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Research Article

Protective Effect of *Hypericum perforatum* Extract on Methotrexate-Induced Osteotoxicity via Reducing Oxidative Stress and MAPK Activity

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Abstract

Backgrounds and Objective: Methotrexate (MTX) is an anti-metabolite and anti-rheumatic drug that is widely used in cancer treatment. It is known that a high dose of MTX chemotherapy causes bone growth disturbance in developing bones. *Hypericum perforatum* (HP) plant is useful against some diseases for local people and regulates apoptosis which is also known as programmed cell death and recent studies also reported that HP reduces the side effects of many toxic drugs. For this reason, we planned a study to reveal the osteoprotective effect of HP extract against MTX induced osteo-toxicity. **Materials and Methods:** For this purpose MLO-Y4 cells were cultured, then these cells were treated with HP and methotrexate and mediators of the apoptotic, MAPK pathway and the oxidative stress parameters were analyzed on these cells. **Results:** Expression of proapoptotic factors increased and expression of antiapoptotic factors were decreased with MTX treatment. Treatment of HP ameliorated the apoptotic effect of MTX on MLO-Y4 cells. HP treatment also inhibited MAPK activity and oxidative stress induced by methotrexate. **Conclusion:** HP attenuated methotrexate-induced osteo-toxicity via reducing oxidative stress and MAPK activity and HP is an effective reducing side effect of MTX.

Key words: MLO-Y4, *Hypericum perforatum,* methotrexate, apoptosis and oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The basis of cancer treatment is chemotherapy and is estimated to treat 70-80% of cancer patients depending on the type of cancer[1]. However, chemotherapy treatment has been associated with severe side effects such as impaired bone growth, osteoporosis in cancer patients[2-4]. The underlying molecular mechanisms of chemotherapy-induced osteo-totoxicity are still unknown. Osteocytes play a major role in bone homeostasis that osteocytes detect mechanical stresses and translate biochemical signals into adjacent osteoblasts and osteoclasts[5,6]. In addition, methotrexate (MTX) is widely used in cancer treatment and causes osteocyte cell death[4,7-9]. Methotrexate, a folic acid antagonist, is an antiproliferative agent that binds the enzyme dihydrofolate reductase and inhibits DNA and RNA synthesis. Because of its anti-proliferative properties, it is used in the treatment of cancer at high doses[10,11]. MTX has many side effects especially nephrotoxicity and hepatotoxicity because of using high dose[12,13]. Oxidative damage caused by reactive oxygen species (ROT) is generally responsible for these side effects. MTX can cause intracellular nicotinamide adenine dinucleotide phosphate (NADPH) levels to decrease. NADPH is also used by the glutathione reductase enzyme, which maintains the level of reduced glutathione (GSH), which is known as an important protective agent against ROT[13]. In one study, they found that MTX application, increased myeloperoxidase (MPO) activity, decreased glutathione levels and marked increase in Malondialdehyde (MDA) levels[14]. In another study, they reported an increase in Nitric Oxide (NO) levels in the kidney tissues of MTX treated rats[15]. Therefore, it is necessary to use it with antioxidant agents in order to protect against MTX oxidation.

St. John's wort (*Hypericum perforatum*) plant has biological active contents and is useful against some diseases for local people[16]. This plant has antioxidant and anti-inflammatory properties and shows these effects by suppressing MAPK[16-18]. The contents of St. John's wort also affect caspase-3, bax and bcl-2 protein which mediate apoptosis is also known as programmed cell death levels and its activity[19]. Recent studies showed that HP is effective in reducing the side effects of many toxic drugs. In a previous study, we showed a protective effect of HP on gentamicin-induced nephrotoxicity by its anti-inflammatory property[16]. In a study it was shown that HP reduced Chromium induced the structural damage in rat's adrenal glands, cellular apoptotic gene expression and chromium aggression. In another study has demonstrated that H. perforatum extracts and its major molecular components can protect against toxic insults through is antioxidant properties. HP has therefore the potential to be neuroprotective therapeutic agent[20].

Therefore, the present study was planned to investigate the apoptotic effects of MTX on MLO-Y4 cells and the protective role of *Hypericum perforatum* extract (HP) by analyzing MAPK pathways and oxidative stress parameters.

MATERIALS AND METHODS

This study was performed in Cukurova University Medicine Faculty Department of Pharmacology, between the dates of 01.01.2018-01.05.2018.

Chemicals: Culture media were purchased from GIBCO BRL (Grand island, NY, U.S.A.) and calf serum was from HyClone Laboratories, Inc. (Logan, UT, U.S.A.). Rat tail collagen type 1was purchased from Becton Dickinson Laboratories (Bedford, MA, U.S.A.). RIPA buffer, fetal bovine serum, PBS, NaCl, TritonX-100, EGTA, dithiothreitol, NaF, Tris-Cl, Na3VO4 were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Inc. bax, bcl-2, wee 1, AIF, gadd153 and grp78 of ELISA kit were purchased from Shanghai Sunred Biological Technology Co., Ltd. p-erk and p-jnk were purchased from My BioSource, Inc.(San Diego, U.S.A.). SOD and GPX assays obtained from BioVision (USA). TAS and TOS assays were purchased from Rel Assay Diagnostics Inc.(Gaziantep, TURKEY).In addition, the Bradford dye reagent was purchased from Bio-Rad Laboratories, Inc.(California, U.S.A.).

Cell culture: The mouse osteocyte-like cell line MLO-Y4 was obtained from Kerafast, Inc. The cells were cultured according to the previously described protocol by Kato and Bonewald[21,22]. MLO-Y4 cells were incubated with MTX (10^{-5} M) and MTX (10^{-5} M) plus HP extract (100 μmol L^{-1}) for 48 h and then cells were homogenized for ELISA experiments

Cell homogenization: Cells (5×10^4 cells/cm^2) were exposed to 100 μmol L^{-1} MTX for 48 h. They were then washed in Phosphate Buffer Solution (PBS) and lysed in RIPA buffer (150 mmol L^{-1} NaCl 0.5%, TritonX-100, 20 mmol L^{-1} EGTA, 1 mmol L^{-1} dithiothreitol, 25 mmol L^{-1} NaF, 50 mmol L^{-1} Tris-HCl [pH 7.4], 1 mmol L^{-1} Na_3VO_4) for 15 min on ice followed by centrifugation at 15000 rpm for 20 min. and supernatants are taken and pellets are discarded[23].

Total protein determination: Bradford method is used to determining of the total protein in homogenized tissues. Protein determination (μg μL^{-1}) was done according to the standard curve drawn in Prism software[23].

ELISA (Enzyme linked immunosorbent assay) tests: ELISA test is used to examine the activity of caspase-3 and expression of bax, bcl-2, wee 1, gadd153 AIF protein which are apoptotic pathway's mediators. Active ERK (p-ERK) and active JNK(p-JNK) also analyzed by ELISA.

The detection of TAS, TOS, SOD and GPx was performed as previously described[24].

Statistical analysis: Results were expressed as Mean±SEM. and n refers to the number of cell culture flask used for each groups. Differences in results between tissues were tested by Analysis of Variance (ANOVA) corrected for multiple comparisons (Bonferroni corrections). P values less than 0.05 were considered to be significant.

RESULTS

Apoptotic mediators: We evaluated mediators of apoptosis which is also known as programmed cell death. While caspase and bax have pro-apoptotic effect, bcl-2 has anti-apoptotic effect. Increased activity of caspase 3 and disruption in the bax/bcl-2 ratio in the direction of bax initiates apoptosis in the cell. MTX treatment increased activity of caspase-3 (Fig. 1a) and expression of bax (Fig. 1b), wee 1, gadd153 and AIF (Table 1) decreased bcl-2 (Fig. 1c) significantly and HP treatment reduced these effects significantly.

MAPK mediators: JNK and ERK are mediators of MAPK signaling pathway which transfer information to the nucleus. The activity of JNK (p-JNK) and ERK (p-ERK) found higher in the MTX group when compared to the control group. HP treatment reduced the MTX induced activity of JNK and ERK. (Fig. 2a-b).

Oxidative stress parameters: The Total Antioxidant Status (TAS) levels were found significantly lower in the MTX group than in the control group ($p<0.05$). And the TAS levels were found significantly higher in HP treated group when compared to MTX group (Fig. 3a).

The total oxidant status (TOS) levels were found higher in the MTX group than the control group ($p<0.05$) and the TOS levels were found significantly lower in HP treated group when compared to MTX group (Fig. 3b).

The SOD and GPx (endogen ROS scavenger) levels were found significantly lower in the MTX group when compared to the control group ($p<0.05$). The SOD and GPx levels were found higher in the HP treated group than in the MTX group ($p<0.05$) (Fig. 4a-b).

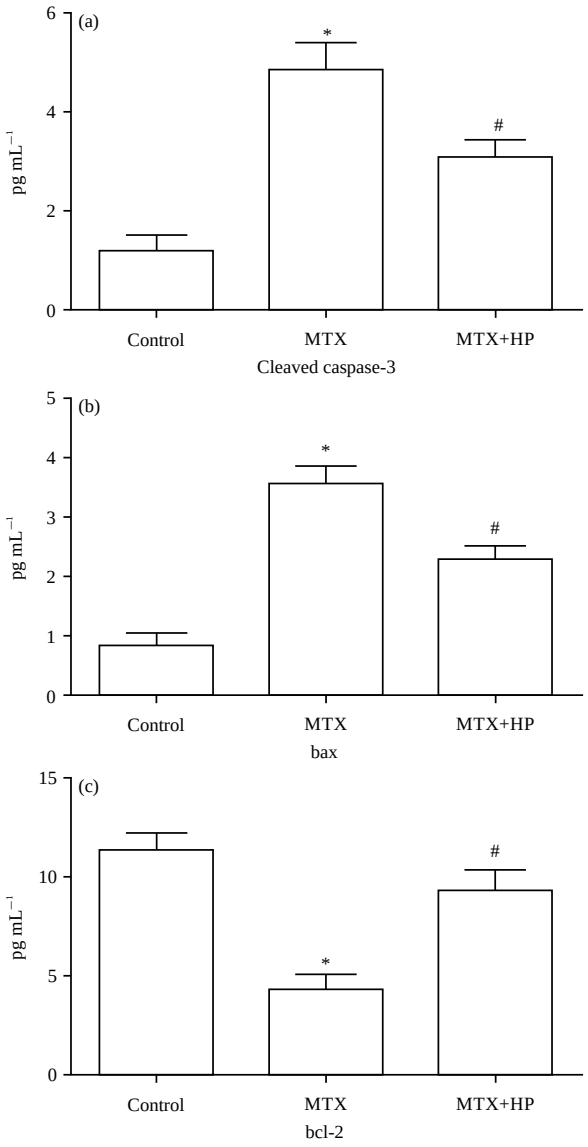

Fig. 1(a-c): Effect of MTX and HP on (a) caspase-3 activity (b) bax expression and (c) bcl-2 expression (n = 8), Statistical analysis: ANOVA, *Post hoc*: Bonferroni
*: For control $p<0.05$, #For I/R $p<0.05$

Table 1: Effects of HP on expression of mitotic division inhibitors (Wee1 and GADD153), proapoptotic AIF and dual function GRP78 proteins

Protein name	Control (pg mL^{-1})	MTX (pg mL^{-1})*	MTX+HP (pg mL^{-1})#
wee 1	0.32±0.01	1.29±0.01	0.75±0.03
AIF	0.95±0.03	1.85±0.05	1.15±0.02
gadd153	0.28±0.019	0.99±0.02	0.63±0.03
grp78	0.35±0.03	1.89±0.03	1.05±0.08

Results are presented as Mean±SE, Statistical analysis: one way anova (Bonferroni corrections), *$p<0.05$ for the comparison between the Control and MTX, #$p<0.05$ for the comparison between the MTX group and MTX+HP, MTX: Methotrexate, HP: *Hypericum perforatum*

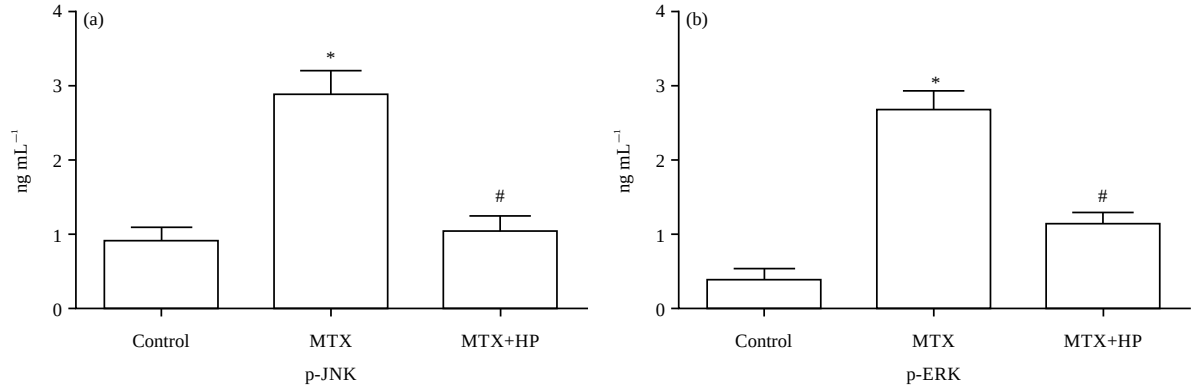

Fig. 2(a-b): Effect of MTX and HP on (a) p-JNK expression and (b) on p-ERK expression (n = 8), Statistical analysis: ANOVA. *Post hoc*: Bonferroni

*: For control p<0.05, #For I/R p<0.05

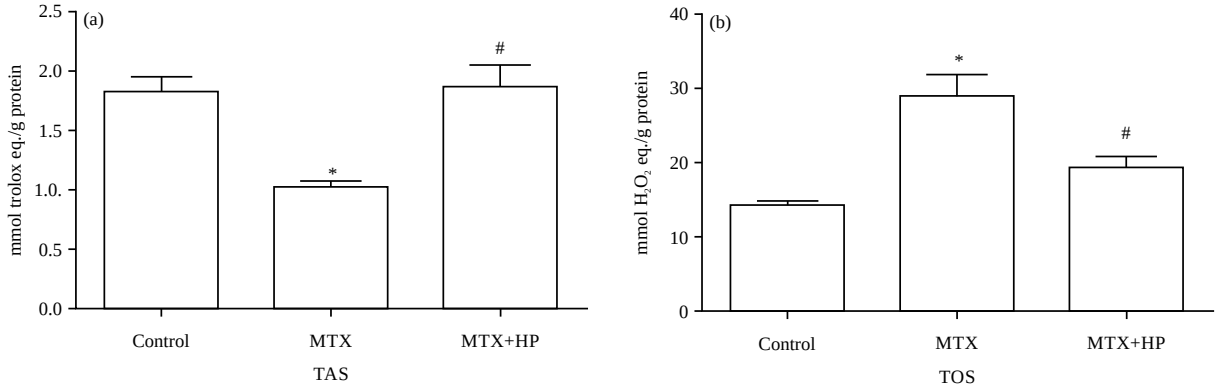

Fig. 3(a-b): Concentration of (a) TAS levels and (b) TOS levels in groups (n = 8), Statistical analysis: ANOVA, *Post hoc*: Bonferroni

*: For control p<0.05, #For I/R p<0.05

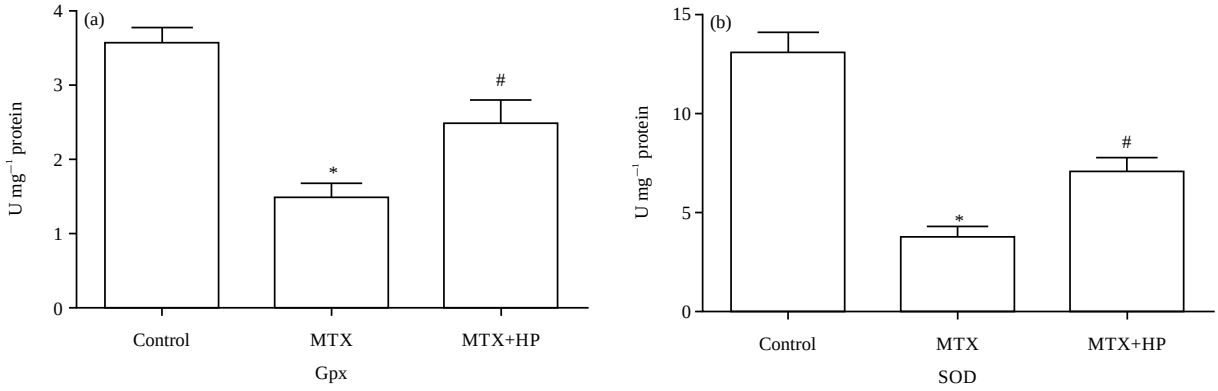

Fig. 4(a-b): Activity of (a) GPx level and (b) SOD level in groups (n = 8), Statistical analysis: ANOVA, *Post hoc*: Bonferroni

*: For control p<0.05, #For I/R p<0.05

DISCUSSION

This study was designed *in vitro* acute chemotherapy with MTX by using osteocyte-like MLO-Y4 cells, which are regarded to be the most compatible *in vitro* model available to clarify the pathophysiological responses of in vivo osteocytes[21,25]. MTX induced apoptosis by increasing caspase-3 activity and expression of pro-apoptotic proteins and decreasing anti-apoptotic proteins. MTX together HP treatment ameliorated the apoptotic effect of MTX via inhibiting MAPKs and oxidative stress.

There are lots of extracellular and intracellular stimuli have been shown to activate MAPK pathways[26]. One of them are Reactive Oxygen Species (ROS) that activates MAPK pathways[27], but the mechanism(s) for this effect is unclear ROS may have important roles as a modulator of cell as signaling molecules. Indeed, lots of evidence supported that ROS has a physiological role as a "second messenger" in intracellular signaling pathways that control cell growth, proliferation, migration and apoptosis[28].

Many protective features of HP have been shown in studies[16,19,29,30]. This plant has both antioxidant and anti-inflammatory effects. Chemotherapeutic drugs, including methotrexate used in cancer treatment, have side effects. Methotrexate-induced toxicity the duration of treatment with methotrexate and the interaction of many factors such as dose schedules, type of disease and patient risk factors as well as genetic and molecular apoptotic factors[31]. MTX therapy is contraindicated in rheumatology authorities[24]. For these reasons, it is emphasized that if the use of medication is necessary for patients with rheumatoid arthritis and cancer, the dose should be reduced by 75% and be given by close monitorisation[32]. MTX treatment induces oxidative stress. Increasing superoxide radicals with oxidative stress cause decrease of endogen antioxidant such as glutathione (GSH). With the reduction of the level of GSH due to methotrexate therapy decrease of the effectiveness of the antioxidant defense system, which protects against reactive oxygen radicals such as superoxide anion, hydroxyl radicals, hydrogen peroxide and hydrochloric radicals[31]. A study has shown that oxidative stress, plays a role in methotrexate-induced small bowel injury due to neutrophil infiltration[33]. The most serious side effect of methotrexate is renal toxicity[31]. Recent studies showed that MTX treatment causes bone loss and the depletion of bone-forming osteoblasts and osteocyte cell death[34]. HP is frequently used by local people in wound healing. Studies have shown that this plant is a potent anti-inflammatory and antioxidant[16]. Studies have shown that the antioxidant properties of HP also contribute synergistically to its anti-inflammatory properties. It has been shown that hypericin is a free radical scavenger and inhibits arachidonic acid release from phospholipids via blocking the 5- and 12-lipoxygenase pathway and IL-la and IL-12 formation. Furthermore, it has been reported that NF- [kappa] B, the regulator of inflammatory mediators and MAPK, are inhibited by the hypericin[35,36]. It has been shown that the formation of another active ingredient, hyperphorine inhibits formation of free oxygen radicals, elastase release, cyclooxygenase-1, 5-lipoxygenase and IL6 release from leukocytes. Hiperosid and isoquercitrin which are flavonoid of HP inhibit the neutrophil elastase which has a role in the pathogenesis of inflammation and nitric oxide synthase. Isoquercitrin has also been shown to inhibit prostaglandin biosynthesis and secretion[37]. In addition, the flavonoid, amentoflanon, has been shown to elicit arachidonic acid release from cyclooxygenase-2, phospholipase A2, iNOS and neutrophils. Recent toxicological studies with anticancer drugs have focused on oxidative stress. Despite these wide indications for use, MTX is confronted with side effects, especially nephrotoxicity and hepatotoxicity and these side effects are often responsible for the oxidative damage caused by reactive oxygen species [32,33]. For this reason, it is suggested that the osteo-protective effect of HP may have resulted from its antioxidant and anti-inflammatory properties. This study has shown the cell-protective effect of the HP, but the studies have also shown the toxic effects of the HP on cancer cells[17,23,38]. In this respect, the importance of HP is increasing. Because chemotherapeutic agents also harm normal cells and there is no chemotherapeutic agent that does not harm normal cells and has no side effects.

CONCLUSION

In conclusion, MTX treatment induces apoptosis and increases MAPK activity in osteocytes via inducing oxidative stress. HP treatment attenuates MTX induced apoptosis by reducing oxidative stress and MAPK activity. HP has a protective effect on MTX induced osteo-toxicity. Our study has shown that it can be beneficial to use MTX with HP, which is frequently used in the clinic and whose toxic effects are known.

SIGNIFICANCE STATEMENT

The protective effect of the HP against the MTX has been discovered. Hypericum perforatum has strong anti-inflammatory and antioxidant agent. This study predicts

that it is effective against other chemotherapeutics that increase oxidative stress. This study will help the researchers to uncover the importance of alternative plant-based remedy based on *Hypericum perforatum* in the treatment of MTX induced osteo-toxicity that many researchers were not able to explore. Thus, a new theory that the extract of *Hypericum perforatum* is effective and sufficient in the fight against MTX induced osteo-toxicity may be arrived at.

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