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Polyphenolic-enriched olive leaf extract attenuated doxorubicin-induced cardiotoxicity in rats via suppression of oxidative stress and inflammation

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Abstract

Background: The therapeutic value of doxorubicin as an effective anti-neoplastic agent is limited by its cardiotoxic side effects. We investigated the effects of ethanolic leaf extracts of olive leaf OL on cardiotoxicity as well as oxidative stress which was induced by doxorubicin (DOX) in Wistar rats. The cardiotoxicity was induced by intraperitoneally injecting a single dose of doxorubicin (10 mg kg⁻¹) after 7 days of OL administration. OL was given by gastric gavage in 250 mg/kg, 500 mg/kg and 1000 mg/kg doses of extract for 10 days.

Results: Cardiac toxicity of DOX was evidenced by histopathological changes in cardiac tissues and an increase in the activities of serum markers of heart damage (AST and CK). DOX caused oxidative stress as evidenced by the elevation of malondialdehyde, protein carbonyl content levels, and catalase activity. That stress was also accompanied by a concurrent depletion of the activity of superoxide dismutase within cardiac tissues. The cardiotoxicity and oxidative stress damages caused by DOX also coincided with an increase of myeloperoxidase activity and iNOS expression. Most of these doxorubicin-induced biochemical and histological alterations were effectively attenuated by prior administration of OL. OL combination with DOX significantly increased its cytotoxicity in HepG2 liver cancer cell line and IC50 dropped from 259.35 to 158.12 µg/ml.

Conclusion: OL potentiated the cytotoxicity of DOX in liver cancer cell line and may play a role in the protection against its cardiotoxicity and thus can be a useful adjuvant therapy where doxorubicin is the common liver cancer-treating drug.

Keywords: Protective effects, Olive leaves, Doxorubicin, Cardiotoxicity

Background

Doxorubicin (DOX) or adriamycin is an effective anti-cancer agent belongs to an anthracycline antibiotic which is a commonly used to treat an abundance of cancers including breast, liver, lung cancer, ovarian

tumors, lymphoma, and leukemia. Despite being a highly targeted molecule for cancer therapy, its clinical efficacy is greatly restricted because of the development of a severe form of cardiomyopathy or congestive heart failure in cancer patients (Li et al., 2009; Thorn et al., 2011). The timing of anthracycline-associated heart failure can be highly variable and has been categorized as acute, early-onset chronic progressive, and late-onset chronic progressive (Cowgill et al., 2019). The molecular mechanisms of DOX-induced cardiotoxicity include the formation of free radicals and direct harm to DNA (Cowgill et al., 2019; Ghigo et al., 2016;

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Raj et al., 2014). DOX enzymatically reduces to its semiquinone radical. This DOX semiquinone radical generates reactive oxygen species (Ghigo et al., 2016; Thorn et al., 2011) which result in oxidative damage to cellular components including lipids, proteins and DNA (Ghigo et al., 2016). Peroxynitrite, a reactive oxidant produced by the rapid reaction of nitric oxide and superoxide, is mainly involved in DOX cardiotoxicity (Ghigo et al., 2016; Mihm et al., 2002).

The search for an effective agent to reduce its cardiotoxicity remains a critical issue. Although synthetic agents (such as antioxidants and metal chelators) have been investigated to decrease DOX-induced cardiotoxicity (Raj et al., 2014; Yagmurca et al., 2003), some of these agents such as vitamin E failed to inhibit DOX cardiotoxicity (Bjelogrlic et al., 2005; Ghigo et al., 2016) and others, such as dexrazoxane (an iron chelator) increased its toxicity (Ghigo et al., 2016; Li et al., 2009). In addition to the classical antioxidants, the protective effect of plant-derived polyphenolic compounds has become a trend for use against DOX cardiotoxicity. Some plants with antioxidant properties have been examined. Among these botanical substances were *Withania somnifera* (Hamza et al., 2008), 6-gingerol (El-Bakly et al., 2012), *Melissa officinalis* (Hamza et al., 2016), curcumin (He et al., 2018), and cranberry and rosemary (El -Desouki et al., 2019) which found to decrease cardiotoxicity of DOX.

In recent years, attention was drawn to the majority of natural phenolic substances possessing antioxidants and anti-inflammatory properties due to their cardioprotective and chemopreventive effects (Shah et al., 2019). The olive leaf (OL) [*Olea europaea* L; family Oleaceae] is commonly used in traditional medicine around European and Mediterranean regions as a diuretic, hypotensive tonic for urinary infections, and antiperspirant (Mosleh et al., 2016). Studies show that the principle active gradients of OL extract; oleanolic and urosolic acids have potent antihypertensive, anti-hyperlipidemic, hypoglycemic, anti-atherosclerotic, and antioxidative properties in animal models (Acar-Tek & Ağagündüz, 2020; Mosleh et al., 2016). The cardioprotective and antioxidant properties of OL may be attributed to the high polyphenolic contents of the leaves, particularly oleuropein, luteolin, rutin, apigenin-7-glucoside, verbascoside, and quercetin (Bali et al., 2014; Wu et al., 2018). The consumption of OL extract in water for 12 days with roughly doses equivalent to 500 and 1000 mg/kg/day in rats induced cardio protective effects in DOX-treated rats (Kumral et al., 2015). Cytotoxicity of OL was also previously observed on different types of cell lines (Makowska-Wąs et al., 2017) and was tested on hepatocellular carcinoma cells (HepG2 cell) with moderate cytotoxicity (Kadan et al., 2013).

However, the effectiveness of DOX in the presence of OL on (HepG2) has not been studied.

The hypothesis is that the effect of the plant on the cardiotoxicity of DOX may be dose dependent and may increase the drug's effectiveness against liver cancer cells. The present study aimed to address the cardioprotective, antioxidant, anti-inflammatory effectiveness of the polyphenolic extract of OL against DOX-induced cardiotoxicity in male rats and to study the mechanism underlying these effects. The second purpose is to study the effectiveness of DOX in the presence of OL on HepG2.

Methods

Reagents

The activity of serum aspartate aminotransferase (AST) and creatine kinase (CK) were assessed using Randox kit (Laboratories Ltd., Country Antrim, United Kingdom). The 2,4-dinitrophenylhydrazine, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), O-dianisidine, Folin's reagent, thiobarbituric acid, epinephrine, super oxide dismutase (SOD) enzyme, hydrogen peroxide, and bovine albumin were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Remaining chemicals were made available from local vendors. DOX (Adriblastina, 50 mg) was purchased from Pharmacia (Pharmacia Italia S.P.A., Italy).

Polyphenolic rich extraction of olive leaves

The olive leaves (OL) were gathered on May 2019, from the Department of Olive and Semiarid Zone Fruits Research at the Center of Agriculture Research in Giza, Egypt. Before extraction, plants were protected in dark bottles at 4 °C. The air-dried and ground OL (100 Gm) were extracted in 70% (v/v) ethanol (1000 mL) by soaking for 2 days at 4 °C. The extracted product was filter-dried in a rotary evaporator under reduced pressure and at 40 °C and then stored at -20 °C for further analysis. Extracted OL was subjected to phytochemical analysis of phenolic contents as well as antioxidant analysis.

Phenolic total makeup of OL

The OL extract's total phenolic content was assessed following Muanda's procedure as detailed in (Muanda et al., 2009).

Total flavonoids content of OL

The total flavonoid content of OL extract was determined following the aluminum trichloride method (Muanda et al., 2009) where flavonoid content was expressed in milligram/gram of rutin per dry weight of OL crude extract.

The OL's ferric reducing antioxidant power

The OL extract's capacity of its total antioxidants was estimated using the ferric reducing antioxidant power (FRAP) technique detailed in (Benzie & Strain, 1996).

The OL-free radical scavenging activity

The ability of the OL extract to provide a H atom was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical as previously described by Ahmeda et al. (2012). Briefly, the inhibition of the free radical scavenging activity was estimated as follows: % = $100 \times (\text{absorbance of the control} - \text{absorbance of the sample}) / \text{absorbance of the control}$. The positive control used was ascorbic acid.

The OL high-performance liquid chromatography analysis of phenolic compounds

Ground samples of OL (5 g) were extracted by maceration for 48 h at 4 °C using (70%, v/v) ethanol/distilled water at pH=3. A rotary evaporator was then used to remove extracted solvents from the filtrate by evaporation under reduced pressure. The phenolic compounds of OL extract were analyzed with a reverse phase thermo-column C18 (250 mm × 4.6 mm i.d. 4 μm), Alliance High-Performance Liquid Chromatography (HPLC) (Milford, MA, USA) with a temperature of 40 °C photodiode array detector, which is fitted with a water 996 photodiode detector for phenolic compound isolation by HPLC using previously described methods (Ghomari et al., 2019). Gradient elution has been achieved using two solvents—(A) was water acidified with 0.2% phosphoric acid, PH=2.9 and (B) was acetonitrile/ methanol (50:50, v/v). A linear gradient was run from 96% (A) and 4% (B) to 50% (A) and 50% (B) during 40 min; then this was changed to 40% (A) and 60% (B) for 5 min; and finally during 15 min it was changed to 0% (A) and 100% (B). The flow rate was 1 mL/min in the mobile phase and 20 μl were injected in each sample. For each phenolic compound, the times were determined with respect to those of standards (rutin, quercetin, gallic acid, Caffeic acid apigenin, and oleuropein).

Animals

Thirty Wistar male albino rats (150–200 g) were acquired from the National Organization for Drug Control and Research (NODCAR) animal facility (Cairo, Egypt). The adopted procedure was in agreement with standard guidelines for the welfare and use of experimental animals (Canadian Council of Animal Care 1993) and following the ethical standards approved by the NODCAR institutional Animal Ethics Committee for animal welfare in research. Rats were given ad libitum access to

tap water and maintained on standard pellet diet. Rats were housed in a room with controlled humidity and temperature and a 12 h light–dark cycle in polycarbonate cage. Rats were randomly divided into 5 groups ($n=6$ per cage) on the top of a wood chip bedding with a room temperature 22–24 °C. Animals were acclimatized to the new cage environment for a full week prior to their use for experiments.

Treatment regimen

Doxorubicin (DOX; also known as Adriamycin) solution was freshly prepared in a saline solution. Prior administration, samples of one gm of OL crude extract were dissolved in 5 ml of distilled water. This aqueous solution was orally administered at doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg body weight (b. wt.) in a volume of 5 ml/kg b. wt. Rats were randomly divided into 5 groups ($n=6$), then they were subjected to treatments as follows. In the DOX-treated group, animals were orally given a dose of (5 ml / kg b. wt.) distilled water for 10 days. Following the first 7 days, this group's rats received each a single intraperitoneal (i.p.) dose of (10 mg/kg b. wt.) DOX. The used DOX dose was designated because it was previously used to successfully induce cardiotoxicity in rats (Hamza et al., 2008; Yilmaz et al., 2006). Animals of the protective groups (DOX+OL 250 mg, DOX+OL 500 mg and DOX+OL 1000 mg) were orally administered 250 mg/kg, 500 mg/kg, and 1000 mg/kg OL extract, respectively, for 10 days. Similar to the previous group, cardiotoxicity was induced by injecting the same single dose of DOX after 7 days of OL administration. Selected OL doses were determined based on multiple previous reports (Abdel-Sattar et al., 2012; Kaeidi et al., 2011; Kumral et al., 2015). Animals treated with distilled water (5 ml/kg b.wt.) for 10 days and injected with single dose of saline after 7 days of water administration were designated as control group. Following all treatments and after the last administration of OL/vehicle solution, blood and heart tissues were collected from all rats and stored at – 20 °C for later analyses.

Sample preparation

Rats were euthanized by cervical dislocation under diethyl ether, and blood was collected from the retro-orbital plexus of all animals. Collected blood samples were centrifuged at 3000 r.p.m. for 20 min at 4 °C and sera were collected. The hearts were dissected out. Heart tissues were sliced frontally into two halves (each half includes parts of all chambers of the heart) and for histopathological examination, samples of cardiac tissues were immediately fixed in 10% buffered formalin. Cardiac tissue samples from the other half were homogenized in ice-cold KCl (150 mM) for all biochemical assays. The

ratio of tissue weight to homogenization buffer was 1:10. Proper dilutions from that were then prepared to determine the biomarkers' levels of oxidative stress. Aliquots were prepared and utilized for the estimation of various biochemical markers.

Biochemical assays and histopathology

Cardiotoxicity indices

The AST and CK activities were estimated in serum samples using appropriate kit (Randox Laboratories Ltd., Country Antrim, United Kingdom) and following the kit's specific instructions.

Histopathological examination

Collected tissues were fixed in 10% neutral formalin for 24 h. Fixed pieces of hearts were embedded in paraffin after being dehydrated by ethanol. Thin, 5- μ m, sections were stained with Hematoxylin and Eosin (H&E). The sections were studied with light microscope (Olympus CX31, Honduras St., London, United Kingdom). They were categorized based on average severity of disorganization of the normal myofibrillar pattern, focal necrosis, degeneration, and inflammation as: 0, no change; 1, mild; 2, moderate; and 3, severe (Li et al., 2009). These ratings were represented as follows: mild being $0 \pm 10\%$ of total myocardium, moderate being $10 \pm 30\%$ total myocardium, and severe being more than 30% of total myocardium.

Oxidative stress biomarkers

We determined lipid peroxidation by assessing malondialdehyde (MDA) level as described in (Gerard-Monnier et al., 1998). Catalase activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm as described in (Aebi, 1984). Catalase (Nicolini et al.): the reaction mixture consisted of 2 ml phosphate buffer (pH 7.0), 0.95 ml of hydrogen peroxide (0.019 M), and 0.05 ml of supernatant in a final volume of 3 ml. Absorbance were recorded at 240 nm every 10 s for 1 min. One unit of CAT was defined as the amount of enzyme required to decompose 1 μ mol of H_2O_2 per min, at 25 °C at pH 7.0, then expressed in units/mg of protein.

Myeloperoxidase (MPO) activities in cardiac homogenates were determined as described in Hillebrand et al., (1990). Briefly, 100 μ l of supernatant was mixed with 2.9 mL of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured with a spectrophotometer. One unit of MPO activity is defined as that which degrades 1 μ mol of peroxide per minute at 25 °C.

SOD activity in heart homogenates was determined according to the method described in Nandi and Chatterjee (1988). This method is based on the ability of SOD

to inhibit the auto-oxidation of pyrogallol at alkaline pH. In 2 ml of a solution containing 50 mM Tris-cacodylate buffer, pH 8.5, 5 μ l of liver sample was applied. The reaction began with the addition of 100 μ l of freshly prepared 2.6 mM pyrogallol solution to 10 mM HCl. Absorbance at 420 nm from 1 to 3 min was observed for 2 min. The findings were expressed in tissue homogenate units per mg of protein. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

Protein carbonyl (P.carbonyl) contents were determined according to the method of Reznick and Packer (1994). This method is based on spectrophotometric detection of protein hydrazones at 370 nm which consists of the reaction of 2, 4-dinitrophenylhydrazine (DNPH) with P.Carbonyl. With cold trichloroacetic acid (TCA, 20 percent final concentration), 400 μ L of liver samples are precipitated and then collected for 3–5 min by centrifugation. To give a final protein concentration of 1–2 mg / ml, a solution of 10 mM DNPH in 2 N HCl is added to the protein pellet of each sample, with 2 N HCl only added to the corresponding sample of aliquot reagent blanks. For 1 h with vortexing every 10 min, samples are allowed to stand in the dark at room temperature; they are then precipitated with 10–20% TCA (final concentration) and centrifuged for 5 min. The supernatants are discarded, the protein pellets are washed with 10–20% TCA again, and then washed three times with 1 ml ethanol/ethyl acetate portions (1:1, v/v) to remove any free DNPH. The samples are then resuspended with vortex mixing in 6 M guanidine hydrochloride (dissolved in 2 N HCl or 20 mM phosphate buffer, pH 2.3) for 15 min at 37 °C. The results were expressed as nmol of carbonyl group per milligram of protein with molar extinction coefficient of 22,000 M/cm.

The total protein contents of heart tissues were determined according to the Lowry method as modified by Peterson (1977). In all the estimations, absorbance was reported using a PerkinElmer, Lambda 25 UV/VIS spectrophotometer.

Immunohistochemical analysis for iNOS

To conduct immunohistochemical analysis of the inducible nitric oxide synthase (Andreadou et al.), mounted sections were placed in sodium citrate buffer (0.1 M, pH 6) and kept in a water bath for 15 min to unmask antigen epitopes. Then, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. Rabbit anti-rat primary antibodies (Ab-1) of anti-iNOS was obtained from Thermo Fisher Scientific (Anatomical Pathology, Fremont, USA, 1:100 dilutions). Polyclonal anti-rabbit antibody (1:200) was bought from Santa Cruz Biotechnology (CA, USA). The primary antibody

was incubated with slides overnight at 4 °C. After washing the slides with phosphate buffered saline, sections were kept in 1: 200 dilution of polyvalent biotinylated goat anti-rabbit secondary antibody at room temperature for 60 min. Following a typical staining protocol using Universal LSAB plus kit and a DAB plus substrate kit as the chromogen, sections were counter-stained with hematoxylin. Tissue images were captured by optical microscopy (Olympus DP71, Olympus, Tokyo, Japan). Positive areas were quantified as optical density in 10 randomly selected fields (at 400 × magnification) per individual samples. These optical densities were then quantified using computer-assisted image analysis software (Image-Pro plus 6.0 imaging software, (Media Cybernetics, Inc. MD, USA).

Cell culture

HepG2 was obtained frozen in liquid nitrogen from American Type Culture Collection (ATCC). HepG2 cells were maintained in RPMI-1640. Cells were supplemented with 10% fetal bovine serum and 1% of 100 U/mL penicillin and 100 µg/mL streptomycin and incubated in a humidified incubator at 37 °C with 5% CO₂. Experiments were initiated when the cells reached 80% confluence.

Cytotoxicity activity

The cytotoxicity of OL, DOX, and OL + DOX was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on HepG2 cells. Cells were seeded in a 96-well plate (1 × 10⁴ cells/well) and then incubated for 24 h in the absence or presence of OL. OL and DOX were dissolved in dimethylsulfoxide (DMSO) the final concentrations of DMSO in culture medium adjusted to 0.1% in all experiments. Cells were treated with OL, DOX and DOX + OL with concentrations (31.25, 62.5, 125, 250, 500, 1000 µg/ml) for 24 h. Cell viability was measured after 24 h using MTT. A total of 20 µl MTT solution (5 mg/ml) was added then cells were incubated for 3 h. After media removal, 200 µl of DMSO was added to each well to extract the formazan products formed by viable cells. The absorbance of the solutions was measured on a Bio-Rad 550 microplate reader at 560 nm. Cell viability (%) was then determined by comparing the absorbance at 595 nm with control (treated with DMSO). The 50% inhibitory concentrations (IC₅₀) for DOX were evaluated according to the dose response curves derived from the results of MTT assay.

Table 1 Total antioxidant capacity of ethanol extract from OL

Extraction yield (%)	Total antioxidant capacity	
	FRAP assay mmol ascorbic acid/g	DPPH scavenging IC ₅₀ µg/ml
25.1 ± 0.25	1.05 ± 0.38	42.90 ± 0.30

Table 2 Amount of total phenolics^a, flavonoids^b and the qualitative–quantitative analyses^c of the ethanol extract from OL carried out using an HPLC–DAD

Peak number	Compounds	Concentration	Retention time (Min)
–	Total phenolic content	53.75 ± 2.33	
–	Total flavonoid content	32.15 ± 1.13	–
1	Gallic acid	37.03	3.1
2	Caffeic acid	15.79	4.5
3	Rutin	45.32	6.6
4	Apigenin	27.35	8.2
5	Oleuropein	19.21	9.8

Results are stated as mean ± SEM of three experiments. a Total phenolic content is represented as mg gallic acid equivalents/g dried extract. b Total flavonoid content is represented as mg rutin equivalents/g dried extract. c The amount of compounds is shown as µg/mL

Statistical analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL, USA) was run to carry out a one-way analysis of variance (ANOVA) on the presented results. When significant differences by ANOVA were detected, analysis of differences between the means of treated and control groups were performed using the Dunnett's t test. Values of *P* < 0.05 were considered statistically significant.

Results

Total antioxidant capacity of OL

The data for total antioxidant capacity of OL crude extract are presented in Table 1. The tabulated data were determined by both the FRAP assay and DPPH-scavenging assay. In the present investigation, each gram of dried OL extract has a high FRAP value, 1.05 mmol ascorbic acid equivalent. The OL extract also showed high scavenging activity against DPPH radical with an IC₅₀ value of 42.9 µg/ml, while that of standard ascorbic acid was found to be 11.23 µg/ml.

The extraction yield, total phenol content, FRAP assay, and DPPH scavenging IC₅₀ for the OL extract. Values are declared as mean ± SEM (*n* = 3). FRAP was expressed as mmol ascorbic acid equivalents/g dried extract. IC₅₀ is the concentration of extract that may scavenge DPPH radicals by 50%.

Quantification of total phenol, flavonoid, and HPLC phytochemical compounds.

In the present study, total contents (flavonoid and phenolic) were expressed as gallic acid and rutin equivalent, respectively (Table 2). There is a high total phenolic content in every gram of dried OL extract; that is equal to 53.75 ± 2.33 mg gallic acid. We find a high total flavonoid content for each gram of dried OL, equal to 32.15 ± 1.13 mg rutin equivalent. The key polyphenols from OL were also recognizable and quantified using a validated HPLC–UV technique. Comparisons of retention times and UV species in similar analytical conditions were observed between five highest peaks of gallic acid (3.1 min), caffeic acid (4.5 min), rutin (6.6 min), apigenin (8.2 min), and Oleuropin (9.8 min) (Table 2).

Biochemical and histological effects on heart tissues

Cardio protective effect of OL against DOX-induced cardiac damage

The activities of the serum markers associated with heart injury, AST and CK, were significantly induced in the control group as compared to serum marker levels in the DOX treated group. Treatment with different OL doses significantly diminished this increase in AST and CK levels in the DOX-treated group (Fig. 1A).

Control rats showed normal myocardium architecture (Table 3 and Fig. 2). Pronounced degeneration of the myofibrils, with focal necrosis and vacuolated cytoplasm, was clearly observed in the DOX-treated group (Fig. 1b, c and Table 3). DOX intoxication also induced eosinophilic cytoplasm and focal hemorrhaging with inflammatory cell infiltrations (Fig. 1b,c). The animals that were pretreated with different doses of OL had almost normal myocardium architecture. Despite that, there were still some inflammatory cells in the cardiac tissues, appearances of slight degeneration, and some leukocyte infiltration with a dose dependent effect (Fig. 1d–f and Table 3).

Cardio protective effect of OL against DOX-induced oxidative stress

Activities of MDA and P.Carbonyl were significantly raised ($P < 0.05$) in cardiac tissues of DOX-treated rats compared to their control levels (Fig. 2a, b). Cardiac tissues of rats treated with DOX showed significant depletion in SOD activity. In contrast, the activity of the CAT enzyme increased significantly in heart tissues from this group of rats (Fig. 2). The concurrent administration of OL and DOX significantly diminished the raised levels of such oxidative stress markers. This effect was dose dependent. Both the medium and high doses of OL extract abolished DOX-induced oxidative stress more than the low dose. Moreover, administering medium and high doses of OL prevented the depletions of SOD

activity whereas all doses of extract significantly attenuated the elevated CAT activity of.

Cardio protective effect of OL against DOX-induced inflammation

The DOX-treated group exhibited a significant rise in MPO activity of cardiac tissues in comparison to the control group (Fig. 3). The elevation of the MPO enzyme activity was completely normalized by treatment with OL extract at all the tested doses. The expression of the pro-inflammatory enzyme iNOS was estimated using immunohistochemical staining. Brown staining, representing the positively immunostained cells, was much abundant in the DOX-treated group compared to the control group (Fig. 3c,f). Pretreatment of intoxicated animals with OL, at both the lower and the higher doses, (Fig. 4c and e) significantly prevented this elevation to a large extent.

Effects of OL, DOX, and their combination on cell toxicity in HepG2 cells

Dose-dependent viability of OL, DOX, and OL+DOX (31.25, 62.5, 125, 250, 500, 1000 $\mu\text{g/ml}$) for 24 h in HepG2 cells in Fig. 4. The cell viability indicates that the reduction of cell viability increased significantly with increase concentration of OL, DOX and OL+DOX (31.25, 62.5, 125, 250, 500, 1000 $\mu\text{g/ml}$). The IC₅₀ value of OL was found to be 553.31 $\mu\text{g/ml}$, IC₅₀ with DOX was found to be 259.35 $\mu\text{g/ml}$, and the combination of OL with DOX significantly increased cell death (IC₅₀ dropped to 158.12 $\mu\text{g/ml}$) in comparison with single DOX treatment.

Discussion

Plant-derived polyphenolic compounds possess antioxidants and anti-inflammatory properties that contribute to their cardiovascular protective effects (Shah et al. 2019). Polyphenolic compounds of OL have been reported to have antioxidant and cardio protective effects (Bali et al., 2014; Wu et al., 2018). In line with these observations, this research examined the protection effects of polyphenolic extracted of OL against DOX-induced cardiac damage and oxidative stress in an animal model. The results revealed that treatment with a single dose of DOX (10 mg/kg body weight) caused severe myocardial histopathological damage such as myocyte necrosis, degeneration, and infiltration of inflammatory cells. In parallel to myocardial damage, it was possible to observe a significant elevation in serum CK and AST activities that is a reliable cardiac injury biomarker and indicates their leakage from damaged tissues. These are consistent with many other studies which demonstrated DOX cardiotoxicity in other animal models; (Hamza et al., 2016; Sahu et al., 2016b). In the present work, OL treatment

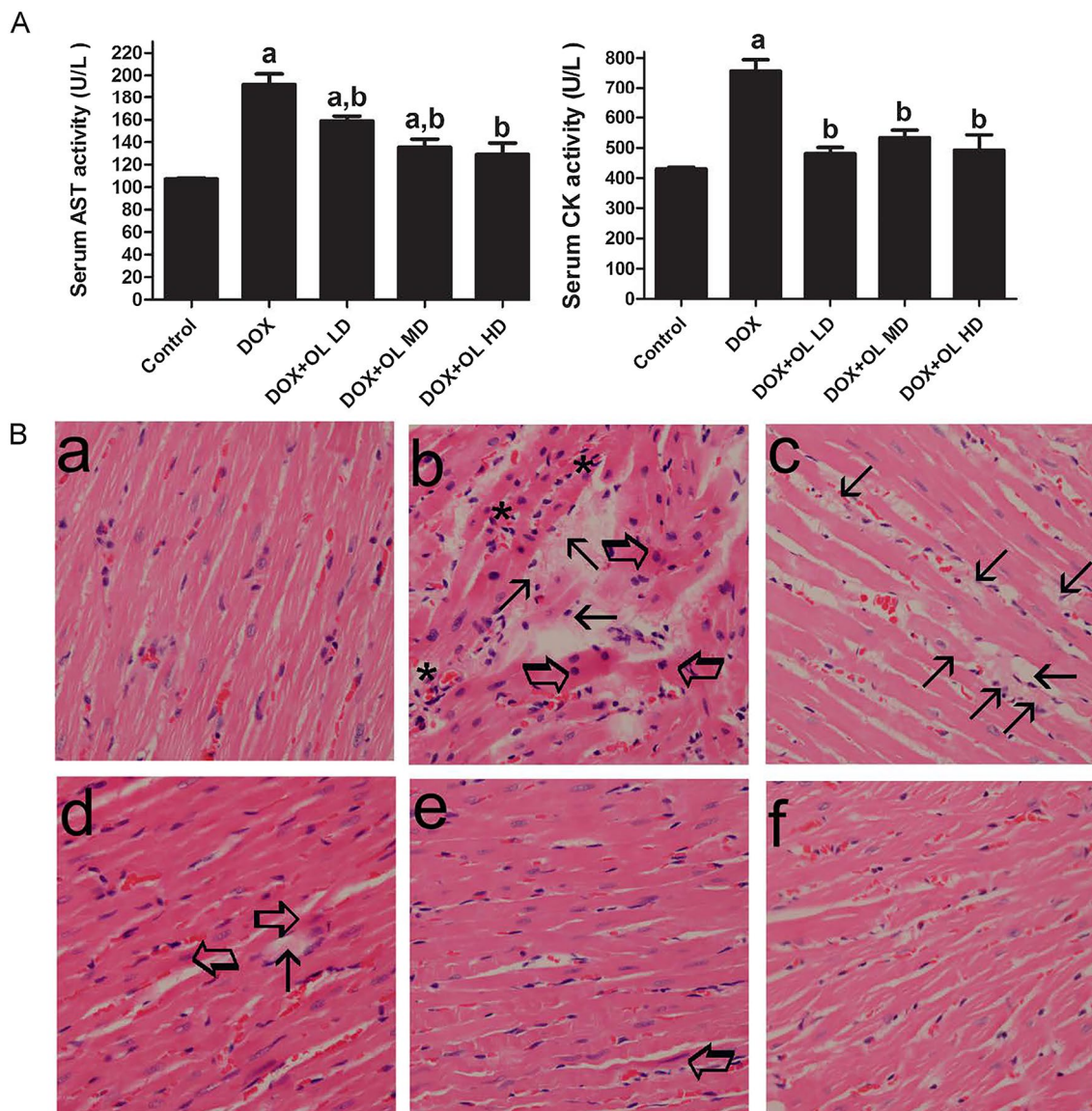


Fig. 1 Cardio protective effect of OL against DOX-induced cardiac damage, **A** serum cardiac injury markers: AST and CK activities in serum. Data are represented as mean \pm S.E.M. of six rats of each of the groups. a $P < 0.05$ versus control; b $P < 0.05$ versus DOX. **B** Histopathological changes. Light micrographs of heart sections from control rats (a) showing normal heart architecture, heart sections from DOX groups (b & c) showing focal necrosis with eosinophilic cytoplasm and pyknotic nuclei (thick arrow) and degeneration of myocardial fibers (thin arrow), and inflammatory cell infiltration (*). In the micrographs from DOX + LD of OL group (d) and DOX + MD of OL group (e) showing some eosinophilic cytoplasm and pyknotic nuclei and degeneration of myocardial fibers. 400 \times magnification

exerts cardio protective effects against DOX-induced cardiotoxicity as evidenced by the attenuation of serum CK and AST activities. These protective properties of OL were further confirmed by the histological findings with a marked improvement in necro-inflammatory score. Interestingly, concurrent administration of high dose (1000 mg/kg) of OL with doxorubicin revealed almost

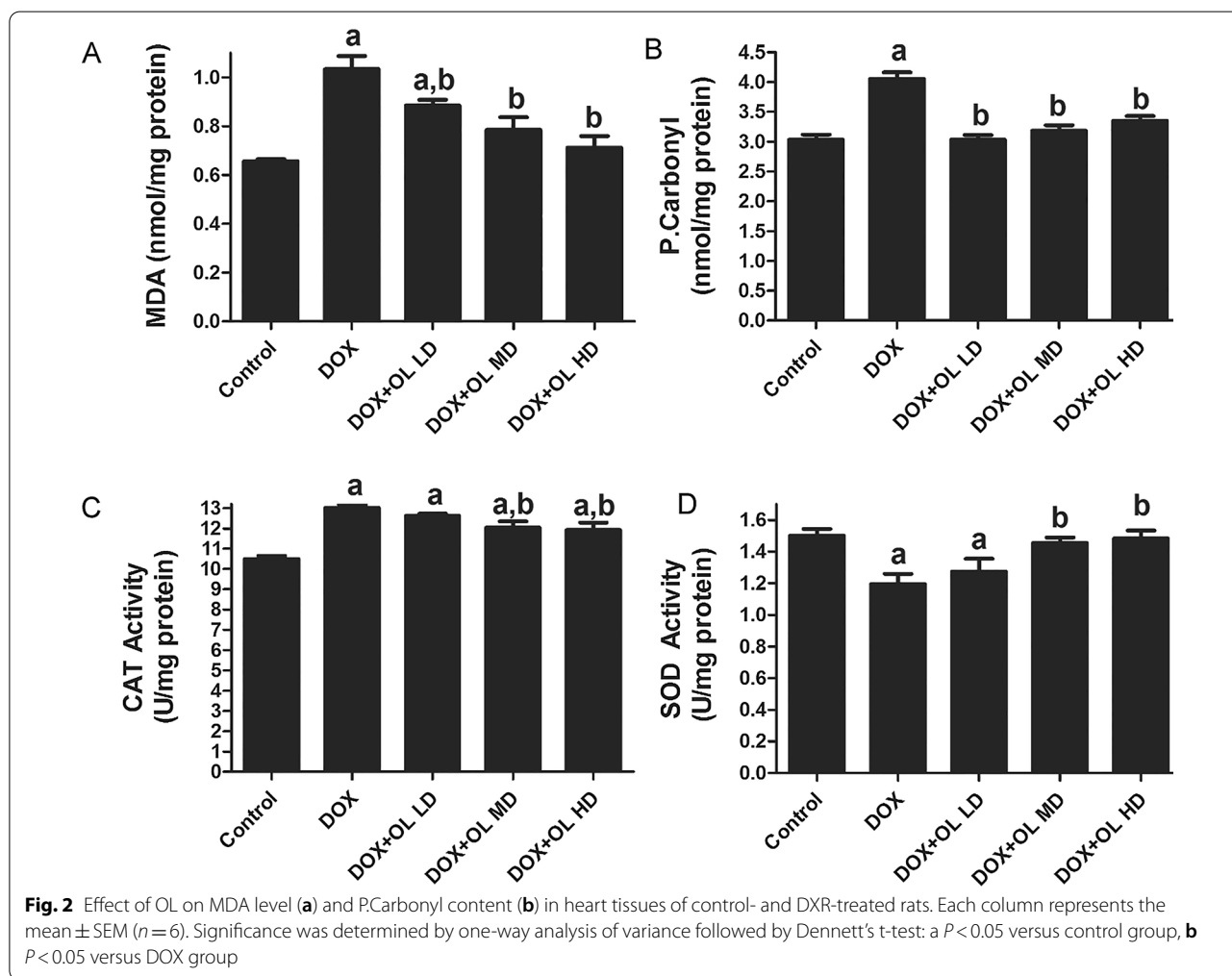
intact cardiac morphology without any vacuolization or degenerative changes.

Generation of ROS and oxidative stress is known to be a major factor in the toxicity of DOX in heart tissue (dos Santos & dos Santos Goldenberg, 2018). The present work confirmed that treatment with DOX-induced oxidations of both lipids (MDA) and proteins (P.Carbonyl) which are important markers of oxidative stress. In

Table 3 Effect of OL administration on severity of histopathologic lesions in DOX-treated rats

Groups	Disorganization	Focal necrosis	Degeneration	Inflammation
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
DOX 10 mg/kg	3.00 ± 0.00 ^a	1.83 ± 0.17 ^a	2.17 ± 0.31 ^a	2.00 ± 0.26 ^a
DOX + OL LD	1.33 ± 0.21 ^a	0.67 ± 0.21 ^a	0.67 ± 0.21 ^a	1.00 ± 0.36 ^a
DOX + OL MD	1.00 ± 0.00 ^a	0.33 ± 0.21 ^a	0.33 ± 0.21 ^a	0.67 ± 0.21 ^a
DOX + OL HD	0.50 ± 0.22 ^a	0.17 ± 0.17 ^a	0.10 ± 0.00 ^a	0.50 ± 0.22 ^a

Effect of DOX and OL on histopathological changes in cardiac tissues. Severity of injury is depicted as mean ± SEM of three scores for six animals in each group. a *P* < 0.05 versus DOX group. Grade 1 < 5% mild. Grade 2 = 16–25% moderate. Grade 3 > 35% severe



addition, DOX reduced the antioxidant activity of SOD enzyme in the heart. Thus, these findings advocate oxidative stress as a major cause for the DOX-induced cardiac tissue deformations. Free radicals have been shown in the heart tissue of DOX-treated rats to exhaust the antioxidants protection mechanism, and thus increase the oxidation process of both lipids and proteins (Andreadou et al., 2007; Hamza et al., 2016; Sahu et al., 2016a). To that

end, both the enzymatic and non-enzymatic single-electron redox cycle releases of ROS from molecular oxygen have been shown as an anthracycline ring structure in DOX (Cowgill et al., 2019; Ghigo et al., 2016). The current study has correlated depletion of the antioxidant defense mechanism with an increase in CAT activity, an enzyme that usually contributes to the removal of H₂O₂ in the heart and other tissues. Similar findings in male Wistar

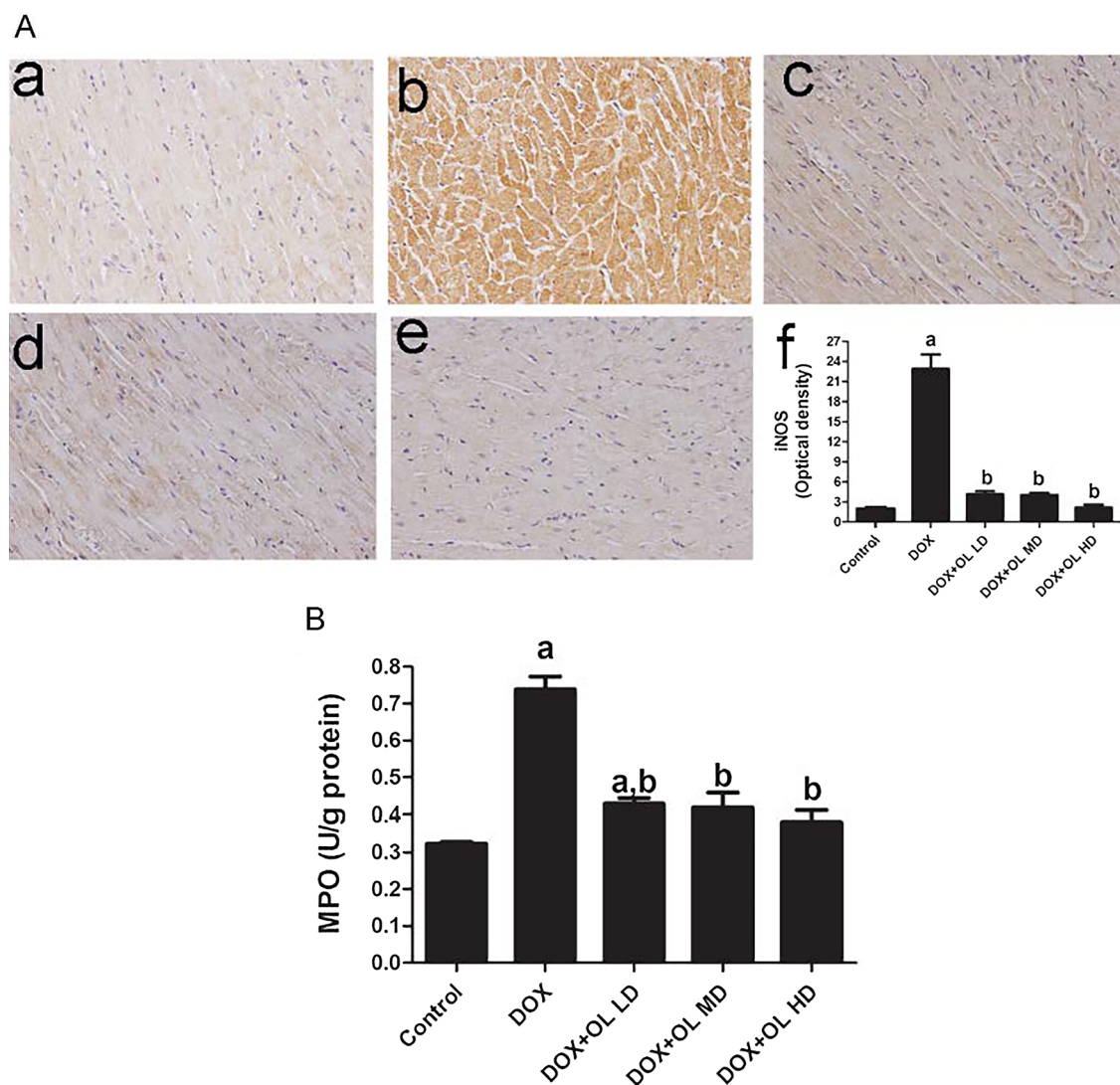
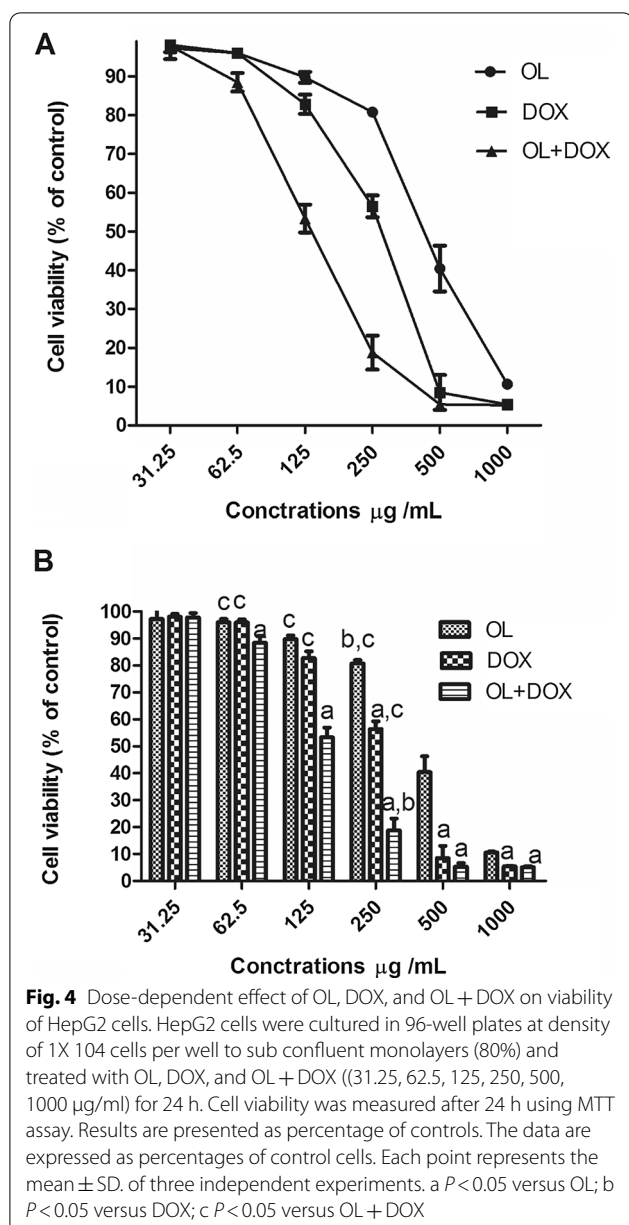


Fig. 3 **A** Representative photomicrographs of immunohistochemical analysis of iNOS of cardiac sections from the experimental groups (a) control, (b) DOX, (c) (DOX + OL LD), (d) DOX + OL MD, and (e) (DOX + OL HD) treated groups (magnification $\times 400$). Brown staining indicates iNOS positive cells. **F** Quantitative image analysis for immunohistochemical staining expressed optical densities (OD) across 10 different fields from each rat section. **B** Cardiac MPO activity. Cardiac iNOS data are represented as mean \pm SEM. for six animals in each group. Significance was determined by one-way analysis of variance followed by Dennett's t-test: a $P < 0.05$ vs. control group, b $P < 0.05$ vs. DOX group. 400 \times magnification

rats treated with DOX have been published (El -Desouki et al., 2019; Hamza et al., 2016; Sahu et al., 2016b). This at least partially illustrates the huge production of H_2O_2 and its important role in the cardiotoxicity of DOX. The DOX redox cycle was also shown to be the main source of H_2O_2 in tissue in the presence of cytochrome P450 reductase and NADPH (Cowgill et al., 2019; Ghigo et al., 2016). In the present work, lipid and protein oxidation induced by DOX was prevented by polyphenolic extract of OL which suggests an antioxidant effect for this herb. Moreover, concurrent OL and DOX treatment reestablished

normal levels of CAT and SOD antioxidants. Plant-derived polyphenolic compounds possess antioxidants and anti-inflammatory properties that contribute to their cardiovascular protective effects (Shah et al. 2019). Polyphenolic compounds of OL have been reported to have antioxidant and cardio protective effects (Bali et al., 2014; Wu et al., 2018). The antioxidant potential of OL extract was established in this study. OL showed DPPH radical scavenging activity which is attributed to its hydrogen donating ability. Also, OL showed substantial power reduction, as seen here in the FRAP assay. The phenolic



compounds of OL which are verified in this extract may be attributed to the antioxidative property of OL. They can work in various ways, such as the direct quenching of ROS, metal ions chelation, and membrane-binding regeneration of antioxidants. Photochemical analysis of OL proved the presence of a high amount of phenolic and flavonoid substances, and the chromatographic analysis of these substances proves the existence of phenolic acids such as gallic acid and caffeic acid and flavonoids such as rutin and apigenin, and polyphenolic compound, oleuropein. This is consistent with previous studies which identified OL polyphenolic compounds such as oleuropein,

luteolin, rutin, apigenin-7-glucoside, and verbascoside (Nicoli et al., 2019) and (Soussi et al., 2019). OL extract has been declared to reduce the oxidative damage to the lipid and protein caused by 4-hydroxynonenal in the myocytes (Bali et al., 2014) and by DOX (Kumral et al., 2015) and isoproterenol (Wu et al., 2018) in the heart of animal models. OL was also shown to be able to scavenge free radicals from bisphenol A and cadmium toxicity in the livers of rats and mice, as demonstrated by lower lipid peroxidation and increased antioxidants enzyme activity (CAT and SOD) in tissues (Jemai et al., 2020; Mahmoudi et al., 2018). However, anti-inflammatory effects of OL in DOX-treated rats have not been reported.

ROS are the major cause of inflammation and ROS in inflammatory cells can also be generated and collected inside the heart tissues and inflammation is one of the major futures of DOX-induced cardiotoxicity (Jemai et al., 2020; Mantawy et al., 2014). Cardiac MPO activity in DOX-treated rats was increased in the present study. This correlates to findings from our previous study (Hamza et al., 2016) and indicates an acute inflammation in DOX-treated animals and leukocyte aggregation in the heart tissues. Activated neutrophils and certain polymorphonuclear leukocytes cause a number of very reactive oxidants, such as hypochlorine acid, which can be oxidized, halogenated, and nitrated by cellular macromolecules throughout inflammation. In this study, OL treatment has prevented an increase in heart MPO activity which shows neutrophil infiltration and inflammation inhibition. OL were stated to have in vivo and in vitro anti-inflammatory and immunosuppressive properties (Jemai et al., 2020). In addition, our study found that, compared with control group, treatment with DOX significantly increased iNOS expression reflecting inflammatory response. INOS is a key mediator of acute inflammation (Cinelli et al., 2020) and overexpression of iNOS was observed in DOX-treated rats (Mantawy et al., 2014; Sahu et al., 2016b). We suggested that inflammation and iNOS may represented as additional source of ROS and NO and DOX cardiotoxicity. On contrary, rats that treatment with OL were attenuated the expression of iNOS and the effect was dose-dependent. We agree with the previous studies that have also shown that treatment with OL-induced anti-inflammatory effects and inhibited the expressions of nuclear factor kappa B (NF- κ B), cyclooxygenase (COX-2) expression, tumor necrosis factor (TNF- α) in in kidney of rats treated with cisplatin (Potočnjak et al., 2016); and in liver of rats treated with bisphenol A (Mahmoudi et al., 2018). In the previous studies, OL administration reduced ROS levels and iNOS expression in the aorta from spontaneously hypertensive rats (Romero et al., 2016). Previously OL extracts have been found to be anti-inflammatory gastritis caused by

HCl/ethanol in rats by investigating MPO activity and mRNA levels of iNOS, IL-1b, and TNF- α in the stomach (Al-Quraishy et al., 2017).

In the present work, the IC₅₀ of DOX when combined with OL decreased to half its value of DOX alone. OL suggested to potentiate or synergies the cytotoxic effects of DOX in hepatic cancer cell lines. OL significantly potentiate the cytotoxicity of DOX in liver cancer cells and protected normal tissues from its cardiotoxicity. The doses of 250 mg, 500, and 1000 mg/kg body weight/day were equivalent to human equivalent doses of 40.5, 81, and 162.2 mg/kg body weight/day respectively. This was calculated according to the formula of human dose equivalent for dose translation based on the body surface area (Reagan-Shaw et al., 2008). The formula of human dose equivalent = the animal dose (mg/kg X the Km factor of Animal/Km factor of human).

Conclusion

OL pretreatment ameliorated DOX-induced cardiotoxicity. The protective effect was mediated through inhibition of oxidative stress and inflammation in DOX-treated rats. This effect could be related to its powerful antioxidant properties. Phenolic compounds of OL could be a promising agent to prevent cardiotoxicity of DOX. Also, olive leaf extract doubled the activity against cancer cells. Further studies are expected to elucidate the pharmacodynamics and pharmacokinetics of OL. In addition, further clinical study is required to explore its clinical efficacy.

Abbreviations

AST: Aspartate aminotransferase; CK: Creatine kinase; OL: Olive leaves; DOX: Doxorubicin; DMSO: Dimethyl sulfoxide; MPO: Myeloperoxidase; MDA: Malondialdehyde; CAT: Catalase; P.Carbonyl: Protein carbonyl; SOD: Superoxide dismutase; ROS: Reactive oxygen species; H & E: Hematoxylin and eosin; iNOS: Inducible nitric oxide synthase; TAC: Total antioxidant capacity; FRAP: Ferric reducing antioxidant power; DPPH: 1, 1-Diphenyl-2-picrylhydrazyl; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

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Authors' contributions

A.A.H., S.O.H, S.H., A.B., and A.A. designed the study. A.H., S.O.H, S.H., A.B., and A.A. performed the experiments and did the statistical analysis. A.A.H., S.O.H, S.H., A.B., and A.A. assisted with methodology and contributed resources. A.A.H and A.A. wrote the first draft of the manuscript, and all authors contributed to the editing of the revised manuscript and approved the manuscript.

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Availability of data and materials

All data of this article are available.

Declarations

Ethics approval and consent to participate

The protocol was conducted in accordance with the standard guide to the care and use of according to the ethical standards approved by the NODCAR institutional Animal Ethics Committee guidelines for animal care and use. The reference number is not available.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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