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Anti-glioma effect of Turkish propolis in C6 cells via regulation of COX-2 and NF-κB mRNA expression and redox homeostasis

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Abstract: This study aims to determine whether Turkish propolis applied to C6 glioma cells has an anti-glioma effect. To investigate this, C6 glioma cells were treated with propolis extract concentrations of 100, 250, and 500 µg/ml. COX-2 and NF-κB mRNA expressions in the cells were detected by using qPCR. The levels of TNFα, MMP-2, -9, and JNK/SAPK in the cells were analyzed by using ELISA. The changes in redox status biomarkers as oxidant and antioxidant status and cell injury biomarkers were detected by using colorimetric methods or ELISA. COX-2 and NF-κB mRNA expression levels were decreased in glioma cells treated with propolis extract at concentrations of 100, 250 and 500 µg/ml as compared to control cells. MMP-2 and -9 levels were unchanged, while TNFα levels decreased in C6 glioma cells treated with propolis extract at concentrations of 100, 250, 500 µg/ml compared to control cells. While the levels of dityrosine, kynurenine, AOPP, advanced glycation end products and lipid hydroperoxides were significantly decreased in C6 glioma cells treated with 100µg/ml propolis compared to control cells, it increased significantly in cells treated with propolis extract at 500µg/ml concentration. These findings suggest that Turkish propolis may exert anti-glioma effects on C6 glioma cells by dose-dependently regulating COX-2 and NF-κB mRNA expressions and promoting oxidative stress-induced cell damage and death.

Keywords: C6 glioma, gene expression, inflammation, propolis, redox status, cell injury

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INTRODUCTION

Propolis is a mixture of resinous compounds produced by bees from plant exudates and tissues. Many variations in types of propolis depending on geographical location, plant and bee species, etc. (Altabbal *et al*., 2023). Since ancient times, this natural material has been known to have therapeutic properties such as antioxidant, anti-inflammatory, anticancer, antibacterial and antifungal (Forma and Bryś, 2021; Yigit *et al.,* 2024). In the present study conducted by Girgin *et al*. (2009) with propolis collected from different regions of Turkey, researchers stated that Turkish propolis has an immunomodulatory effect. According to Ozdal *et al*. (2019), compared to other regions, the highest total phenolic content and antioxidant capacity values were determined in Turkish propolis collected from the Marmara region. Therefore, we preferred to use propolis collected from the Marmara region in our study.

Glioma is a common, aggressive brain tumor that is highly resistant to chemotherapy (Su *et al.*, 2023). Glioma cells have been reported to use reactive oxygen and nitrogen species for growth and invasion (Ostrowski and Pucko, 2022). Wang *et al*. (2022) suggested that cyclooxygenase-2 (COX-2) is important in the pathologic diagnosis and prognostic prediction of glioma patients. Nuclear factor-kappaB (NF-κB) is a transcriptional regulator of inducible expression of genes such as COX-2 and tumor necrosis factor alpha (TNFα) that regulate cell proliferation and inflammatory response (Lim *et al*., 2001; Kumar *et al*., 2021).

In the study, we explored the effects of Turkish propolis in the C6 glioma cells, which are commonly used in neuro-oncology research. In this way, we demonstrated whether Turkish propolis extract (i) contributed to the regulation of the COX-2 and NF-κB mRNA expressions, (ii) affected MMP-2, -9 and JNK/SAPK levels (iii) ameliorated redox homeostasis.

MATERIALS AND METHODS

Cell culture

In this study, C6 glioma cells derived from the brain of a rat with glioma were obtained from the cell line archive of the Demiroglu Bilim University. For cell culture assays, cells were maintained in DMEM/F12 medium supplemented (Sigma Aldrich, D5546/N6658) with 5% FBS (Gibco, 10270), 100µg/ml streptomycin, penicillin (100 U/ml) (Gibco, Pen Strep, 15140-122), 0.2 mM L-glutamine (Wisent Inc., 609-065-EL) and incubated at 37°C in a humidified atmosphere of 5% CO_2 (Sanyo MCO-18M, Japan).

Preparation of propolis extract and formation of groups

The propolis used in this study was collected from Tekirdag/Turkey. Briefly, crude propolis was extracted with 70% alcohol at room temperature. The propolis extract was centrifuged and filtered through Whatman

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filter paper (Saleh *et al*., 2023; Kurek-Górecka *et al*., 2024). The experimental groups were divided into four groups as control (0), 100, 250 and 500µg/ml propolis extract treated cells.

Analysis of mRNA expression

Glioma cells were seeded in plates (TPP^{TM} cell culture microplates, 6-well) at the density of 1 x 10^5 cells per well and treated with propolis extract at concentrations of 100, 250 and 500 µg/ml for 24 hours. Cell lysates were from C6 glioma cells by sonication (Starsonic 35, Liarre, Italy). After that, RNA isolation from cell lysates was performed using a commercial kit (Hibrigen, Turkiye). cDNA was synthesized from isolated RNA (Applied Biosystems, 4388950, Lithuania). Total cDNA was analyzed by real-time PCR (qRT-PCR). qRT-PCR was performed on a CFX96 Touch Real-Time PCR Detection Systems using a Sso Fat Eva Green Supermixes (Biorad, 172-5204, USA). Primers for qRT-PCR were designed as follows (Macrogen, Korea): COX-2, F: 5'-TTAAAACCAGGTG AGCTTCG-3' and R: 5'-TGGTCG ATTTGATGTCAC TG-3', NF-κB, F: 5'-CCGGGAGCCTCTAGTGAGA-3' and R: 5'-CAT TTGTGACCAACTGAACGA-3', Actb, F: 5'-CTAAGGCCAACCGTGAAAAG-3 and R: 5'-TCTGGCGAGTCCATCACAAT-3'. The amounts of target mRNA relative to control were calculated using the Pfaffl method (Pfaffl, 2001).

Proinflammatory biomarker

TNFα concentrations were analyzed with a commercial ELISA kit (BT Lab, China). The absorbance values of samples were read at 450 nm (Epoch Microplate Spectrophotometer, BioTek, USA). The standard curve was used to calculate sample levels.

ELISA

MMP-2, -9, and JNK/SAPK levels were measured using ELISA kits (BT Lab, China). Sample absorbance was measured at 450 nm (Epoch Microplate Spectrophotometer, BioTek, USA). The standard curve was used to calculate sample levels.

Oxidant and antioxidant status biomarkers

Dityrosine (DT), kynurenine (KYN) and advanced glycation end products (AGEs) levels were detected (Sadowska-Bartosz *et al*., 2014). Advanced oxidation protein products (AOPP) levels were performed by Hanasand *et al*. (2012)'s method. Lipid hydroperoxides (LHPs) were analyzed according to the method described by Woff (1994). Total thiol (T-SH) concentrations of cell suspensions were determined by method of the Sedlak and Lindsay (1968). Glutathione peroxidase (GPx) concentrations were determined with commercial ELISA kit (BT Lab, China). The absorbance of samples was read at 450 nm (Epoch Microplate Spectrophotometer, BioTek, USA).

Cell injury parameters

Lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities were detected with commercial ELISA kit (BT Lab, China). The absorbance of samples was read at 450 nm (Epoch Micro Plate Spectrophotometer, BioTek, USA). Sample levels were calculated according to the standard curve.

STATISTICAL ANALYSIS

Experiments were performed in triplicate. GraphPad Prism software version 9 was used for statistical analysis. Normality of the distribution was detected by the Shapiro-Wilk normality test. Kruskal-Wallis and Mann-Whitney U nonparametric tests were used to evaluate the comparisons between groups. All values were expressed as mean ± SD. $P<0.05$ was considered significant.

RESULTS

As seen in the microscopic image in fig. 1, it was found that the number of glioma cells decreased due to the application of increasing doses of propolis.

Changes in COX-2 and NF-κB mRNA expressions

The mRNA expressions of both COX-2 and NF-κB were lower in glioma cells treated with propolis at concentrations of 100, 250 and 500µg/ml compared to the control cells ($P<0.05$ for all). COX-2 mRNA expression was lower in the group treated with 500µg/ml of propolis compared to glioma cells treated with propolis at concentrations of 100 and 250µg/ml ($P<0.05$ for both). However, NF-κB mRNA expression level was similar in the cells treated with 100, 250 and 500µg/ml propolis (fig. 2).

Changes in TNFα levels

As seen in fig. 3, proinflammatory biomarker TNFα levels decreased in glioma cells treated with propolis at concentrations of 100, 250 and 500µg/ml compared to the control cells ($P<0.0001$ for all). However, the lowest TNFα levels were in the 100µg/ml propolis group.

Analysis of MMP-2, MMP-9 and JNK/SAPK levels

When the propolis applied groups were compared with the control cells, no significant difference was observed in MMP-2 and -9 levels (fig. 3). MMP-2 levels in the 100µg/ml propolis applied group were significantly lower than the 250 and 500µg/ml propolis applied groups ($P< 0.05$ for both). On the contrary, the JNK/SAPK level showed a significant increase in the groups treated with 100, 250 and 500µg/ml propolis compared to the control cells ($P<0.05$ for all). JNK/SAPK levels of cells treated with 250 and 500µg/ml propolis were significantly increased compared to cells treated with 100 µg/ml propolis ($P<0.05$ for both, fig. 3).

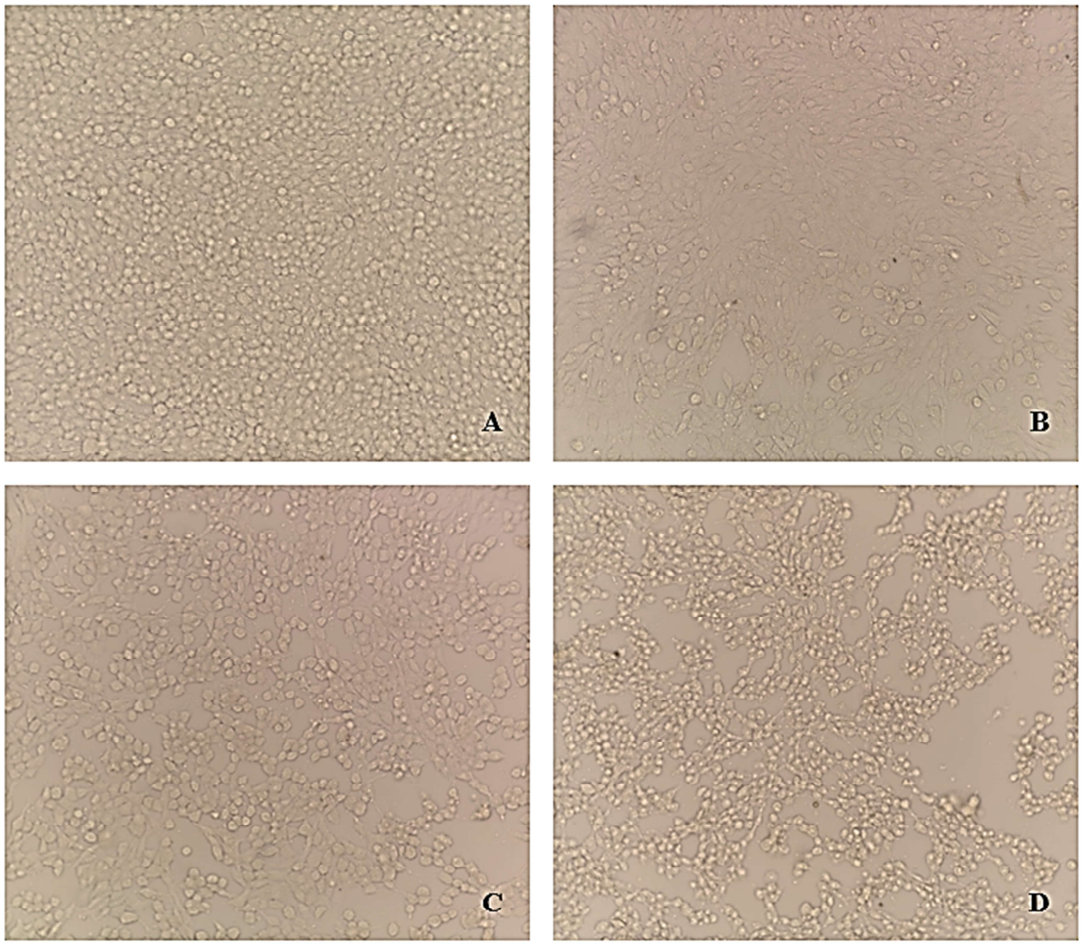

Fig. 1: Inverted phase contrast microscopy image of C6 glioma cells treated with propolis extract (X20 magnification). Control group (A), the cells treated with 100µg/ml (B), 250µg/ml (C) and 500µg/ml (D) propolis.

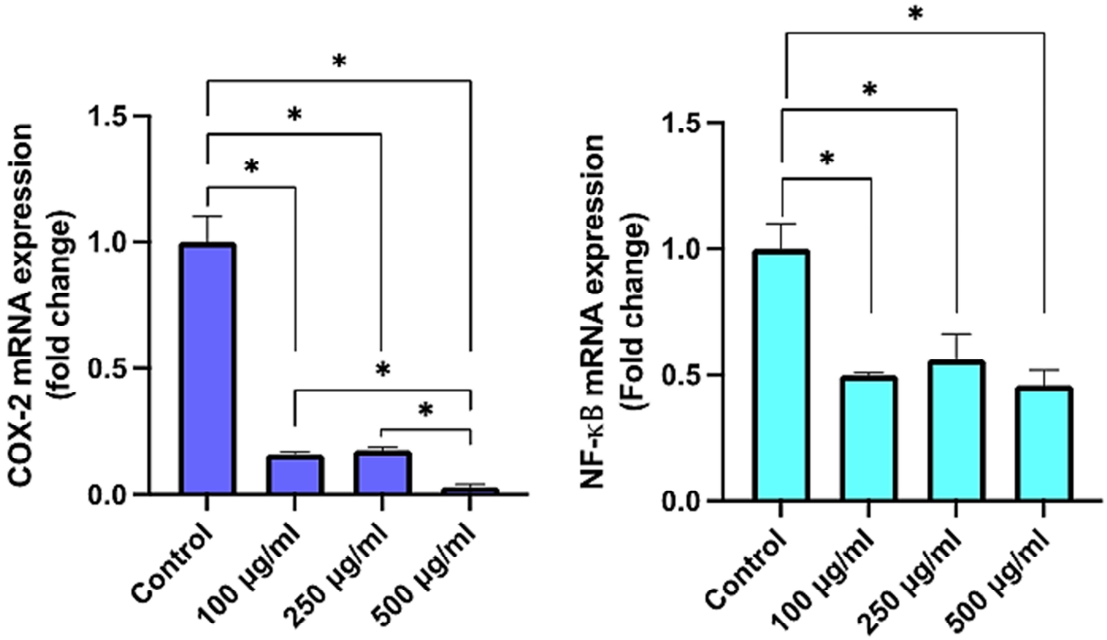

Fig. 2: Cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) mRNA expression levels in C6 glioma cells treated with propolis. Graphical data are expressed as mean ± SD. *P<0.05.

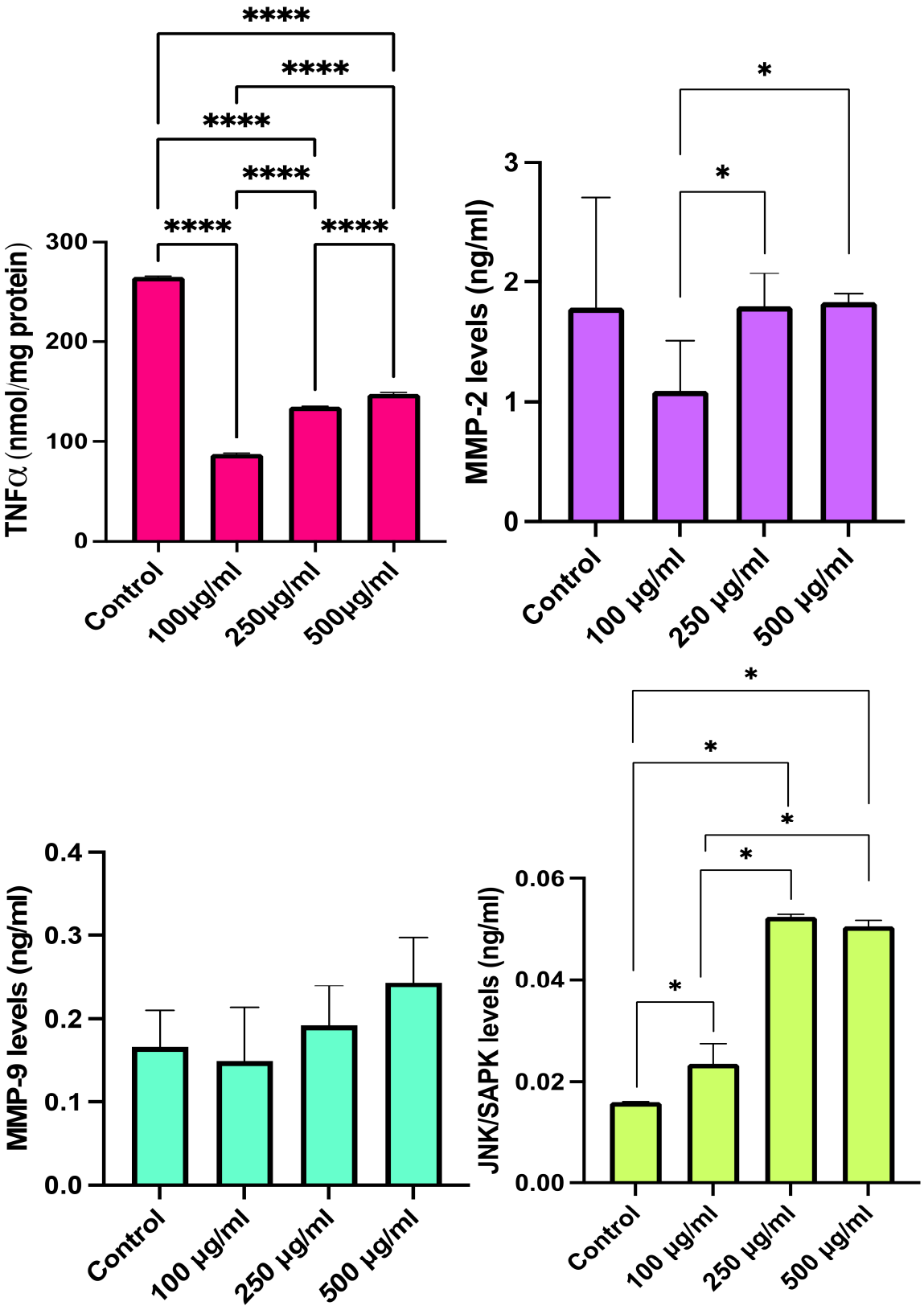

Fig. 3: Tumor necrosis factor-alpha (TNF-α), matrix metalloproteinase (MMP)-2, MMP-9 and c-Jun NH(2)-terminal kinase/stress-activated protein kinase (JNK/SAPK) levels in C6 glioma cells treated with propolis. Graphical data are expressed as mean ± SD. *P<0.05. ****P<0.0001.

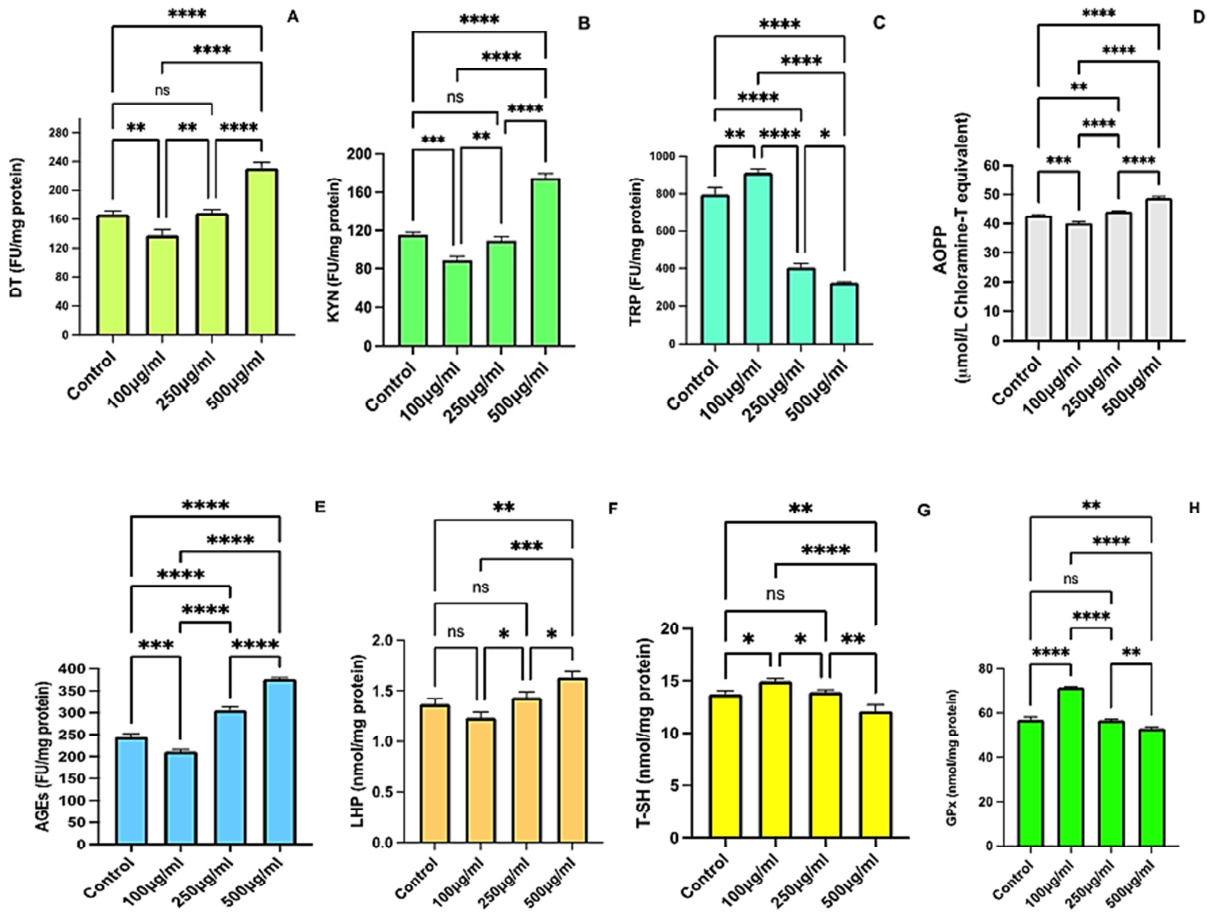

Fig. 4: Levels of oxidant and antioxidant status biomarkers. DT; dityrosine (A), KYN; kynurenine (B); TRP; tryptophan (C), AOPP; advanced oxidation protein products (D), AGEs; advanced glycation end products (E), LHP; lipid hydroperoxides (F), T-SH; total thiol (G), Gpx; glutathione peroxidase (H), FU; fluorescence unit. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001, ns; non-significant.

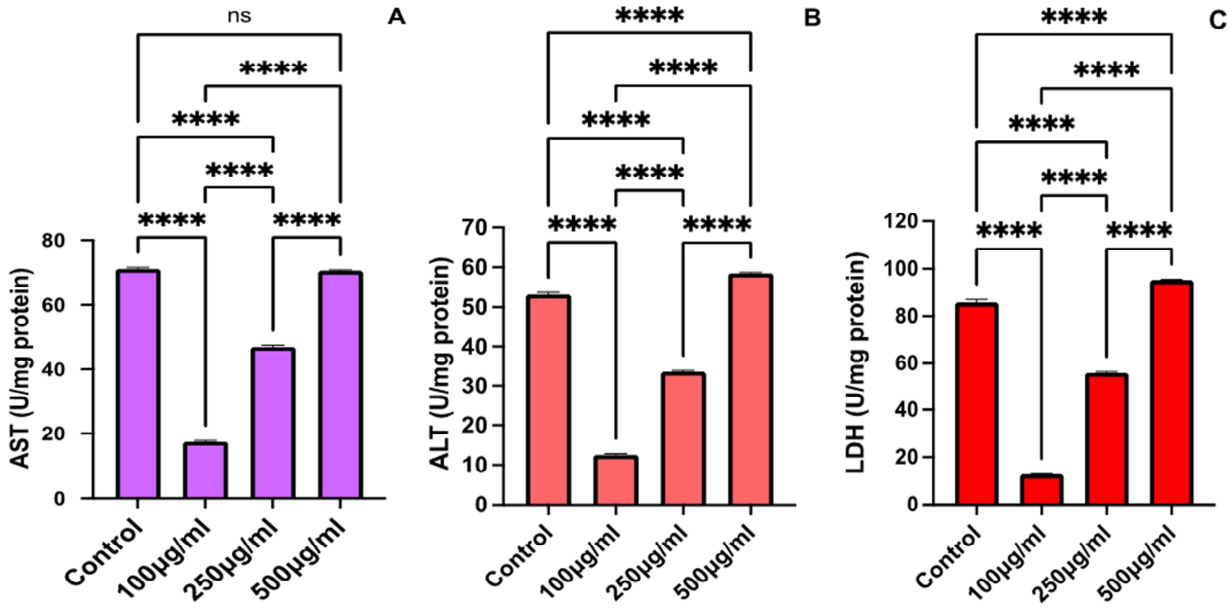

Fig. 5: Cell injury parameters: AST; aspartate aminotransferase (A), ALT; alanine aminotransferase (B), LDH; lactate dehydrogenase (C). ****; P<0.0001, ns; non-significant.

Analysis of oxidant and antioxidant status biomarkers

Oxidant status parameters are shown in fig. 4. Propolis administration affected oxidant status biomarkers and antioxidant status biomarkers and depends on their dosage. Treatment with a low dosage of propolis (100 µg/ml) demonstrated a statistically significant decrease in all of the oxidant status biomarkers levels except tryptophan when compared to untreated glioma cells, moderate (250µg/ml) and high dose (500µg/ml) of propolis treatment. TRP levels were higher in glioma cells treated with low dose of propolis when compared to other groups. Low dose administration of propolis showed antioxidant properties and ameliorated oxidative damage. However, moderate and high dose of propolis showed pro-oxidant effect. Antioxidant status biomarkers are shown in fig. 4. Treatment with low dose propolis resulted in increased levels of TSH and GPx.

Effects of propolis on cell injury

Cell injury biomarkers are shown in fig. 5. Changes in LDH, AST and ALT levels were similar. The lowest levels of these three parameters were found in the group administered with 100µg/ml of propolis.

DISCUSSION

This study provided evidence that Turkish propolis may exert anti-tumor effects by regulating COX-2 and NF-κβ mRNA expression and redox homeostasis in glioma cells. Studies have reported that the overexpression of COX-2 increases angiogenesis, which is associated with tumor growth, metastasis, and invasion, and facilitates the progression of some types of cancer, such as liver, colon and pancreas (Tang *et al*., 2005; Cascinu *et al*., 2007; Othman *et al*., 2022). The study by Xing *et al*. (2019), demonstrated that isoalantolactone inhibited COX-2 expression and induced apoptosis in glioma cells by blocking the NF-κβ signaling pathway. Thus, researchers proposed that isoalantolactone may be an effective antitumor agent in the treatment of glioblastoma.

Similarly, Othman *et al*. (2022) suggested that inhibition of COX-2 and NF-κβ by zinc nanoparticles biosynthesized with berberine caused suppression of carcinogenesis in Caco-2 cells. It has been reported that suppressing the overexpression of NF-κβ, which is known to be abnormally activated in most tumors, may prevent glioma progression. Researchers asserted that an agent called pantoprazole may be an effective anticancer drug for glioma by attenuating NF-κβ signaling (Geeviman *et al*., 2018). In our previous study, we reported that Turkish propolis has cytotoxic and apoptotic effects (Coşkun et al., 2020). In this study, we found that propolis reduced both COX-2 and NF-κβ mRNA expression. Microscopic images confirmed our results.

Wang *et al*. (2015) showed that TNFα, a pro-inflammatory cytokine, induced NF-κβ activation in HeLa cells and used dihydrotanshinone I to inhibit NF-κB activation. MAP kinases such as JNK/SAPK and p38 are known to be activated by TNFα (Yeh *et al*., 2008). In addition, NF-κB, causes tumor progression by regulating invasion-associated factors, such as matrix metalloproteases (Nagendraprabhu *et al*., 2011). In our study, we found that the TNFα level in glioma cells decreased with the application of propolis. On the contrary, it was determined that the JNK/SAPK level was found to be higher in the propolis applied groups. According to Tomite *et al*. (2003), the increase in the JNK/SAPK activation of human leukemia (MOLT4) cells may be due to X-ray-induced apoptosis/rapid cell death. We can say that the increase in JNK/SAPK level in C6 glioma cells caused by propolis application can be considered as an indicator of cell death. On the other hand, no significant change was observed in the propolis treated groups compared with the control group in MMP-2 and -9 levels. Thus, it can be thought that propolis does not show any effect on invasion via MMP-2 and -9.

Although the impaired redox status is known to increase tumor invasion and metastasis in brain cancer, some studies have reported that oxidative stress inhibits the growth of glioma cells and causes apoptotic cell death (Liu *et al*., 2015; Yang *et al*., 2020). Ostrowski and Pucko (2022) proposed that an optimal approach to exploit oxidative stress caused by the impaired redox status for anti-glioma treatment. According to this approach, impaired redox status caused by free radicals should be induced and antioxidant defense mechanisms should be suppressed selectively in glioma cells. That is, optimal treatment of gliomas should be able to both modulate oxidative/nitrosative stress-induced impaired redox status and trigger apoptotic cell death mechanisms (Ostrowski and Pucko, 2022). Despite the antioxidant capacity of propolis, which is known to have anti-inflammatory and antioxidant activities, it has been reported that it can trigger oxidative DNA damage due to some flavonoids it contains (Tsai, 2012). In our study, we evaluated the levels of DT, KYN, TRP and AOPP levels, which are biomarkers of global and specific protein oxidation as well as the levels of AGEs and LHP, which are other biomarkers involved in impaired redox homeostasis. According to our findings, it was observed that propolis applied to glioma cells at a concentration of 100µg/ml showed an antioxidant effect and reduced the above-mentioned biomarkers except TRP. On the contrary, it was observed that these markers of propolis applied at concentrations of 500µg/ml, except for TRP, increased. In addition, the opposite effect was observed in the levels of antioxidant markers T-SH and GPx. Thus, we can say that Turkish propolis, which has an antioxidant effect, can increase oxidative stress by showing a pro-oxidant effect above a certain dose and cause the death of glioma cells. In our study, cell damage biomarkers such as LDH, ALT and AST also supported the findings of impaired redox status.

CONCLUSION

In our study, Turkish propolis was found to decrease COX-2 and NF-κβ mRNA expressions and TNFα levels, increase JNK/SAPK levels, and affect redox status biomarkers in C6 glioma cells depending on the dose. However, no effect of propolis on MMP-2 and MMP-9 was found. Thus, evidence emerged that Turkish propolis may have an anti-tumor effect on C6 glioma cells by causing cell damage and death in a dose-dependent manner, but it has no effect on preventing invasion. However, more research is needed on the role of propolis in the treatment of glioma.

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