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ORIGINAL ARTICLE

Nephroprotective and therapeutic potential of *Helichrysum orientale* (L.) Gaertn. on hydrogen peroxide-injured kidney cells

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Abstract

Acute kidney injury (AKI) is a severe form of kidney disease that is characterized by dysfunction. Excessive production of reactive oxygen species (ROS) is a major contributing factor in AKI pathogenesis. This study aims to investigate the cytoprotective effects of *Helichrysum orientale (L.)* Gaertn.'s capitulum aqueous extract against hydrogen peroxide (H_2O_2)-induced oxidative stress and apoptosis of 293 T cells (in vitro AKI model). The polyphenolic content of the extract was determined using ultra-high-performance liquid chromatography (UHPLC) revealing substantial amounts of cinnamic acid derivatives as well as notable quantities of polyphenolic compounds. Cell viability assays demonstrated that H_2O_2 induced dose-dependent cytotoxicity, whereas the extract significantly mitigated these effects and increased cell viability up to 2.43-fold. The antioxidant activities of the extract were evidenced by 5.1-fold increase in catalase (CAT) activity, 3.3-fold increase in superoxide dismutase (SOD) activity, and 2.3-fold reduction in malondialdehyde (MDA) levels. These results indicate its capacity to counteract H_2O_2-induced oxidative damage. Additionally, the extract prevented apoptotic cell death by significantly downregulating Bax expression by 16% and suppressing caspase-3 activation to control levels in damaged cells. These findings demonstrate that the aqueous extract of *H. orientale's* capitulum exhibits robust antioxidant and antiapoptotic properties in H_2O_2-injured kidney cells, offering preliminary pre-clinical substantiation.

Keywords *Helichrysum orientale* (L.) Gaertn. · Kidney injury · Antioxidant and antiapoptotic effects

Introduction

Acute kidney injury (AKI) is a common clinical complication characterized by kidney disfunction, and is often caused by renal ischemia–reperfusion (IR) and nephrotoxins. Excessive production of ROS and deficiency of antioxidants are major contributing factors in AKI pathogenesis (Tomsa et al. 2019; Makris and Spanou 2016; Pavlakou et al 2017; Ronco et al. 2019). Previously, it has been proven that the excessive ROS generation causes ischemia–reperfusion-induced renal injury and immune-mediated glomerular injury. Failure to take necessary precautions, AKI can result in end-stage and chronic kidney diseases with high morbidity and mortality (Polipoch, 2013; Makris and Spanou 2016).

Antioxidant system studies in patients diagnosed with chronic renal failure unveiled significant alleviations of SOD, CAT, glutathione peroxidase (GPx), and glutathione (GSH) levels in blood samples. ROS overproduction and insufficient antioxidant activity are supposed to generate oxidative stress in chronic kidney failure (Podkowińska and Formanowicz 2020; Ratliff et al 2016). Antioxidants have

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traditionally been used to deal with oxidative stress. Efficient ROS scavenging could alter the renal microenvironment spectrum and suppress the progression of AKI. Considering multiple pathophysiological mechanisms, there is no influential pharmacological method to prevent acute kidney injury or adverse tissue damage (Tomsa et al. 2019; Gyurászová et al. 2020).

Antioxidants prevent the uncontrolled free radical formation and its interaction with cellular structures (Flieger et al 2021). Medicinal plants are a source of a wide variety antioxidant properties, including phenolic compounds. (Palipoch 2013; Sofowora et al 2013). They are widely used for the treatment of various diseases. Lately, constant attention has been paid to the effectiveness of medicinal plants in preventing of various complications, especially liver and kidney diseases (Nasri et al. 2013). Phytotherapeutic strategies were shown to be effective, safe, and inexpensive approaches. Recent studies highlight their therapeutic potential, especially their antioxidant effects in protecting kidneys from oxidative stress (Palipoch 2013; Liao et al 2022).

The target medicinal plant of the current study is *Helichrysum orientale* (L.) Gaertn. blooming from mid-April to June. *H. orientale* is a perennial plant that wild-growing chasmophyte on limestone sea cliffs. It has hairy green-grayish foliage and greenish-yellow flowers. This plant is endemic to western and southwestern coasts of Turkey, and the Aegean islands of Greece and Crete (Hind et al. 2007; Strid 2016; Tanker and Sezik 1978). *Helichrysum* species, known as golden grass, immortal flower or evergreen flower, belongs to the Asteraceae family. It is stated that there are over 600 *Helichrysum* species worldwide. *Helichrysum* species are represented in Turkey by 27 taxa, 15 of which are endemic (Węglarz et al. 2022; Albayrak et al. 2010). These are aromatic plants that are widely used as tea for the treatment of kidney stones, gastrointestinal, and urogenital disorders in Anatolia (Umaz and Umaz 2020; Eroglu et al., 2010). The oil obtained by steam distillation from the aerial parts of *H. orientale* notably contains linear hydrocarbons, such as nonacosane, caryophyllene epoxide, β-selinene, and γ-curcumene, which are analyzed by GC–MS. Additionally, it was previously analyzed by HPLC that the phenolic composition of the plant consisted of chlorogenic, caffeic, ferulic, *p*-hydroxybenzoic, syringic acids, apigenin, naringenin, and resveratrol (Roussis et al. 2000; Albayrak et al. 2010). *H. orientale* did not exhibit in vitro genotoxic effects that include triggering micronucleus formation, decreasing % mitotic and replication indexes of human lymphocytes, unlike other *Helichrysum* species in Turkey. For this reason, it has been reported that it can be used as an alternative medicine (Eroglu et al., 2010).

A face-to-face study conducted in the Aegean region of Turkey (Köyceğiz, Muğla) reported the consumption of *H. orientale* in tea for the treatment of kidney stones and pain (Akbaş, et al. 2024). In addition, studies have demonstrated that tea derived from capitulum samples of certain *Helichrysum* species indigenous to Turkey can reduce the formation of calcium oxalate crystals in an animal model of kidney stone formation. Furthermore, *H. orientale* has been documented as a miscellaneous plant in the Marmaris region of Turkey used against urinary tract diseases, gallstones, kidney stones, gastritis, jaundice, stomach pain, and kidney diseases. (Orhan, et al. 2015; Gurdal and Kultur 2015; Kultur, et al. 2021; Sarı, et al. 2010). In this context, a series of pharmacological and phytochemical investigations are necessary to support the traditional use of *H. orientale* and to elucidate its mechanism of action.

The present study aims to investigate the protective effects of *H. orientale* (L.) Gaertn. capitulum sample's aqueous extract in an in vitro acute kidney injury model. Hydrogen peroxide (H_2O_2) was utilized to enhance oxidative stress in 293 T kidney cells. To the best of our knowledge, this is the first pre-clinical evidence indicating the antioxidant and antiapoptotic properties *of H. orientale* (L.) Gaertn. and its protective effects on AKI.

Materials and methods

Reagents and materials

The dried capitulum samples of *Helichrysum orientale* (L.) Gaertn. were obtained from Ahmet Arifoglu Biomedical Cosmetic Food Industry and Trade Inc. in a single lot (Batch no. 500 12 004). Hydrogen peroxide solution 30% Suprapur®, the reagents required for MDA, SOD, and CAT activities and PVDF membrane for immunoblotting were purchased from Merck (IPVH00010, Darmstadt, Germany). Dulbecco's modified Eagle's cell culture medium (DMEM HG, 01-052-1A, Sartorius), fetal bovine serum (FBS, A5256701, ThermoFisher Sci.), penicillin, streptomycin, and amphotericin B (1X) solution (A5955, Merck) and Tripsin–EDTA (1X) (T4049, Merck) for in vitro cell culture, MTT reagent (M5655, Merck) for testing cell viability, cell lysis RIPA buffer (9806, Cell Signaling Tech.), bovine serum albumin (BSA, A8806, Merck), ECL reagent (WesternBright, Advansta) for Western blot analysis, and cell culture plates were purchased from Thermo Fisher Scientific, Inc. Western blotting primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Myco-Alert assay kit for mycoplasma detection was purchased from Lonza (Basel, Switzerland). Ultrapure water was obtained via water purification system (Milli-Q Ultrapure) with the water outlet operating at 18.2 MΩ (Millipore, Bedford, MA).

Preparation of aqueous extract of Helichrysum orientale (L.) Gaertn

The dried capitulum samples (5 g) of *H. orientale* plant were incubated in boiled 100 mL distilled water for 1 hour (h) at room temperature. Filtration through Whatman no. 1 filter paper was performed to eliminate solid wastes from the aqueous extract. Then sterilization of the aqueous extract samples were performed using 0.22 µm filter for use in in vitro studies. The amount of substance dissolved in 1 mL was determined by evaporation of the aqueous extract.

Determination of polyphenolic content of the extract using ultra-high-performance liquid chromatography (UHPLC)

The UHPLC method was used to determine the phenolic composition of *Helichrysum orientale* (L.) Gaertn. It was performed on a Dionex UltiMate 3000 system (Thermo Sci., USA) equipped with a quaternary rapid separation pump (LPG-3400RS) and a photodiode array detector (DAD-3000RS). Separation was performed on Acclaim® C18 (5 µm) Dionex column (4.6 × 250 mm) at 30 °C with a flow rate of 1 mL/min and an injection volume of 20 µL. The UV detector was set to 250 nm, 270 nm, and 280 nm for 22.0 min, while the diode array detector was set to an acquisition range of 200 nm to 700 nm. Prior to UHPLC analysis, a solution of the extract (50 mg/mL) was prepared in a sterile distilled water. All the solutions (mixed standards, samples, and spikes solutions) were filtered through a 0.20 µm syringe filter and then degassed in an ultrasonic bath for 5 min. Data acquisition, peak integration, and calibrations were calculated with the help of the Dionex Chromeleon software (Version 6.80 RS 10).

Cell culture

The 293 T cells (CRL-11268, Manassas, VA) were obtained from the American Type Culture Collection (ATCC). Mycoplasma testing of the cells was performed using the MycoAlert assay (LT07-710, Lonza, Basel, Switzerland). Authentication of the subcultured 293 T cell line was conducted via Short Tandem Repeat (STR) analysis using Applied Biosystems GeneMapper Software 5 (Waltham, Massachusetts). The cells were cultured in Dulbecco's modified Eagle's medium high glucose with L-glutamine (DMEM-HG, 01-052-1A, Sartorius AG, Göttingen) supplemented with 10% fetal bovine serum (FBS, 10099141, Gibco, Waltham, Massachusetts) and penicillin, streptomycin, and amphotericin B (PSA) (A5955, Sigma-Aldrich, Louis, Missouri). They were incubated in a humidified atmosphere of 5% CO_2 at 37 °C and fed every other day. When the cultures reached 80–90% confluency, they were passaged by trypsinization (0.25% Tripsin–EDTA solution, 2520056, Gibco, Waltham, Massachusetts). To simulate an acute kidney cell injury in vitro, the cells were treated with hydrogen peroxide (H_2O_2) in a dose-dependent manner.

Cytotoxicity assay

Human embryonic kidney 293 T cells (5×10^3 cells/well) were seeded onto 96-well plates at an appropriate density. The experiments were carried out after overnight the cells were plated. First, the cells were treated for 24 h by different concentrations of *Helichrysum orientale* (L.) Gaertn. aqueous extract (ranging from 16 to 2000 ppm) and H_2O_2 (ranging from 62.5 to 1000 µM) to determine the optimum dose range (Tian Y, et al. 2023). H_2O_2 was freshly prepared from a stock solution (30%), and directly added to the cell culture medium at determined concentrations. In addition, the highest safe doses of *H. orientale* aqueous extract (250 and 500 ppm) were applied to the cells in combination to examine its counteractive effect on H_2O_2-induced cytotoxicity. After treatment with *H. orientale* aqueous extract and H_2O_2 for 24 h, the cells were incubated in 100 µL solution of MTT (final concentration 0.5 mg/mL) after aspiration of the culture medium. After incubation at 37 °C for 3 h, the MTT solution was aspirated. Then, the produced cellular insoluble formazan was solubilized in 100 µL dimethylsulfoxide (DMSO). After 15 min incubation, the absorbance of each well was measured at 570 nm using a microplate reader MultiscanGO, Thermo Fisher Scientific Inc. (Waltham, Massachusetts). Cell viabilities were expressed as percentages of the control culture values. Cell viability rates of the cells were calculated using the following formula:

$$Cellviability(\%) = AbsofSample/AbsofControl \times 100$$

Morphological observation of 293 T cells

The protective effects of *Helichrysum orientale* (L.) Gaertn. aqueous extract on the damaged cells by H_2O_2 were followed up cytomorphologically. The cells were treated with *H. orientale's* aqueous extract and H_2O_2 for 24 h, jointly and severally. The daily morphology of cells in each group was observed under an inverted microscope at 20X and 40X objectives (Primovert, Zeiss, Germany).

Detection of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) activity assay

The MDA levels of the samples were determined at 532 nm by double heating method (Draper and Hadley, 1990). The basic principle of this method is to spectrophotometrically evaluate

the reaction between thiobarbituric acid (TBA) and MDA. Results were expressed as nmol/µg protein.

The SOD activity was estimated at 560 nm by a defined method by Sun (Sun et al., 1988). The spectrophotometric SOD measurement is basically based on the principle of reaction between xanthine and xanthine oxidase to form superoxide radicals. Then, these radicals react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and form a red formazan dye. The obtained optical density results were expressed as U/µg protein.

The CAT activity levels of the samples were determined at 240 nm according to the method of Aebi (Aebi, 1984). The principle of this method is based on the specification of a rate constant (k, per second) of hydrogen peroxide decomposition into water and oxygen through CAT enzyme activity. Results were expressed as kU/µg protein.

Western blot

Protein concentrations of the samples were determined by the Bradford method, and 50 µg of each sample was loaded on 10% polyacrylamide gel and electrophoresed at 120 V for 1 h (Bio Rad, Hercules, CA). Afterward, the proteins were transferred to the PVDF membrane (10600023, Amersham, Chicago). Following, the membrane was blocked for 2 h in TBST buffer (J77500, Thermo Fisher Sci., Waltham, Massachusetts) containing 1% BSA, and then primary antibodies (1:1000 dilution; Active Caspase-3, sc-22171; Bax, sc-65532; and β-Actin, sc-517582; Santa Cruz, CA) and secondary antibodies (1:5000 dilution; Anti-mouse, sc-2005; Anti-rabbit, sc-2004; Santa Cruz, CA) were performed for 2 h. After immunoblotting, the membranes were sealed dropwise with ECL reagent (WesternBright, Advansta, San Jose, CA) and chemiluminescence signals were detected by the Chemidoc system (BioRad, Hercules, CA). The band intensities of the samples were quantified by the imageJ software (https://imagej.nih.gov).

Statistical analysis

Graphpad Prism 9 software was used to analyze the results. All experimental data were repeated at least three times. The results are expressed as mean ± standard deviation values. Followed by a post hoc protected Tukey test, one-way analysis of variance (ANOVA) test was applied to analyze the difference between groups. A value of $p < 0.05$ was considered statistically significant.

Results

Polyphenolic content of *Helichrysum orientale* (L.) Gaertn. capitulum aquous extract

This study used a UHPLC system to identify and quantify the phenolic compounds in the aqueous extract of the examined *H. orientale* capitulum samples. The content of each compound was calculated from the corresponding calibration curve and presented as the mean of triplicates as shown in Table 1. The extract contained a number of compounds from the most to the least including, trans-cinnamic acid, *o*-coumaric acid, resveratrol, chlorogenic acid, rutin, apigenin 7-glucoside, trans-*p*-coumaric acid, hesperidin, gallic acid, and others (Table 1).

Helichrysum orientale (L.) Gaertn. extract reverses the cytotoxic effect of hydrogen peroxide (H_2O_2) on 293 T cells

The *Helichrysum orientale* (L.) Gaertn.'s extract was found to be non-cytotoxic and extremely safe for 293 T cells in the range of 0–500 ppm. Toxic effects were observed at concentrations of above 1000 ppm. Therefore, 250 and 500 ppm were selected as the maximum safe and non-toxic doses for the extract. However, H_2O_2 exhibited dose-dependent toxic effects as expected. The viability of cells treated with 125 µM H_2O_2 was 76%, while it decreased to 29% for cells treated with 250 µM H_2O_2. Concentrations of 500 and 1000 µM of H_2O_2 were completely cytotoxic for 293 T cells (Fig. 1a). Interestingly, the combination of the extract and H_2O_2 resulted in the extract exerting a curative effect and contributing to the viability of tested cells. This aqueous extract increased the viability from 76 to 91% in cells treated with 125 µM, and from 29 to 70.5% in cells treated with 250 µM H_2O_2. The toxic effect of H_2O_2 on 293 T cells was reduced and reversed more than twice by the aqueous extract of *H. orientale* (Fig. 1b).

Cytoprotective effects of *Helichrysum orientale* (L.) Gaertn. extract on hydrogen peroxide (H_2O_2)-damaged cells

H_2O_2 attenuates cell viability and induces cellular apoptosis of 293 T cells in a concentration-dependent manner. A significant decrease in cell number was detected compared to the control when 293 T cells were cultured in the presence of H_2O_2 for 24 h. Additionally, the cells exhibited common apoptotic morphologies, such as rounding and blebbing. However, the confluence and the morphology of the cells were not adversely affected by the presence of *H. orientale*

Table 1 Molecular formula, molecular weight, retention time, maximum absorbance, and amount of polyphenolic compounds detected in the aqueous extract of *H. orientale* by UHPLC

Polyphenolic compound	Molecular formula	Molecular weight g/mol	Retention time min	Absorbance mAU	Amount µg/mL
Apigenin	$C_{15}H_{10}O_5$	270.24	11.5	0.52	4.60 ± 0.5
Apigenin 7-glucoside	$C_{21}H_{20}O_{10}$	432.4	8.1	567.13	441.84 ± 38
Catechol	$C_6H_6O_2$	110.11	1.7	70.31	92.49 ± 28
Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	6.6	53.59	483.57 ± 33
Epicatechin	$C_{15}H_{14}O_6$	290.27	6.9	93.31	171.30 ± 36
Ferulic acid	$C_{10}H_{10}O_4$	194.18	7.3	369.70	130.50 ± 18
Gallic acid	$C_7H_6O_5$	170.12	0.9	225.88	300.50 ± 17
Hesperidin	$C_{28}H_{34}O_{15}$	610.6	7.7	223.07	321.97 ± 29
Luteolin	$C_{15}H_{10}O_6$	286.24	9.7	4.97	3.40 ± 0.3
Naringenin	$C_{15}H_{12}O_5$	272.25	8.9	1.62	14.74 ± 3.3
Naringin	$C_{27}H_{32}O_{14}$	580.5	7.5	240.71	249.10 ± 23
o-Cumaric acid	$C_9H_8O_3$	164.16	7.5	912.53	737.10 ± 21
Quercetin	$C_{15}H_{10}O_7$	302.23	9.1	2.67	8.05 ± 3
Resveratrol	$C_{14}H_{12}O_3$	228.24	7.8	358.39	675.42 ± 27
Rutin	$C_{27}H_{30}O_{16}$	610.5	7.7	477.16	470.00 ± 20
Syringic acid	$C_9H_{10}O_5$	198.17	6.7	218.64	66.66 ± 9
Trans-Cinnamic acid	$C_9H_8O_2$	148.16	8.4	1525.23	1398.00 ± 36
Trans-*p*-Cumaric acid	$C_9H_8O_3$	164.16	7.1	1089.92	378.90 ± 34
Vanillic acid	$C_8H_8O_4$	168.15	4.3	4.26	30.31 ± 8
Vanillin	$C_8H_8O_3$	152.15	6.6	58.28	45.61 ± 14

Trans-cinnamic acid, *o*-coumaric acid, and resveratrol were identified as the most abundant compounds

aqueous extract. Remarkably, the combination of the extract and H_2O_2 showed similar confluence and morphology to the untreated cells in the control wells. This result suggests that *H. orientale's* capitulum aqueous extract is effective in protecting cells from cellular death and antiproliferative effects caused by H_2O_2 (Fig. 2).

Antioxidant properties of *Helichrysum orientale* (L.) Gaertn. extract are regulated by SOD, CAT, and MDA activities

As expected, the amount of MDA, which is a major active aldehyde indicating cell damage, increased by 56% in H_2O_2-induced cells compared to the control. Additionally, there were significant reductions of 80% and 88.5% in the activities of endogenous antioxidant enzymes, such as SOD and CAT, respectively. However, the extract restored the MDA levels, and SOD and CAT activities in 293 T cells despite the administration of H_2O_2 and were consistent with those of control cells. This extract, reduced MDA levels by 2.3-fold and enhanced SOD and CAT activities by 3.3- and 5.1-fold, respectively, even in H_2O_2 treated cells. This suggests that *H. orientale's* capitulum aqueous extract suppressed the oxidative stress triggered by H_2O_2 by decreasing the intracellular levels of MDA and supporting SOD and CAT activities (Fig. 3).

Antiapoptotic activity of *Helichrysum orientale* (L.) Gaertn. extract on hydrogen peroxide (H_2O_2)-injured kidney cells

The apoptosis status of the tested cells was investigated by determining the level of caspase-3 activation and Bax protein. Bax contributed to form pores in the mitochondrial outer membrane by oligomerization, releasing cytochrome-c into the cytosol and subsequently triggering activation of apoptotic effector caspase-3. As expected, H_2O_2 increased caspase-3 activation in 293 T cells by a statistically significant 1.7-fold compared to the control. However, despite the presence of H_2O_2, the aqueous extract of *H. orientale* reduced the active caspase-3 to basal level. Similar results were also observed at the Bax protein level. Bax expression was increased by 1.8-fold by H_2O_2, while the extract decreased it by %16 in the presence of H_2O_2 (Fig. 4).

Discussion

Helichrysum species have been used in folk medicines regarding their antibacterial, antifungal, antiviral, antioxidant, and anticancer activities. Among these species, *H. orientale*, *H. sanguineum*, *H. pamphylicum*, *and H. noeanum* are some of the medicinal plants in Turkey that play an important role in human health as a tea substitute. There

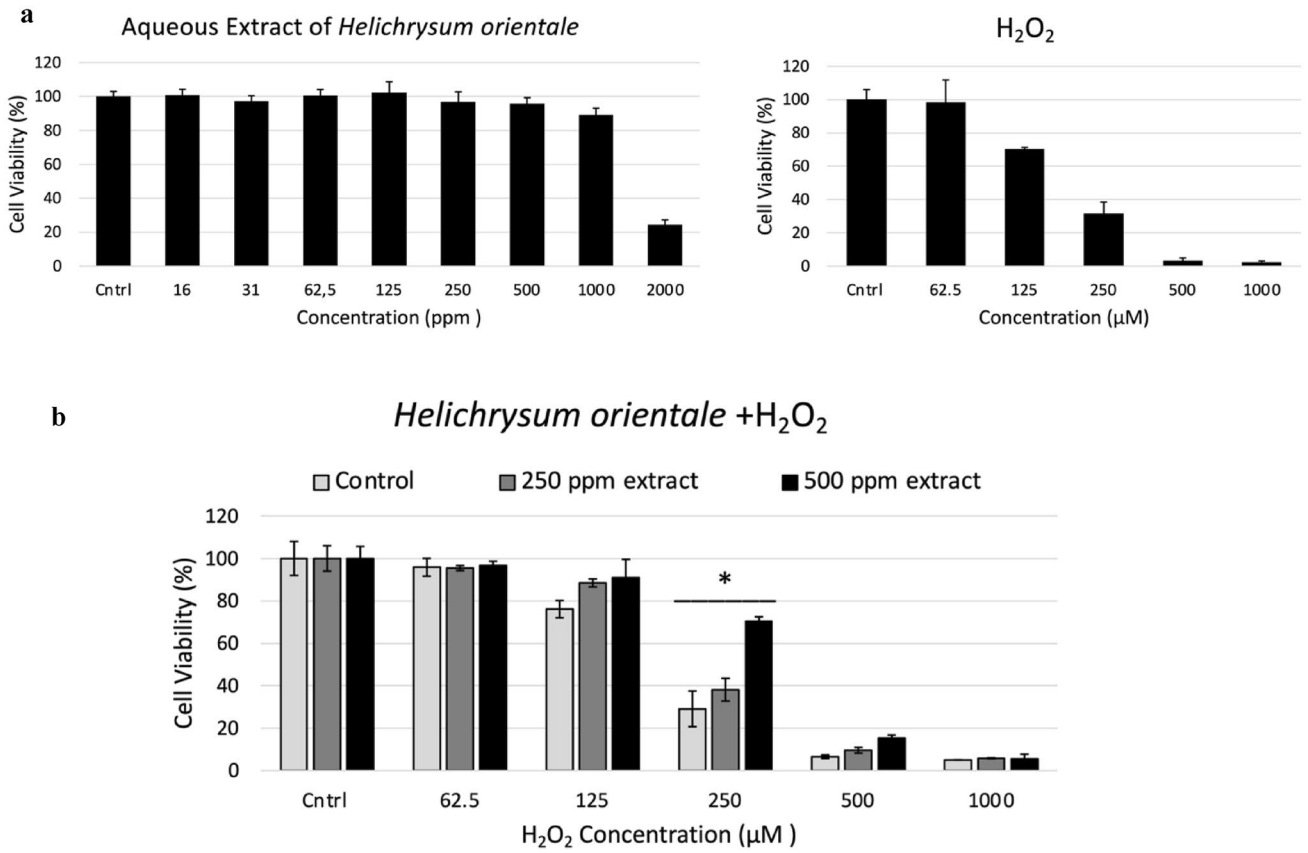

Fig. 1 Dose-dependent cytotoxic effects of *H. orientale* extract and H_2O_2 either separately (**a**) or in combination (**b**) on 293 T cells (values presented are the mean ± standard deviation. $*p < 0.05$). The extract was found to be non-cytotoxic for 293 T cells at concentrations up to 1000 ppm. While H_2O_2 reduced cell viability in a dose-dependent manner, co-treatment with the extract increased viability from 76 to 91% at 125 µM and from 29 to 70.5% at 250 µM, demonstrating a curative effect

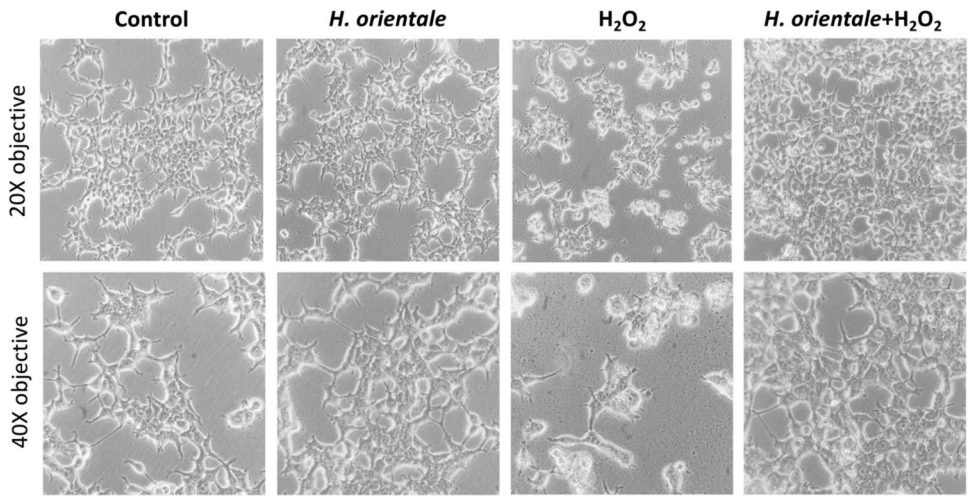

Fig. 2 Morphological characteristics of 293 T cells treated with *H. orientale* extract (500 ppm) and H_2O_2 (250 µM). (Control: untreated cells; *H. orientale:* treated cells with the extract; H_2O_2: treated cells with hydrogen peroxide; *H. orientale* + H_2O_2: combined treatment). H_2O_2 caused apoptotic changes (cell rounding and blebbing), but co-treatment with the extract maintained healthy cell morphology and confluence, indicating a protective effect against oxidative stress

are some strong evidences regarding cytotoxic, genotoxic, and mutagenic effects of *Helichrysum* species. Unlike *H. orientale, H. sanguineum, H. pamphylicum,* and *H. noeanum* extracts increased the percentage of micronucleus (MN), and decreased mitotic and replicative index of the human lymphocytes in vitro. The micronucleus test is used for the determination of the genotoxic effects of compounds. There were no significant evidences about H. orientale's extract on

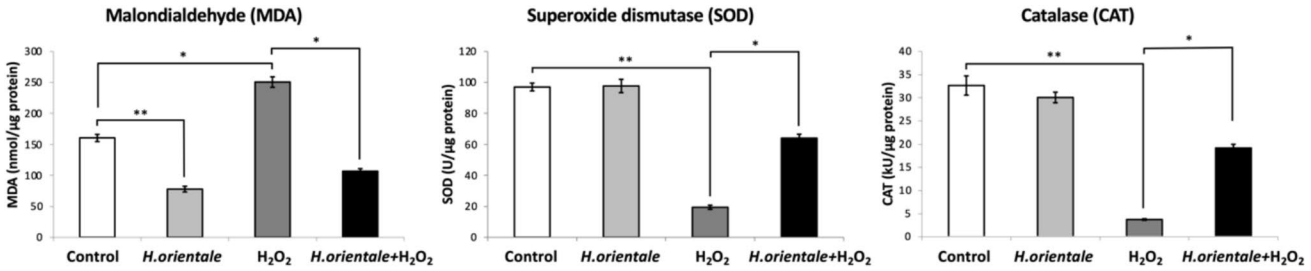

Fig.3 Comparison of MDA, SOD, and CAT levels in 293 T cells treated with extract (500 ppm) and H_2O_2 (250 µM). (Values presented are the mean ± standard deviation. $*p < 0.05$, $**p < 0.01$). H_2O_2 increased MDA and decreased SOD and CAT activities, but the extract reversed these effects by lowering MDA levels and restoring antioxidant enzyme activities (SOD and CAT) to near-control levels in 293 T cells

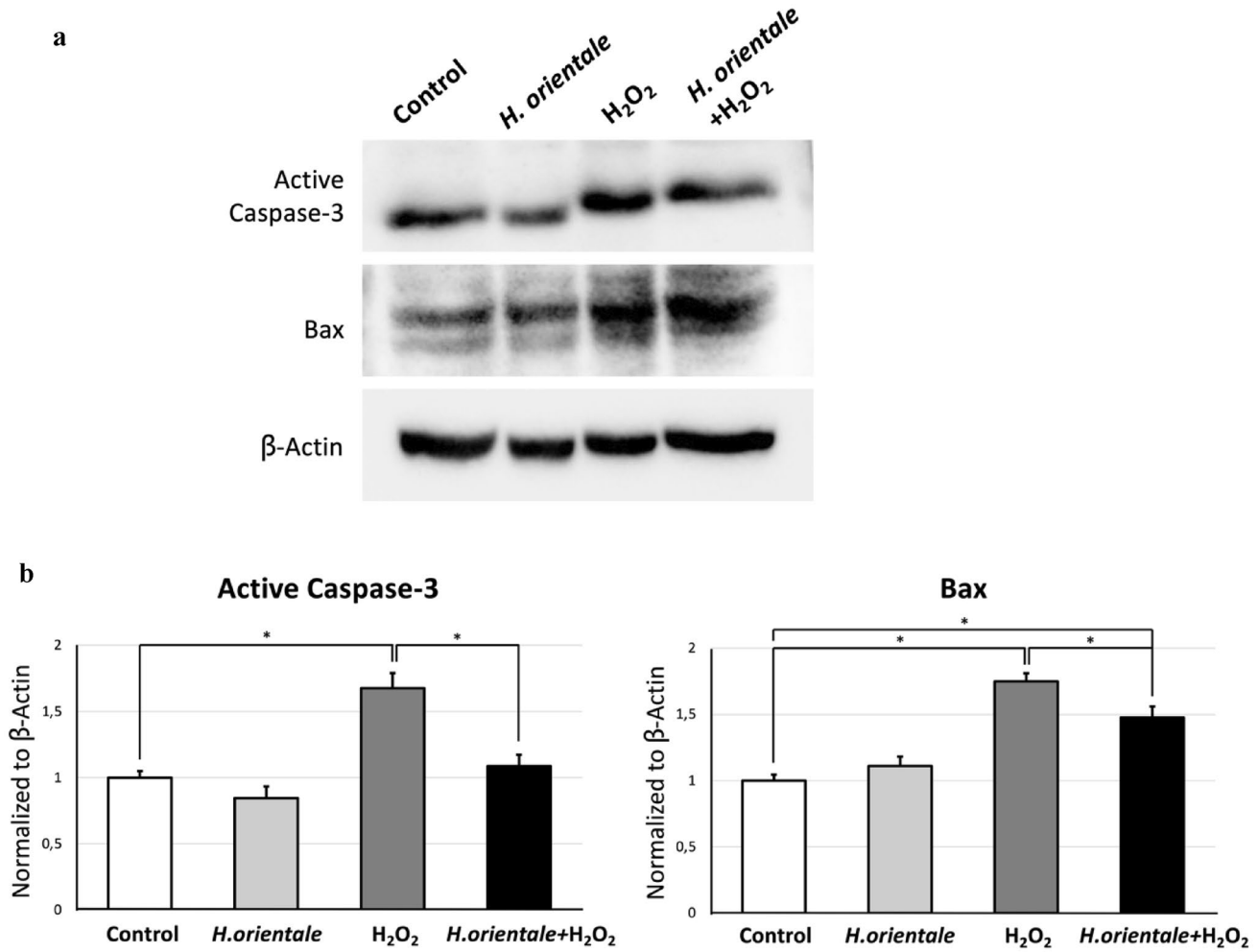

Fig. 4 *H. orientale* extract (500 ppm) reduced H_2O_2 (250 µM)-induced apoptosis in 293 T cells by modulating Bax and active caspase-3. (values presented are the mean ± standard deviation. $*p < 0.05$). Exposure to H_2O_2 caused a significant increase in caspase-3 activity (1.7-fold) and Bax expression (1.8-fold), confirming apoptotic cell death. Notably, treatment with the extract counteracted these effects by suppressing caspase-3 activation to control levels and reducing Bax expression by 16%, highlighting its role in protecting cells from H_2O_2-induced apoptosis

MN and cell proliferation frequency (Eroglu et al., 2010). Due to these featured reassuring effects, we investigated the potential beneficial effects of *H. orientale* among other *Helichrysum* species.

In the present study, the potential curative role of *H. orientale's* capitulum aqueous extract was preliminarily investigated in H_2O_2-induced 293 T kidney cell damage in vitro. To the best of our knowledge, there has been no scientific evidence regarding the antioxidant and antiapoptotic effects of the aqueous extract of *H. orientale*. Previous studies have indicated that the methanolic extract of *H. orientale* has the lowest total antioxidant property among the *Helichrysum* species in Turkey (Albayrak et al. 2010). Therefore, in this study, the aqueous extract of capitulum samples was preferentially used instead of the methanolic extract from the whole plant body.

The total phenolic content of some of *Helichrysum* species (*H. arenarium*, *H. armenium*, *H. plicatum*) in Turkey was measured previously using HPLC coupled with photodiode array (DAD) detection. According to these results, chlorogenic acid was the most abundant compound and also resveratrol and naringenin were also notably detected in the methanolic extract of the aerial parts of the plants. Besides that, chlorogenic acid, caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, syringic acid, naringenin, and resveratrol, which are known as antioxidants, have been detected previously (Albayrak S. et al. 2010).

This study revealed the polyphenolic content of *H. orientale* capitulum samples for the first time. UHPLC analysis data showed that the highest levels of trans-cinnamic acid, and also apigenin 7-glucoside, chlorogenic acid, gallic acid, hesperidin, naringin, *o*-coumaric acid, resveratrol, rutin, and trans-*p*-coumaric acid were present in the extract (Table 1). Polyphenolic compounds are secondary metabolites that accumulate in plant leaves and flowers to protect plants against diseases, infections, and damage. The polyphenolic family is divided into several major classes, including flavonoids, phenolic acids, stilbenes, and lignans. Flavonoids are the largest group of polyphenolic compounds. We have identified several flavonoids in this group, including quercetin (flavonol), apigenin (flavone), naringenin and hesperetin (flavanones), and catechol (flavanol). The second largest group is the phenolic acids, which have a benzoic or cinnamic acid derivative in their structure. The resveratrol molecule, which is present in high amounts in the extract composition, is a polyphenolic compound with a stilbene structure (Amawi H, 2017). Our findings confirm that trans-cinnamic acid, present in the highest levels in the *H. orientale* aqueous extract, has been proven to alleviate high-fat diet-induced kidney damage. It has been established previously that trans-cinnamic acid prevents obesity-related chronic kidney disease by reducing the phosphorylation levels of ERK, JNK, and p38 MAPK proteins in HEK293T and HK-2 cells (Jia K, 2025). Second, *o*-coumaric acid, which was detected at the higher level in the *H. orientale* aqueous extract, is an ortho isomer of hydroxycinnamic acid (monohydroxycinnamic acid). In a previous study, *o*-coumaric acid and rutin were observed to reduce oxidative stress and glutathione disulfide (GSSG) content, while increasing the levels of GSH, GPx, glutathione reductase (GRd), and glutathione S-transferase (GST) in liver tissue of rats with HFD-induced obesity. The findings have been confirmed from evidences that the ingestion of *o*-coumaric acid and rutin may confer benefits in the context of a high-fat diet-induced oxidative stress, dyslipidemia, and hepatosteatosis (Hsu CL 2009). We have identified another cinnamic acid derivative, trans-*p*-coumaric acid, in abundance in the aqueous extract of *H. orientale*. *p*-Coumaric acid is a para isomer hydroxy derivative of cinnamic acid (4-hydroxycinnamic acid), which has three different isomers. The most common form is *p*-coumaric acid. *p*-Coumaric acid is a powerful phenolic compound found naturally in various plants, grains, fruits, and vegetables. *p*-Coumaric acid and its derivatives have been proven to have different bioactive properties, including antioxidant and antimicrobial effects. It has been proven to have anticancer, antiarthritic, anti-inflammatory, anti-gout, antidiabetic, antimelanogenic, skin regeneration, gastroprotective, antiulcer, cardioprotective, hepatoprotective, renoprotective, bone formation, antiangiogenic, and antiplatelet properties (Kaur J, 2022). The data of this study indicate that the *H. orientale* extract contains a substantial quantity of cinnamic acid derivatives. Based on the literature and the well-established antioxidant properties of cinnamic acid and it derivatives, it can be posited that the *H. orientale* extract exerts a considerable protective effect against oxidative stress-based kidney damage. Our analysis revealed that resveratrol was the third most abundant compound in *H. oriantale* extract. Resveratrol (RSV) is a well-known antioxidant phenolic compound. RSV is one of the most extensively studied polyphenols and is emerging as a promising nutraceutical for the management of acute kidney injury (AKI) due to its potent antioxidant properties. The antioxidant properties of RSV are attributable to its capacity to scavenge a range of ROS and numerous secondary organic radicals. The potential health benefits associated with RSV include antimicrobial, neuroprotective, anti-aging, anti-inflammatory, anticancer, cardioprotective, and blood sugar-lowering properties. RSV has been demonstrated to increase the expression of a number of antioxidant defense enzymes, including HI-1, CAT, GPx, and SOD. Additionally, it has been shown to elevate GSH levels, which are essential for maintaining cellular redox balance. These protective mechanisms are mediated by the sirtuin 1 (Nrf2) and nuclear factor B signaling pathways. It seems

that RSV exerts its renoprotective effects in AKI by modulating the SIRT, NF-κB, and mitochondrial bioenergetics pathways. The majority of studies have demonstrated that RSV is a protective agent in AKI, and have suggested that its antioxidant properties are mechanistically important. *In vitro* experiments with human renal proximal tubule epithelial cell lines (HK-2 cells) have provided evidence that supports the renal protection provided by RSV in radiocontrast-induced nephropathy. RSV has been shown to significantly attenuate cytotoxic effects through several mechanisms, including the alleviation of oxidative stress (Rashid H, 2024).

According to the obtained data, the capitulum extract of *H. orientale* reduced oxidative stress levels in the cells by downregulating the activity of the MDA enzyme, and promoting the activities of SOD and CAT enzymes significantly, even in H_2O_2 treated cells (Fig. 3). These findings strongly suggest that *H. orientale's* extract prevents cell damage caused by H_2O_2. MDA is a major reactive aldehyde originated from the peroxidation of cellular membranes. It is used for the prediction of cellular damage level caused by reactive oxygen species (ROS) (Siddique et al. 2012). One of the most abundant antioxidant enzymes in aerobic organisms is SOD. It plays a crucial role in neutralizing superoxide radicals. By catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide, SOD helps prevent the harmful effects of superoxide radicals, which can cause oxidative damage to cellular components, such as proteins, lipids, and DNA. Therefore, the SOD activity is essential for maintaining cellular redox balance and protecting cells from oxidative damage (Karmakar et al. 2022). It has been demonstrated that oxidative stress can facilitate the onset of inflammation. Furthermore, oxidative stress has been shown to enhance the expression of TNFα-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and suppress the expression of SOD. In this regard, cellular oxidative stress may be suppressed by triggering intracellular SOD expression and activation (Li et al. 2020). CAT is a prevalent antioxidant enzyme with the highest turnover rate observed in all aerobic organisms. It is renowned for its ability to catalyze the conversion of hydrogen peroxide into water and oxygen in an energy-efficient manner. CAT is present in all major sites of hydrogen peroxide production within the cellular environment, including cytosol, peroxisomes, and mitochondria. CAT is a pivotal biomarker for oxidative stress and progression of diverse diseases and infections (Mahomoodally et al. 2022). Normal metabolism and proliferation of cells are adversely affected by the occurrence of oxidative stress. In severe cases, cell survival and proliferation rates significantly decrease. Excessive oxidative stress can result in the disruption of cell structures and the induction of apoptosis. A notable decline in this area has significant implications for human health. By reducing cellular apoptosis caused by oxidative stress, the cells can be protected and rendered more resilient to disease (Li et al. 2020).

In addition to its antioxidant effects, the *H. orientale* extract was observed to suppress apoptosis in H_2O_2-injured cells. This was achieved by ameliorating Bax overexpression and robustly returning the initial activation level of caspase-3. Furthermore, the extract promoted survival rates and metabolic activities of the treated 293 T cells. Bax is a member of the proapoptotic Bcl-2 family, which controls the regulation of apoptotic cell death. (Westphal et al. 2014). The expression of Bax is decreased by antioxidants, which consequently results in a reduction in apoptosis and an increase in survival rates in cells (Li et al. 2020). Caspase-3, the principal apoptotic executioner caspase, activates death proteases and initiates the proteolytic cleavage of intracellular structures (Pehlivanoglu et al. 2023). It was determined that the *H. orientale* extract exhibited a marked inhibitory effect on effector caspase-3 activity, which is responsible for initiating apoptosis, and also demonstrated the ability to repress Bax expression, thereby providing further evidence of its antiapoptotic properties (Fig. 4).

Helichrysum species has been used widely in traditional medicines for the treatment of various diseases. It has been found that the aqueous extract *H. arenarium* showed toxic effects on lymphoma and human colorectal adenocarcinoma cell lines (Akinfenwa et al. 2022). Also, biosynthesized silver nanoparticles of *H. arenarium* exhibited antioxidant, anticancer, and antimetastatic activities on SK-MEL30 melanoma cell line (Aydin Acar et al. 2023). *Helichrysum* species like *H. aureum, H. caespititium, H. italicum, H. nudifolium, H. odoratissimum, H. petiolare,* and *H. platicum* had low-to-high toxic effects in various cancer cells (Akinfenwa et al. 2022). It has been proven that the isolates of the mostly studied species *H. italicum* and *H. arenarium* had significant antioxidant effects (Judzentiene et al. 2022). In this context, for example, the use of *H. italicum* essential oil has recently drawn attention to its tissue regeneration and anti-inflammatory effects. This could be used as a reconstructive agent in the cosmetic industry, anti-aging in surgery, and possibly as a wound-healing agent. (Węglarz et al. 2022). Recently, *H. arenarium* was investigated for its healing role in cardiomyocyte injury triggered by high-glucose. After treatment with *H. arenarium* extract, the casein kinase and lactate dehydrogenase activities and the pro-inflammatory cytokine levels (TNF-α, IL-1β, and IL-6) were significantly reduced in damaged cardiac myocytes. Besides that, the levels of Bax were significantly down-regulated while the Bcl-2 proteins were significantly up-regulated (Liu and Lan 2022). Although the antioxidant and antiapoptotic effects of *H. orientale* in an in vitro kidney injury model have been demonstrated for the first time, previous literature findings related to other

Helichrysum species supported and confirmed the findings of the present study.

Conclusions

In conclusion, the aqueous extract of *Helichrysum orientale* (L.) Gaertn.'s capitulum samples exhibits promise in the prevention of acute kidney cell damage and apoptosis induced by hydrogen peroxide. The aqueous extract of *H. orientale* was found to contain substantial quantities of cinnamic acid derivatives, including trans-cinnamic acid, *o*-cumaric acid, and trans-*p*-cumaric acid. Furthermore, the extract contains notable quantities of polyphenolic compounds, including resveratrol, chlorogenic acid, apigenin 7-glucoside, rutin, gallic acid, and hesperidin. The robust antioxidant characteristics of these compounds enabled the extract to reverse H_2O_2-induced damage in 293 T kidney cells. This protective effect is attributed to its ability to enhance SOD and CAT activities while reducing MDA, Bax, and active caspase-3 levels. Nevertheless, further research is required to identify the active pharmaceutical ingredients responsible for its antioxidant properties and elucidate the underlying mechanism of action. In terms of future perspectives, this will require *in vivo* studies in animal models of nephropathy and also clinical studies to evaluate the precise cellular mechanisms of kidney protective effects. A comprehensive and integrative investigation is required to assess its potential antioxidant role in the management of various diseases.

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Data availability The data of this study are available from the corresponding author upon conceivable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval This study does not require an informed consent form or approval from for experimental animals.

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