compensation of free radicals generated at a single dose level (20 mg/kg) upon

the treatment of MTX at the indicated dose. Indeed, the increase in the MDA level also confirmed this situation. The decrease in the activities of the enzymes can be explained either with their induction during the conversion of free radicals into inactive metabolites or secondarily with the direct inhibitory effect of MTX on enzyme activity. Among relevant studies that have been conducted in rats, Jahovic *et al. (*3), reported MTX to increase MDA levels in blood, liver, and kidney tissues. Similarly, we reported a significant increase in liver homogenates MDA levels and decrease in SOD and CAT activities (34).

Regarding the results of the present study, it is likely that free radical scavenging and antioxidant properties of propolis protected the liver against MTX-induced injury. Furthermore, it was demonstrated in previous studies that propolis has antibacterial activity and it may has some beneficial effects by accelerating healing ability.

In conclusion, exogenous propolis administration is capable of reversing the oxidative toxic effects of MTX. These data suggest that propolis, by preventing liver injury, may enhance the tolerability of MTX in clinical studies.

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Table 1. Oxidative stress parameters of rat liver within 4 study groups

Variables	MTX (n=10)	Propolis-MTX (n=10)	Propolis (n=12)	Control (n=12)	p
MDA $\overline{X} \pm SD$ (nmol/mg prot)	3.17 ± 0.43[a]	2.07 ± 0.17[b]	1.21 ± 0.25[c]	1.32 ± 0.34[c]	<0.001
SOD $\overline{X} \pm SD$ (U/mg prot)	5.28 ± 0.69[a]	6.54 ± 0.73[a]	11.50 ± 1.95[b]	11.88 ± 1.49[b]	<0.001
GSH-Px $\overline{X} \pm SD$ (U/g prot)	13.88 ± 2.00[a]	17.18 ± 1.17[b]	28.60 ± 2.12[c]	24.88 ± 1.94[c]	<0.001
CAT $\overline{X} \pm SD$ (U/mg prot)	0.42 ± 0.13[a]	0.58 ± 0.12[a]	1.06 ± 0.21[b]	0.97 ± 0.14[b]	<0.001

$\overline{X} \pm SD$: mean ± standard deviation, MTX: Metothrexate, MDA: malondialdehyde, SOD: superoxide dismutase, GSH-Px: glutathione peroxidase, CAT: catalase, U: unit, prot: protein

Same superscripts refer to similarity, different superscripts refer to difference in groups