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Cinnamon's effect on apoptosis, inflammation and trpm$_2$ channels in methotrexate induced renal damage

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Abstract
Introduction: In this study, it was aimed to investigate the potential protective effects of cinnamon (CIN) against methotrexate (MTX) induced kidney damage.
Materials and Methods: Spraque dawley male rats were divided into five groups (n = 7) as: control group received no treatment; oil group was administered 1ml/kg olive oil (OO) by oral gavage during the experiment; MTX group was given OO by gavage once a day for 14 days and a single dose of 20 mg/kg MTX was injected intraperitoneally on the third day; CIN group was given 100 mg/kg CO (CIN bark oil) by oral gavage throughout the experiment; MTX+CO group received 100 mg/kg CO throughout the experiment and injected with a single dose of 20mg/kg MTX IP on the third day. In the end of the experiment, the animals were decapitated and the kidneys were removed.
Results: MTX administration caused glomerular atrophy, tubular enlargement and increased TRPM$_2$ immunoreactivity on kidney tissue and CIN reversed these changes significantly.
Conclusion: In this study, it was determined that MTX caused histological damage on kidney tissue and CIN reduced this damage.

Keywords: Methotrexate, kidney, apoptosis, TRPM$_2$, cinnamon

Introduction
Methotrexate (MTX) is a folic acid antagonist that acts by inhibiting dihydrofolate reductase enzyme and is used as an antineoplastic and in autoimmune/ inflammatory diseases and some gynecological pathologies such as ectopic pregnancy [1]. Serious side effects limit the use of MTX. Side effects can be observed in various organs and tissues, including the gastrointestinal tract, liver, kidneys and nervous system. These side effects have been associated with oxidative stress, inflammatory apoptotic processes [2].

Transient receptor potential (TRP) channels are members of the cation permeable ion channel superfamily, which is involved in essential cell functions. Transient receptor potential melastatin (TRPM$_2$) channels act as Ca^{+2} channels in the plasma membrane or modify Ca^{+2} channels. The activation of active TRPM$_2$ cation channels is known to be influenced by three factors: oxidative stress, ADP ribose/nicotinamide adenine dinucleotide metabolism, and tumor necrosis factor alpha (TNFα). Increased intracellular Ca^{+2} ions cause harmful cellular processes such as mitochondrial dysfunction and potentially fatal cascades. TRPM$_2$ inhibition reduces oxidation state, and TRPM$_2$-targeted treatments may be effective in reducing renal injury [3].

Cinnamon (CIN), a member of the Lauraceae family, is widely used as both a spice and a medicine. In traditional medicine, it has been used as a flavoring, tonic, stomachic, febrifuge, and carminative agent. It has antiinflammatory, antiallergic, anticancer, antiulcerogenic, antipyretic, antifungal, antibacterial, acaricidal, larvicidal, and insulin-potentiating properties. According to reports, CIN's fruits, flowers, leaves, roots, and bark have medicinal properties [4].

Caspase-3 protein is an apoptotic cell marker that can be detected using immunohistochemical staining [5].

Tumour necrosis factor alpha is a proinflammatory cytokine that is produced by macrophages/monocytes during acute inflammation and is responsible for a variety of signaling events within cells that lead to necrosis or apoptosis [6].

In this study, we aimed to investigate the potential protective effects of CIN against MTX-induced kidney damage/ nephropathy (MIN).

For this purpose, we examined histologically and immunohistochemically the kidney tissue of animals treated with MTX and CIN in addition to MTX.

Materıals and Methods

Study design and animals: For the use and care of laboratory animals, ethical requirements were followed (Adiyaman University Animal Experiments Local Ethics Committee, protocol number. 2020/057). Metotrexate (Koçak Farma, 500 mg/20 ml) and CIN bark oil (CO*)* (Art de Huile Aromatherapy Services Industry and Trade Company) was provided in the highest purity.

35 Spraque dawley male rats were used. During the study, feed and water were given ad libitum to the rats, which were kept in polypropylene cages at 21 °C ambient temperature and 12 hours light-dark cycle. Rats were divided into 5 groups (n=7). Control group received no treatment, oil group was administered 1ml/kg olive oil (OO) by oral gavage during the experiment. The rats in the MTX group were given OO by gavage once a day for 14 days and a single dose of 20 mg/kg MTX was injected i.p. on the third day. CIN group was given 100 mg/kg CO dissolved in OO (a total of 1 ml volume) by oral gavage throughout the experiment period and MTX+CIN group received 100 mg/kg CO throughout the experiment period and injected with a single dose of 20 mg/kg MTX i.p. on the third day. In the end of the experiment period, the animals were decapitated under anaesthesia and the kidneys were removed.

Histological study: Kidney tissues fixed with 10% formaldehyde were embedded in paraffin blocks after routine tissue follow-up procedure. Sections of 4-6 µm taken from paraffin blocks were stained with hematoxylin-eosin (H& E), and the prepared samples were examined under a light microscope (Leica DFC295) and photographed.

Immunohistochemical analysis: Immunohistochemical staining method was applied with minor modifications of the avidin-biotin-peroxidase (ABC) complex [7].

Sections of 4-6 µm thickness were taken from the tissues blocked and deparaffinized. Primary antibodies Caspse-3 (Rabbit polyclonal IgG, Abcam, ab184787, London, UK), anti-TNFα (Rabbit polyclonal IgG, abcam, ab220210, London) diluted 1/200 with the Thermo Scientific™ TP-015-HA commercial kit, UK) and anti-TRPM2 (Rabbit polyclonal IgGantibody, ab101738 Abcam, London, UK) were used. Positive and negative controls were performed as recommended by the manufacturers. After AEC chromogen was applied, staining was done with Mayer Hematoxylin and examined under light microscope. The prepared samples were examined under the Leica DM500 microscope, evaluated and photographed (Leica DFC295). Histoscore was established based on the extent (0.1: <%25, 0.4:%26-50, 0.6:%51-75, 0.9:%76-100) and severity (0:no, +0.5: very slightly, +1:slightly, +2: medium, +3:severe) of immunoreactivity in staining. Histoscore = prevalence x severity.

Statistical analysis: Statistical analyzes were performed using SPSS package program version 22.0 (IBM, Armonk, NY). Descriptive data were reported as median (min-max). Kruskal Wallis H test was used to compare continuous variables between more than two independent groups, and then Dunn-Bonferroni post-hoc test was used for pairwise comparisons to determine which group the difference originated from. Statistical significance was accepted as $p<0.05$.

Results

Histopathological results: In the histopathological examination, control (A), oil (B) and oil + CIN (C) groups showed normal histology. Significant glomerular atrophy and tubular enlargement were detected in the methotrexate group (D) when compared to the control, oil and oil+CIN groups. In the MTX+CIN (E) group, tubular dilatation and glomerular atrophy were significantly reduced compared to the MTX group (Figure 1).

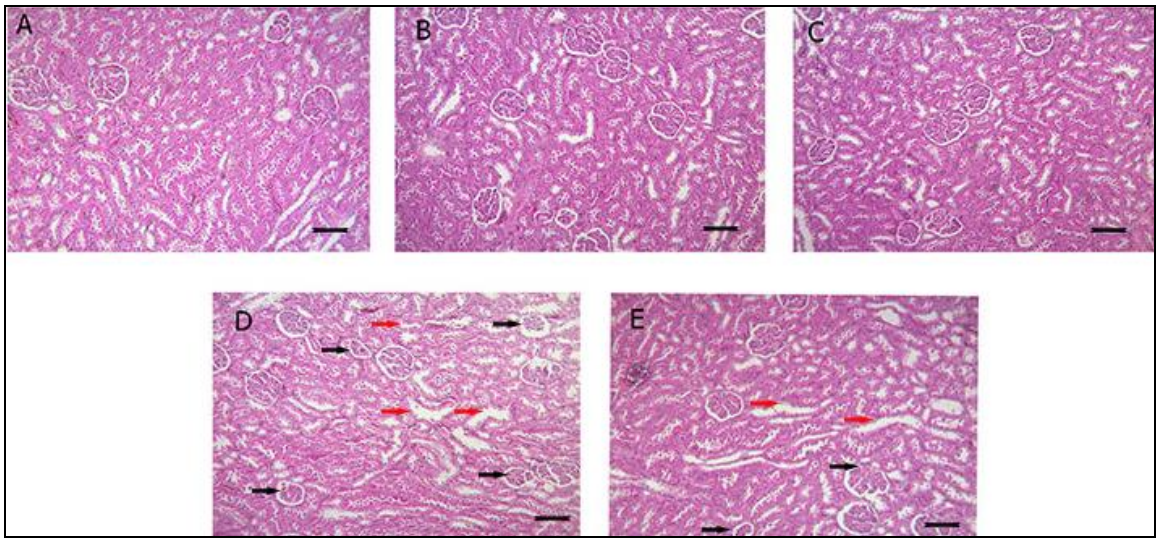

Fig 1: Histopathological findings of study groups (Control (A), oil (B), oil+CIN (C), Mtx (D), Mtx + CIN group (E)).

Immunohistochemical results: $TRPM_2$ immunoreactivity of the MTX group was significantly higher than control, oil and CIN+oil groups ($p=0.001$, $p=0.006$, $p<0.001$). The $TRPM_2$ immunoreactivity of the MTX+ CIN group was found to be significantly reduced compared to the MTX group ($p=0.045$) (Figure 2 and Table 1).

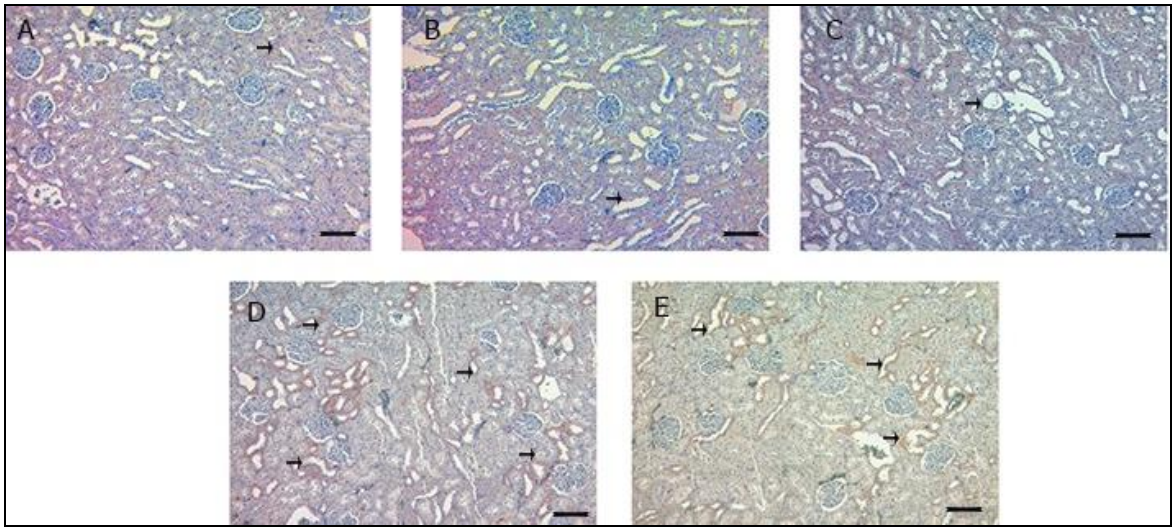

Fig 2: TRPM2 immunoreactivity of study groups (Control (A), oil (B), oil+CIN (C), Mtx (D), Mtx + CIN group (E)).

Caspase-3 immunoreactivity of the MTX group was significantly higher than the control, oil and CIN+oil groups ($p<0.001$, $p<0.001$, $p=0.002$). There was a significant decrease in caspase-3 immunoreactivity of the MTX+ CIN group compared to the MTX group ($p=0.036$) (Figure 3 and Table 1).

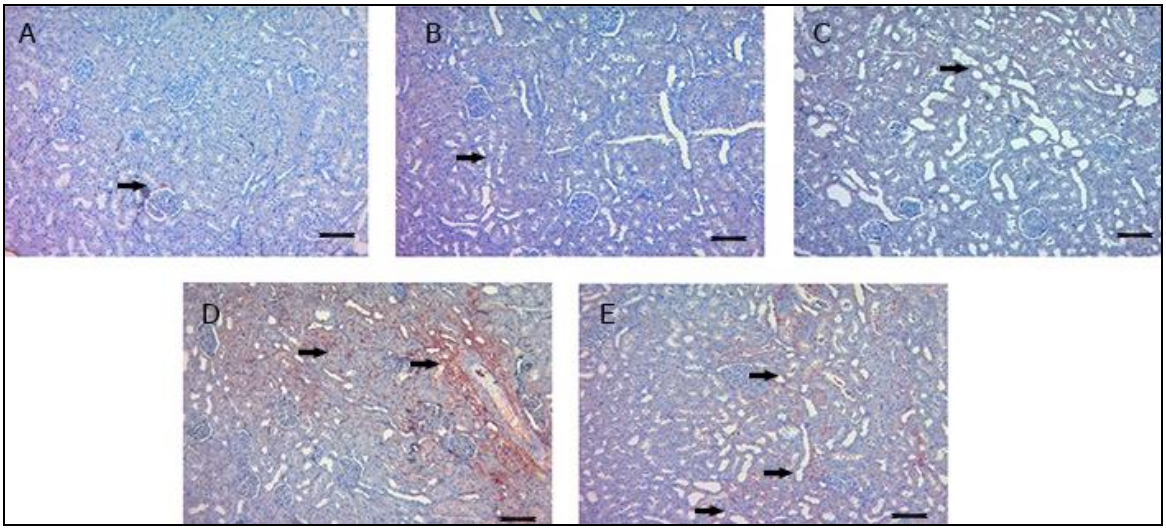

Fig 3: Caspase immunoreactivity of study groups (Control (A), oil (B), oil+CIN (C), Mtx (D), Mtx + CIN group (E)).

TNFα immunoreactivity of the MTX group was significantly higher than control, oil and CIN+oil groups ($p<0.001$, $p<0.001$, $p=0.002$). TNFα immunoreactivity of the MTX+ CIN group was found to be significantly decreased compared to the MTX group ($p=0.047$) (Figure 4 and Table 1).

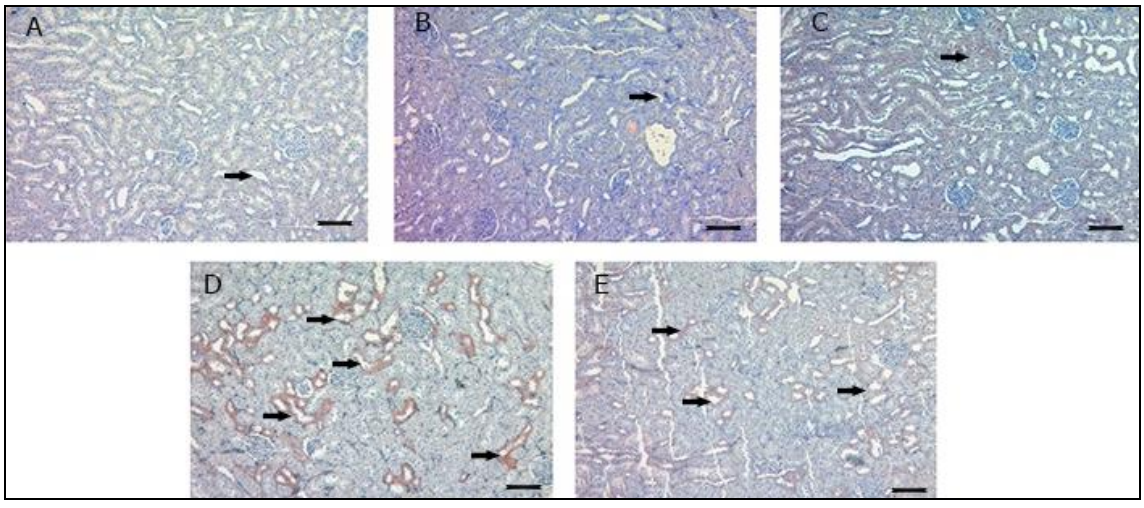

Fig 4: TNFα immunoreactivity of study groups (Control (A), oil (B), oil+CIN (C), Mtx (D), Mtx + CIN group (E)).

Table 1: Immunohistochemical results of study groups

	Control	Oil	Cinnamon+ oil	Mtx	Mtx+ cinnamon	p Value
$TRPM_2$	$0,51^{a}(0,36-0,62)$	$0,52^{b}(0,45-0,65)$	$0,43^{cd}(0,25-0,52)$	$2,96^{abde}(2,92-2,99)$	$0,60^{ce}(0,53-0,62)$	<0,001
Caspase-3	$0,85^{a}(0,79-0,95)$	$0,81^{b}(0,65-0,98)$	$0,88^{c}(0,67-1,01)$	$2,29^{abcd}(2,12-2,57)$	$0,94^{d}(0,87-0,96)$	<0,001
TNFα	$0,44^{ab}(0,32-0,54)$	$0,46^{c}(0,31-0,54)$	$0,51^{d}(0,35-0,62)$	$2,94^{bcde}(2,89-2,98)$	$0,54^{ae}(0,49-0,57)$	<0,001

Values with the same superscripts in the same row are significantly different ($p<0.05$).

Dıscussıon

In this experimental study, we determined that CIN administration reversed/ reduced tubular dilatation, glomerular atrophy and increased $TRPM_2$ immunoreactivity that caused by MTX.

Methotrexate, a cytotoxic antineoplastic agent, is a folate antimetabolite used in the treatment of many malignancies. It is also used in the treatment of rheumatoid arthritis and some other rheumatic and inflammatory diseases due to its immunosuppressive/ modulatory effect [8]. MTX-polyglutamates formed after entering the cell, potently inhibit the dihydrofolat reductase enzyme. Inhibition of this enzyme stops protein synthesis by blocking the synthesis of thymidines and purines, which need tetrahydrofolate for their synthesis [9]. Methotrexate causes serious side effects that require dose reduction / discontinuation in many tissues and organs, especially in tissues with high proliferation properties. Hepatotoxicity, nephrotoxicity, pulmotoxicity, hematotoxicity,neurotoxicity, cardiotoxicity, gastrointestinal and gonadal toxicity and neocarcinogenic effects are the main side effects. Side effects depend on dose, route of administration, frequency of administration and concomitant use of folinic acid [10].

The kidneys are the organs responsible for homeostasis, toxin/metabolite/drug elimination and complex metabolic functions in the organism. Methotrexate-induced nephrotoxicity has been attributed to the pathological processes of oxidative damage, inflammation and apoptosis [11].

A study with 140 patients to evaluate the anthropometric and glycemic effects of CIN found that taking 1 g of CIN per day for three months significantly improved body fat, insulin resistance, glycosylated hemoglobin, fasting plasma glucose, and cholesterol levels [12]. Similarly, many studies have been conducted on the effect of CIN on fasting plasma glucose levels and insulin resistance in type 2 diabetes and prediabetes patients [13]. According to Sadeghi S *et al*. [14] impaired apoptosis plays a significant role in cancer biology, and cinnamon has anti-cancer effects by affecting numerous apoptosis-related pathways in cancer cells. A recent meta-analysis involving 641 subjects found that oral CIN supplementation significantly reduced systolic and diastolic blood pressure in the adult population [15]. Cinnamon's tolerable daily intake ranges from 1 to 6 g, or about a teaspoon per day for humans. Higher doses may result in diarrhoea, warmth, sweating, nausea, and vomiting [16].

$TRPM_2$ cation channels are critical for cancer cell survival. $TRPM_2$ knockdown promoted cancer cell death in gastric cancer cells by impairing autophagy [17].

This study demonstrated that CIN administration reversed MIN. CIN demonstrated therapeutic potential by regulating the $TRPM_2$ channels and could be combined with chemotherapy to combat its toxic side effects. Increased $TRPM_2$ expression in the MTX group may be involved in the pathophysiology of MIN. $TRPM_2$-mediated Ca^{+2} influx was inhibited by CIN. $TRPM_2$ channel inhibition appears to be the mechanism by which CIN protects kidney tissue from MIN. Finally, it is possible that $TRPM_2$ could be used as a therapeutic option to prevent MIN.

Conflict of interest: None.

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