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RESEARCH ARTICLE

TURKISH PROPOLIS MITIGATES OXIDATIVE STRESS AND ENDOPLASMIC RETICULUM STRESS IN CARDIAC DAMAGE CAUSED BY DOXORUBICIN IN RATS

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ABSTRACT

Purpose: This study examined the effects of propolis extracted with ethanol (EPE) or olive oil (OPE) on the endoplasmic reticulum (ER) and oxidative stress in doxorubicin (DXR)-induced cardiac damage in rats.
Material and Methods: Six groups of Sprague–Dawley rats were used in this research: Control, EPE, OPE, DXR, EPE+DXR, and OPE+DXR. The extracts were administered orally for two weeks (50 mg/kg/day), and DXR was injected 48 hours before sacrifice (15 mg/kg). Cardiac malondialdehyde (MDA) and glutathione (GSH) levels and catalase (CAT) activity were assayed spectrophotometrically, while cardiac glucose-regulated protein (GRP)78, pro-caspase 12, and serum troponin I levels were determined via ELISA.
Results: The DXR group presented elevated serum troponin I, indicating cardiac injury, increased MDA, decreased CAT activity, and reduced GSH, indicating oxidative stress, along with elevated GRP78 and decreased pro-caspase 12, indicating ER stress. Pretreatment with EPE or OPE significantly prevented DXR-induced increases in troponin I and MDA, as well as decreases in GSH, CAT activity, and pro-caspase 12, but did not significantly alter cardiac GRP78 levels. Compared with the EPE+DXR group, the OPE+DXR group presented higher levels of CAT activity ($p<0.01$).
Conclusion: Pretreatment with EPE or OPE may provide protection against DXR-induced cardiotoxicity by suppressing both oxidative and ER stress.

Keywords: cardiac injury, ethanol, GRP78, MDA, olive oil, pro-caspase 12, propolis

INTRODUCTION

Doxorubicin (DXR) is a potent chemotherapeutic agent employed in the management of a wide range of neoplasms. However, its clinical utilization is predominantly restricted by its cardiotoxicity, which has been observed in approximately 25% of patients undergoing therapy (1,2). The basic cellular processes responsible for DXR-induced cardiotoxicity are multifactorial and include mitochondrial damage, increased production of reactive oxygen species (ROS), oxidative damage to cellular components, calcium dysregulation,

endoplasmic reticulum (ER) stress and apoptotic cell death (3,4).

ER stress initiates a cellular response when unfolded or misfolded proteins accumulate within the ER lumen in response to various threats, such as oxidative stress, nutritional deprivation, calcium imbalance, genetic mutations, or viral infection (5). It has been reported that DXR therapy can induce ER stress in cardiomyocytes through the induction of ROS production and disruption of redox balance, which negatively affects protein folding processes in the ER lumen (6). To restore cellular homeostasis, the ER stress response stimulates ER-associated protein degradation and suppresses the protein synthesis machinery, but it increases the synthesis of chaperone proteins such as glucose-regulated protein (GRP)78 and GRP94, which are involved in protein folding processes in the ER. However, extended or severe ER stress can trigger apoptosis via three main pathways: caspase 12, C/EBP homologous protein and c-JUN NH2-terminal kinase (5).

Researchers are intensively investigating strategies to reduce the toxic side effects of DXR while maintaining its anticancer activity (7,8). Propolis, a natural bee product, has been shown to possess antioxidant, anti-inflammatory, and antitumour benefits and has emerged as a potential agent to mitigate or prevent DXR-induced cardiotoxicity (9). Previous studies have shown that propolis, administered intraperitoneally or orally, reduces DXR-induced cardiotoxicity primarily by decreasing oxidative stress (10-12). Its high polyphenolic content, which scavenges free radicals and prevents lipid peroxidation, contributes significantly to its therapeutic potential. Propolis and its biologically active constituents, including caffeic acid phenethyl ester (CAPE), chrysin, and artepillin C, have demonstrated efficacy in attenuating ER stress in various cell lines (13-15). Although the impact of propolis on ER stress within the context of DXR related cardiotoxicity remains to be fully clarified, CAPE has been shown to mitigate ER stress in rat cardiomyoblast H9c2 cells under in vitro DXR exposure (13).

As propolis is a complex mixture of phenolic acids and flavonoids, the cellular benefits of propolis are likely different from those of individual compounds (9). Therefore, the content, biological activity and protective efficacy of propolis extracts may be affected by regional variations in propolis and the solvents used in its extraction (16-18). Ethanol is the preferred solvent for propolis extraction, but alternatives such as water, olive oil, and propylene glycol are also used (19). Olive oil, a key constituent of the Mediterranean diet, attenuates oxidative damage and the inflammatory response and has been shown to reduce DXR-induced cardiotoxicity on its own (20, 21).

To date, studies on the cardiotoxicity of DXR have focused on ethanolic extracts of propolis, and no data are available on extracts prepared in olive oil. In our recent study, we reported that olive oil-based propolis was more effective at preventing DXR-induced hepatorenal oxidative stress than ethanol-based extracts were (22). The present study therefore focused on the influence of propolis extracts obtained using olive oil and ethanol on DXR-induced cardiotoxicity and compared their efficacy in alleviating ER stress and oxidative stress in myocardial damage.

MATERIAL AND METHODS

Ethical Considerations

This study was reviewed and approved by the Institutional Animal Experiments Ethics Committee of Ordu University (Date: 11.01.2023, Decision No: 2023/09).

Propolis extraction procedures

The raw Turkish propolis used in this study was obtained from accredited beekeepers operating in the Black Sea region of Turkey. The propolis was kept at -20°C until the extraction procedure was carried out. For the preparation of ethanol-based propolis extract (EPE), the propolis was ground and mixed with ethyl alcohol (96%) for a period of 36 hours. The procedure was performed in the dark at a temperature of 20–22°C. The aforementioned mixture was then filtered through filter paper (Whatman No. 1). Following evaporation of the solvent, a quantity of water was added until the ethanol concentration reached 2%. This resulted in the formation of a homogeneous mixture. For the preparation of olive oil-based propolis extract (OPE), the propolis was ground and stirred with extra-virgin olive oil for a period of 72 hours. This procedure was also carried out in the dark at 35°C. Thereafter, the sample was cooled to ambient temperature and subjected to filtration (18, 23).

Animals and study design

In this study, young adult Sprague–Dawley rats (aged between 2.5 and 3 months) were utilized. The animals were randomly allocated to six groups, with a total of seven animals per group.

- Control (n=7): The animals were given drinking water once a day for fourteen days by intragastric gavage (ig).
- EPE (n=7): Rats were given a daily dose of propolis extract prepared in ethanol (50 mg/kg, ig) for two weeks (12).
- OPE (n=7): Rats were given a daily dose of an extract of propolis processed in extra-virgin olive oil (50 mg/kg, ig) for a period of two weeks (12).
- DXR (n=7): For fourteen days, the animals were given daily drinking water (ig), and DXR injection (15 mg/kg) was performed intraperitoneally 48 hours before the termination of the study (24).
- EPE+DXR (n=7): Rats were given a daily dose of propolis extract prepared in ethanol (50 mg/kg, ig) for fourteen days. DXR was injected 48 hours prior to the termination of the study, in the same manner as in the DXR group.
- OPE+DXR (n=7): Rats received a daily dose of propolis extracted with olive oil (50 mg/kg, ig) for fourteen days. The administration of the DXR injection was conducted 48 hours prior to the conclusion of the study, employing the same methodology as was utilized in the DXR group.

The animals involved in the study were given unrestricted access to a regular laboratory diet as well as water. At the conclusion of the experiment on day 15, the rats were euthanized under general anesthesia (ketamine and xylazine, 80:10 mg/kg, ip). The animals were sacrificed after the heart and blood samples were taken quickly. Blood samples were maintained at a temperature of 20-22°C for about 30 minutes. Following this, centrifugation at 1000 g for 20 minutes was performed to obtain the serum. The serum samples and the heart tissue samples were preserved in a deep freezer (-80°C). On the day of the experiment, the serum samples were used for the measurement of troponin I, whereas the tissue samples were used for the following parameters after homogenization.

Troponin I measurement

To estimate the serum levels of cardiac troponin I (TNNI3/cTn-I), a commercially available ELISA kit was used (Elabscience, China), and the assay was conducted in strict accordance with the manufacturer's instructions. Serum troponin I concentrations were estimated via a standard curve.

MDA measurement

Prior to the measurements, the tissue was weighed and homogenized in 1.15% KCl (1:9). The homogenate was subjected to centrifugation at a speed of 1000 × g (10 minutes). The resulting top layer was then used to measure the MDA and GSH contents.

MDA measurements were performed via a spectrophotometric method (25). This method relies on the interaction between MDA and thiobarbituric acid (TBA), resulting in the formation of an MDA-TBA2 adduct that strongly absorbs at 535 nm. The results were calculated via a standard graph and expressed as a ratio to wet tissue weight.

GSH measurement

The spectrophotometric method for GSH involves the oxidation of GSH by the Ellman reagent (5,5′-dithiobis (2-nitrobenzoic acid)), which produces a yellow derivative (5'-thio-2-nitrobenzoic acid) that can be read at 412 nm (26). The results were determined from a standard graph and are presented relative to wet tissue weight.

CAT activity measurement

To determine the enzymatic capacity of CAT, the heart tissue was initially homogenized in 1:9 phosphate buffer. Next, the mixture was subjected to centrifugation at 1000 × g (10 minutes). The activity of the enzymes was determined by monitoring the decrease in hydrogen peroxide (H2O2) concentration at a wavelength of 240 nm over time via a spectrophotometer (27). The data are expressed as ratios relative to milligrams of wet tissue.

Measurement of GRP78 levels

Prior to the execution of the ELISA experiments, the cardiac tissues were homogenized in phosphate buffer solution (1:9 ratio), and then, the samples were subjected to a centrifugation process (5000 × g, 10 minutes) to yield the supernatant. GRP78 concentrations in tissue homogenates were evaluated by ELISA via appropriate commercial kits (BT-Lab, China). The assay procedure was conducted following the manufacturer's instructions. The results were calculated using a standard graph and are presented as the ratio of the total protein, which was determined according to the Bradford

method in the same homogenates (Thermo Scientific, USA).

Measurement of the pro-caspase 12 level

Cardiac levels of pro-caspase 12, the inactive form of caspase 12, were measured by ELISA method via a suitable commercial assay kit (BT-Lab, China) in tissue supernatants prepared by homogenization in phosphate buffer. The assay was carried out as described in the supplier's instructions. The amount of pro-caspase 12 was determined by means of a standard curve and is related to the amount of total protein in the sample.

Statistical evaluation

All the data are shown as the means ± standard deviations (SDs). The Kolmogorov–Smirnov test was utilized to confirm the normality of the variables. Statistical analysis was performed using one-way analysis of variance and Tukey's test (GraphPad Prism 4.0 software). Pearson's correlation analysis was employed to assess the interrelationships between the specified parameters. Statistical significance was considered for p values less than 0.05.

RESULTS

The impact of propolis extracts on the serum levels of troponin I in DXR cardiotoxicity

The serum concentration of troponin I in the control group was 823.41±71.75 pg/ml and did not change significantly in the EPE and OPE groups ($p>0.05$). DXR injection caused a significant increase in the serum troponin level in the DXR group (1730.34±184.39 pg/ml, vs control $p<0.001$). Two weeks of treatment with propolis extract significantly reduced DXR-induced troponin increases in the EPE+DXR group (1251.80±220.71 pg/ml, vs DXR $p<0.001$) and OPE+DXR group (1234.08±210.59 pg/ml, vs $p<0.001$ DXR); however, the values did not completely return to the control levels. The findings revealed that troponin levels in the EPE+DXR and OPE+DXR groups were comparable ($p>0.05$) (Figure 1).

Influence of propolis extracts on the oxidative stress response in DXR-induced cardiotoxicity

The oxidative stress status in cardiac tissue was evaluated by the MDA level, GSH level, and CAT activity. Two weeks of treatment with propolis extract prepared in ethanol or olive oil did not significantly affect cardiac MDA or GSH levels or CAT activity ($p>0.05$). As demonstrated in Figure 2, DXR injection significantly induced cardiac oxidative stress, as evidenced by elevated MDA levels (from 191.62±39.89 to 523.63±68.80 nmol/g tissue, $p<0.001$), GSH depletion (from 3.75±0.51 to 1.10±0.22 µmol/g tissue, $p<0.001$) and decreased activity of CAT (from 3.64±0.34 to 1.34±0.37 µmol H_2O_2 /min/mg tissue, $p<0.001$) compared with those in the control group. The alterations in these parameters that were induced by DXR approximated the control values to a significant degree in both the EPE+DXR and OPE+DXR groups. However, none of the parameters fully returned to the control levels (Figure 2). The effectiveness of the two extracts in reducing DXR-induced oxidative stress was similar, except for CAT activity. A comparison of the OPE+DXR and EPE+DXR groups revealed a significantly greater level of CAT activity in the OPE+DXR group ($p<0.01$) (Figure 2C).

The impact of propolis extracts on the ER stress response in DXR-induced cardiotoxicity

In the control rats, the GRP78 level in the heart tissue was 0.62±0.08 ng/mg protein and did not significantly change in the EPE- or OPE-treated rats (0.59±0.07 and 0.62±0.17 ng/mg protein, respectively). Compared with the control, DXR injection significantly increased the level of GRP78 in cardiac tissue to 0.81±0.09 ng/mg protein ($p<0.001$). Pretreatment with EPE slightly reduced the DXR-induced increase in GRP78 level in the EPE+DXR group (0.73±0.04 ng/mg protein); however, this decrease was not statistically significant ($p>0.05$). A comparable response was detected in the OPE+DXR group (0.69±0.10 ng/mg protein) (Figure 3A).

The cardiac level of pro-caspase 12, the inactive form of caspase 12, was 17.36±4.01 ng/µg protein in the control group, and two weeks of treatment with EPE or OPE did not cause any significant change in its level (16.02±2.78 and 16.72±4.29 ng/µg protein, respectively, $p>0.05$). A substantial decrease in pro-caspase 12 levels was evident in the DXR group compared with the control group (9.96±1.36 ng/µg protein, $p<0.01$). However, this value did not differ from that of the control in the EPE+DXR and OPE+DXR groups ($p>0.05$), both of which had significantly higher levels of pro-caspase 12 than the DXR group (14.62±1.91 and 14.78±2.21 ng/µg protein, respectively) (Figure 3B).

The correlations between the data

To evaluate the relationships between cardiac damage and stress parameters, correlation analyses between troponin I and both oxidative stress and ER stress parameters were performed. Significant correlations were detected between the serum troponin I concentration and the cardiac MDA level ($r = 0.8569$, $p < 0.0001$, positive), GSH concentration ($r = -0.8217$, $p < 0.0001$, negative), and CAT activity ($r = -0.7916$, $p < 0.0001$, negative) (Figure 4A-C).

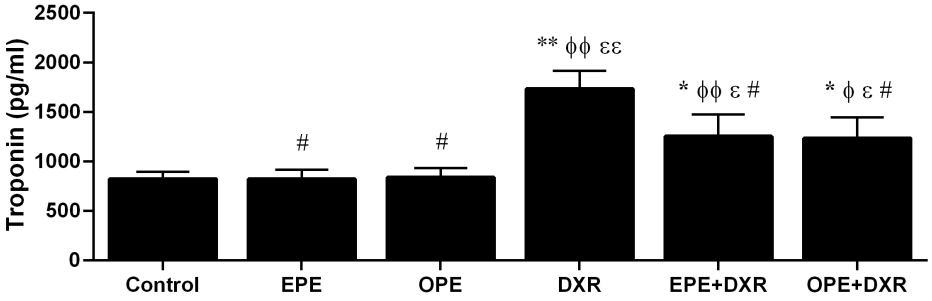

Figure 1. Serum troponin I levels in the control, ethanolic propolis extract (EPE, 50 mg/kg), olive oil propolis extract (OPE, 50 mg/kg), doxorubicin (DXR, 15 mg/kg), EPE+DXR and OPE+DXR groups. The data are presented as the means ± SDs (n=7). Statistical difference from the control *p<0.01, **p<0.001; from the EPE ϕp<0.01, ϕϕp<0.001; from the OPE εp<0.01, εεp<0.001; from the DXR #p<0.001.

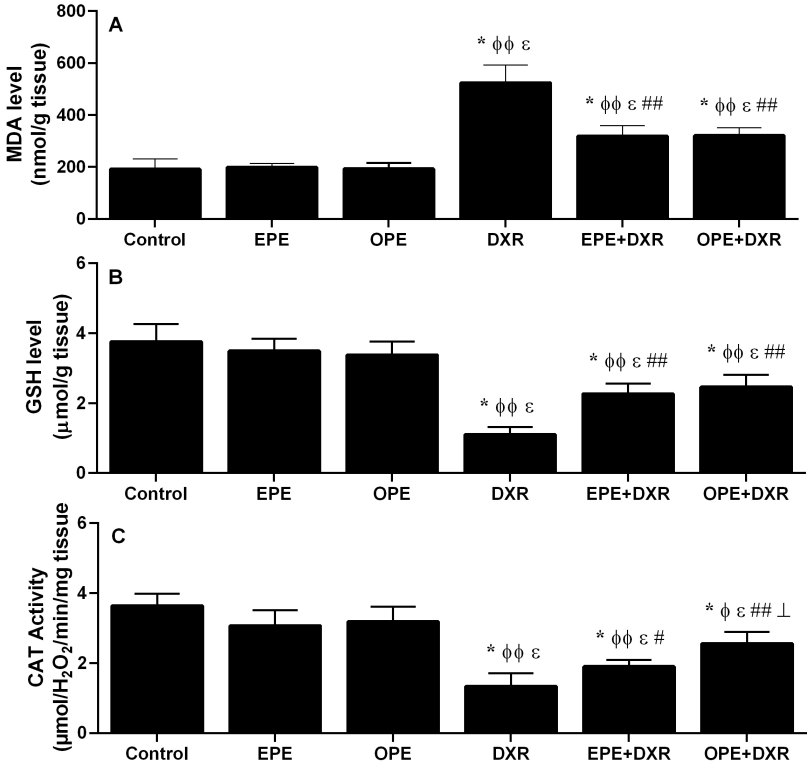

Figure 2. Changes in the cardiac oxidative stress response in the control, ethanolic propolis extract (EPE, 50 mg/kg), olive oil propolis extract (OPE, 50 mg/kg), doxorubicin (DXR, 15 mg/kg), EPE+DXR and OPE+DXR groups. The oxidative stress response was assessed by the tissue levels of MDA (A) and GSH (B) and the activity of CAT (C). The data are presented as the means ± SDs (n=7). Statistical differences from the control *p<0.001; from the EPE ϕp<0.01, ϕϕp<0.001; from the OPE εp<0.001; from DXR #p<0.05, ##p<0.001; from the EPE+DXR ⊥p<0.01.

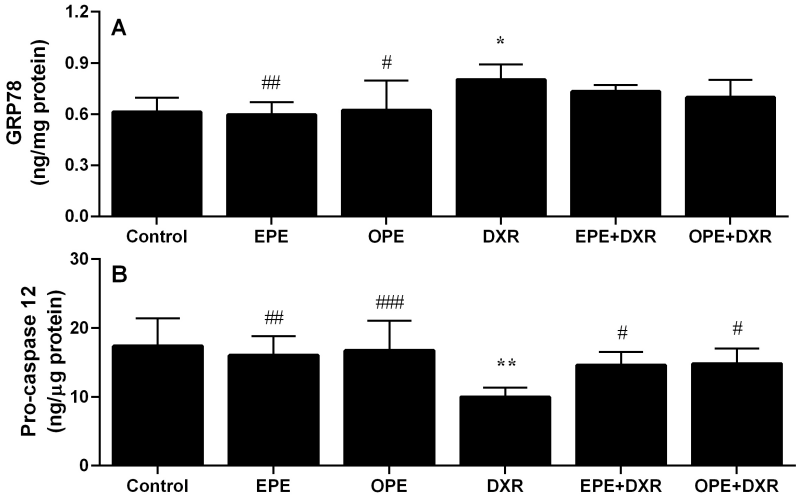

Figure 3. Changes in the cardiac ER stress response in the control, ethanolic propolis extract (EPE, 50 mg/kg), olive oil propolis extract (OPE, 50 mg/kg), doxorubicin (DXR, 15 mg/kg), EPE+DXR and OPE+DXR groups. The ER stress response was evaluated by determining the tissue levels of GRP78 (A) and pro-caspase 12 (B). The data are presented as the means ± SDs (n=7). Statistical difference from the control *p<0.05, **p<0.01; from the DXR #p<0.05, ##p<0.01.

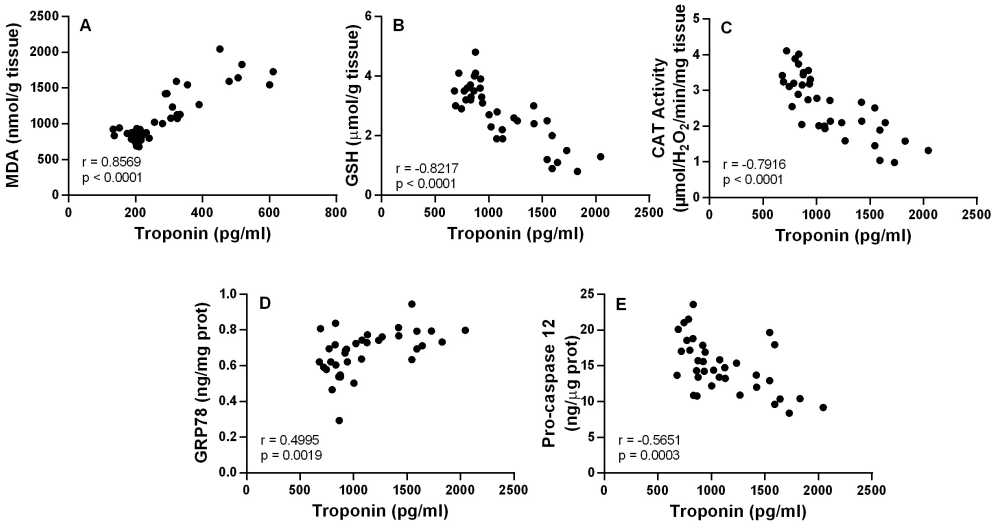

Figure 4. Correlations between serum troponin I concentrations and cardiac MDA levels (A), GSH levels (B), CAT activity (C), GRP78 levels (D) and pro-caspase 12 levels (E). Correlation analyses were performed via Pearson's correlation analysis.

According to the correlation analysis between serum troponin I and ER stress parameters, there was a positive correlation with GRP78 (r = 0.4995, p = 0.0019) and a poor correlation with pro-caspase 12 (r = -0.5651, p< 0.0003) (Figure 4D-E).

DISCUSSION

Several reports to date have indicated that ethanol based propolis can reduce DXR cardiotoxicity by decreasing oxidative damage, inflammation and cell death. However, its effects on ER stress have not yet been studied (3, 4, 28). In this study, the effects of Turkish propolis extracted with ethyl alcohol and olive oil on endoplasmic reticulum stress and the oxidative stress response during DXR-induced cardiotoxicity were investigated.

Given that the serum troponin level is a sensitive marker for DXR-induced cardiotoxicity (29), this study

utilized the serum troponin I level to evaluate cardiac damage. Consistent with expectations, the DXR group presented a substantial increase in troponin I levels. The groups treated with EPE for two weeks presented a lower level of DXR-induced troponin I increase. Although histological examination was not performed, which is a limitation of this study, the dose of propolis used and the treatment duration are effective in reducing DXR-induced cardiac damage. The attenuation of DXR-induced oxidative stress, as evidenced by lower levels of lipid peroxidation, higher levels of GSH and increased CAT activity after pretreatment with EPE, demonstrates the antioxidant properties of propolis as one of the protective mechanisms by which it protects against DXR-induced cardiotoxicity, which is consistent with previous studies (10-12).

The effects of propolis extracted with olive oil on cardiac damage during DXR toxicity were also investigated in this study. Our results demonstrate, for the first time, that OPE provides protection against DXR-induced cardiotoxicity and is as effective as EPE in reducing serum troponin I levels and oxidative damage. Importantly, the OPE treatment improved CAT activity better than the EPE treatment. In our previous study comparing the efficiency of olive oil and ethanol-based propolis extracts in DXR-induced hepatorenal damage, we demonstrated that the reduction in lipid peroxidation and the increase in SOD activity in the kidney and liver, as well as the increase in GSH levels in the liver, were greater in the oily extract group; however, the increase in CAT activity was similar in both extract treatment groups (22). These results suggest that the efficacy of OPE may vary depending on the tissue, with cardiac tissue providing less benefit than hepatorenal tissues from the treatment. The variations in cardiac CAT activity levels observed in the EPE+DXR and OPE+DXR groups may be attributed to differences in the biologically active components of the two extracts, as the active components of propolis not only scavenge free radicals but also possess the ability to activate antioxidant enzyme systems, including CAT, at the transcriptional level (34). However, content analyses of the extracts were not performed, which may be another limitation of the present study. On the basis of the literature knowledge that olive oil alone provides cardioprotection against DXR damage, the difference in the effectiveness of the extracts can also be attributed to the direct effect of olive oil (21). Nonetheless, the olive oil vehicle group (4 ml/kg/day) showed no changes in any of the parameters we assessed, including CAT activity (data not shown). The inefficiency of extra virgin olive oil in the vehicle group might be dependent on the lower volume of olive oil administered to the subjects than in the literature (10 ml/kg/day) (21). However, despite the higher levels of CAT activity, the similarity in lipid peroxidation and cardiac damage to those of the ethanolic extract group suggests that there was not a sufficient increase in the total antioxidant capacity of the tissue to reduce oxidative damage in the present study.

In our study, we also investigated how different propolis extracts affect ER stress in DXR-induced cardiac injury. Previously, it has been shown that propolis from different geographical regions or its components (e.g., artepillin C and chrysin) can inhibit ER stress (35-37). However, the effect of propolis on the cardiotoxicity of DXR has not been studied thus far. Consistent with previous reports (30,31,38,39), DXR-induced ER stress in this study was evidenced by increased GRP78 and decreased pro-caspase 12 levels. Compared with those in the DXR group, both the EPE+DXR and OPE+DXR groups presented slight decreases in GRP78 levels accompanied by significant increases in pro-caspase 12 levels, indicating alleviation of the ER stress response. Altgough Turkish propolis has demonstrated efficacy in mitigating ER stress in a lung cancer cell line (36), its impact on ER stress in nontumor cells remains unexplored. This study, therefore, represents the first attempt to elucidate the effect of Turkish propolis on ER stress in this context.

The dose of propolis used in our study is the lowest dose reported in the literature to be effective in treating DXR-induced cardiotoxicity (12). According to the present results, treatment with a low dose of propolis has been sufficient to alleviate oxidative stress and ER stress to some extent but is inadequate to completely prevent them. Nevertheless, the suppression of these two damage mechanisms by propolis appears to be successful in partly reducing tissue damage. The strong correlations between serum troponin I levels and lipid peroxidation ($r = 0.8569$), GSH levels ($r = -0.8217$), and CAT activity ($r = -0.7916$) indicate the role of oxidative stress inhibition in propolis-mediated cardioprotective effects (Figure 4). Similar relationships have been observed with ER stress parameters. The positive correlation between troponin I and GRP78 ($r = 0.4995$) and the negative correlation between

troponin I and pro-caspase 12 (r = -0.5651) suggest that the suppression of the ER stress response may also play a role in the cardioprotective effects of propolis (Figure 4). Although the correlations between troponin I and ER stress parameters were found to be statistically significant, the correlations between troponin I and oxidative stress parameters appeared to be much stronger. Therefore, while the suppression of ER stress may contribute to the cardioprotective effects of propolis, its antioxidant effect may be the predominant mechanism.

CONCLUSION

This study investigated the effects of treatment with various propolis extracts on oxidative stress and ER stress in DXR-induced cardiotoxicity. These results indicate that propolis treatment provides protection against DXR-induced cardiotoxicity by suppressing both oxidative stress and the ER stress response. Another finding of the study is that the propolis extract prepared in olive oil is as effective as the ethanolic extract and even more successful in preserving endogenous CAT activity. These results may have several potential clinical implications. First, propolis extracts could be developed as cardioprotective adjuvants for patients undergoing DXR chemotherapy, potentially allowing higher therapeutic doses while minimizing cardiac damage. Second, the comparable efficacy of the olive oil-based extract offers opportunities for use in pediatric patients or those with alcohol sensitivity or religious restrictions. Future research should focus on determining optimal dosage regimens and investigating whether these protective effects are replicated in humans.

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Author contribution: Conception: SC; Design: SC, EKC; Supervision: SC, EKC; Fundings: SC; Materials: SC, EKC, GH, EGGP; Data Collection and/or Processing: SC, EKC, GH, EGGP; Analysis- Interpretation: SC, EKC, GH, EGGP; Literature Review: SC, EK; Writing: SC, EKC, GH, EGGP; Critical Review: EKC, GH,
Conflict of interests: The Authors declare that there is no conflict of interest.
Ethical approval: This study was reviewed and approved by the Institutional Animal Experiments Ethics Committee of Ordu University (Date: 11.01.2023, Decision No: 2023/09).
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