Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Cardio protective effect of methanolic extract of chlorella vulgaris against in domethacin-induced cardiotoxicity in zebrafish

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ABSTRACT

Zebrafish (*Daniorerio*) have emerged as a valuable vertebrate model to study cardiovascular development over the last few decades.In this study, the protective effect ofmethanolic extract of *chlorella vulgaris* against cardiotoxicity induced by Indomethacin in zebrafish was evaluated. Studies were performed on eight groups of six animals each, including-control,Indomethacin,SN,*chlorella vulgaris*, Indomethacin plus SN, Indomethacin plus *chlorella vulgaris* at different concentrations of 20, 40 and 100 µg/g of feed. Zebrafishreceived SN, indomethacin and *chlorella vulgaris* daily thereafter throughout the study. Cotreatment with SN and *chlorella vulgaris*attenuated indomethacin-induced alteration in the levels of antioxidant status and cardiac marker enzymes such as creatine kinase, Lactate dehydrogenase (LDH) as well as histopathological changes in cardiac tissues. In conclusion, the matahnolic extract of *chlorella vulgaris* may have protective effects against cardiotoxicity induced by indomethacin by reducing lipid peroxidation, renewing the activities of antioxidant enzymes, and preventing apoptosis.

KEYWORDS: *chlorella vulgaris*, cardiotoxicity, indomethacin ,silymarin, zebrafish.

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INTRODUCTION

Zebrafish(Daniorerio) is a small freshwater teleost species, reaching only 3 cm in length. During the embryonic and larval stages, zebrafish is only 1 mm to 4 mm long. Zebrafish larva can live for seven days in a single well of a standard 96-well or 384-well plate, surviving on nutrients stored in the yolk sac. The zebrafish larva develops rapidly, and its organogenesis is complete within 48 h post-fertilization.

The adult zebrafish is a feasible option for a variety of studies and drug screens, and has proven to be an economic alternative to other animal models. zebrafsh have been extensively used to study cardiotoxicity[1]. Cardiotoxicity is defined as the toxicity that damages the heart muscle and other cardiac tissues and/or disrupts the electrophysiology of the heart. As a result of cardiotoxicity, the heart maynot be able to pump adequate blood throughout the body[2]. If severe, cardiotoxicity may lead to cardiomyopathy in other terms cardiac muscle dysfunction. Cardiotoxicity might occur as a side effect of chemotherapeutical drugs or might develop due to exposure to certain chemicals. zebrafish heart has a simpler structure than the human counterpart (two chambers instead of four chambers), it possesses analogs of the major components of the human heart and utilizes similar cellular and molecular strategies to assemble the heart[3,4]. Due to the transparency of the embryos, the morphology and function of the developing hearts can be directly observed by light microscopy. This optical transparency can also be leveraged by the use of transgenic reporters in which cardiac cells are labelled with fluorescent markers[5-9].

*Chlorella*is one of the most promising microalgalgenuses from both a scientific and a commercial point of view. *Chlorella vulgaris* was first described by Beijerinck in 1890[10]. After that, a large number of Chlorella species were isolated and characterised[11]. *Chlorella vulgaris* and *Chlorella pyrenoidosa* werereported as high protein containing species among other microalgae belonging to Chlorophytes[12]. The *Chlorella vulgaris* is a genus of single-celled green algae, belonging to the phytumChlorophyta, which contain green photosynthetic pigments chlorophyll a and b in its chloroplast. The bioactive components of this unicellular alga make it an excellent candidate for various medical uses. The *Chlorella vulgaris* exhibit medical properties such as antitumor effect, hepato-protective, antioxidant, and antibacterial properties. These algae also include many dietary antioxidants such as lutein, α -carotene, β-carotene, ascorbic acid and α –tocopherol.
Indomethacin is in a group of drugs called nonsteroidal anti-inflammatory drugs (NSAIDs). Indomethacin works by reducing hormones that cause inflammation and pain in the body. Indomethacin is used to treat pain or inflammation caused by many conditions such as arthritis, gout, ankylosing spondylitis, bursitis, or tendinitis.In particular, it has been found to be associated with an increased risk of heart failure in several randomised clinical trials[13] and observational

studies[14,15].Silymarin (SN), a polyphenolic flavonoid, is a standardized extract obtained from the seeds and fruits of milk thistle. It is a mixture of some isomeric flavonolignans including silybin, isosilybin, silydianin, and silychristin[16]. SN indicates effective antioxidant properties [17] in addition to anti-inflammatory [18]and anticarcinogenic actions[19]. Additionally, it has emerged that in animal models, silymarin can protect the heart, brain, liver and kidneys against ischemia reperfusion injury, probably by preconditioning. The mechanisms of preconditioning are, in general, well studied, especially in the heart. On the other hand, the mechanism by which silymarin protects the heart from ischemia remains largely unexplored.

MATERIALS AND METHODS

Source and extraction material:

The microalgae *Chlorella vulgaris* was cultivated in f/2 basal mediumfor 45 days and the biomass was collected by centrifugation[20]. The filtrate was continuously washed with distilled water to remove salt remnants. Further, the samples were dried, lyophilized and used for analysis

Maintenance of Zebrafish:

Zebrafish were collected from local aquarium, acclimatized for 15 days, stocked and bred under laboratory conditions. The aquaria were continuously aerated through stone diffusers connected to a mechanical air compressor. Water temperature was 25±2ºC and pH was maintained between7.0 ± 0.5. The fish were fed twice daily. The experimental fishes were exposed to different drugs and concentration.10 fishes for each concentrations were used. In the experimental condition, aquaria water was replaced daily with fresh treatment. After the end of the experimental periods (3, 7 and 15 days), required number of treated fish was taken out from both experiment and control groups and their tissues were dissected.

Group Distribution:

Group I-Control Adult Zebra fish

Group II –Adult Zebra fish subjected to indomethacin

Group III- Adult Zebrafish subjected to *chlorella vulgaris* 20 µg/ml

Group IV- Adult Zebra fish subjected to silymarin

Group V-- Adult Zebra fish + indomethacin +silymarin

Group VI- Adult Zebra fish+ indomethacin + *chlorella vulgaris* 20 µg/ml

Group VII- Adult Zebra fish+ indomethacin + *chlorella vulgaris* 40 µg/ml

Group VIII- Adult Zebra fish+ indomethacin + *chlorella vulgaris* 10 0µg/ml

Preparation of Fish Extracts for Enzyme Assays:

Preparation of fish extracts for enzyme assays was performed as described by Ahmad *et al*[21] with some modifications.The fish heart tissue were homogenized in chilled 0.1 M phosphate buffer (pH 7.4)containing KCl (1.17%) [w/v]. The homogenate was filtered through Miracloth R and centrifuged at 10,000 g for 15 minutes at 4° C. The supernatant was then taken and centrifuged again (13,000 g) at room temperature for 20 minutes to obtain post-mitochondrial supernatant of the fish extract, which was used for the enzyme assays.

Estimation of Anti-oxidant enzyme

Assay of Catalase (CAT):

Catalase activity was assayed by the method[22]with some modifications. The fish extract (100 µl) was added to the reaction mixture containing 1.7 ml of 50 mM phosphate buffer (pH 7.0) and 1.2 ml of 40 mM H2O2 in atotal volume of 3ml. The decomposition of H2O2 was measured spectrophotometrically at 240 nm, using a 1.0 ml quartz cuvettein a Beckman DU 650 spectrophotometer at 25°C. The activity was calculated using the Beer-Lambert law.

Assay of Superoxide Dismutase (SOD):

SOD activity was determined according to the method of Beau-champ and Fridovich[23] with little modifications. The reagents used included phosphate buffer (0.1 M, pH 7.5),riboflavin (24 M), nitro blue tetrazolium (NBT) (840M),Na2 EDTA (1.2 mM), and methionine (150 mM).The reaction mixture containing 1.95 mL phosphate buffer, 0.25mL riboflavin, 0.25mL methionine, 0.25mL EDTA, 0.25 mL NBT, and 0.05 mL tissue extract was pipetted in 4 glass tubes. Another set of 4 tubes was pre-pared adding 0.05mL of phosphate buffer instead of enzyme extract. Three tubes from each set were then placed on shaker at 25 ∘ C in fluorescent light for 15 minutes and the last one was kept in dark at 25∘ C (reference sample, in darkness free radicals are not generated). After the incubation period the change in the absorbance was measured at 560 nm using respected dark-incubated sample as reference for test samples for each series. The SOD activity was expressed in terms of relative enzyme activity U/mg protein.

Assay of Glutathione peroxidase (GPX):

The method of Wood, J.L [24] was used to assay glutathione peroxidize. 13 About2.0 ml of phosphate buffer (75 mmol/L, pH 7.0), 50µl of (60mmol/L) glutathione reductase solution, 50µL of (0.12 mol/L) NaNO3, 0.1 ml of (0.15mmol/L) Na2 EDTA ,100µL of (3.0 mmol/L) NADPH, and 100µL of fish heart cell extract were mixed in a tube. Water was added to make a total volume of 2.9 ml. The reaction started by the addition of 100µL of (7.5 mmol/L) H2O2, and the conversion of

NADPH to NADP monitored by a continuous recording of the change of absorbance at 340 nm at 1-min interval for 5 min.

Assay of Lipid peroxidation (LPO):

The level of Lipid peroxides was estimated by Thiobarbituric acid reaction method described by Ohkawa et al[25]. To 0.2 ml of fish heart cell extract, 0.2 ml of SDS, 1.5 ml of acetic acid and 1.5 ml of Thiobarbituric acid were added. The mixture was made up to 4.0 ml with water and then heated in a water bath at 95°C for 60 minutes. After cooling, 1.0 ml of water and 5.0 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was taken and its absorbance was read at 532 nm. The activity of lipid peroxides expressed as nanomoles

Estimation of cardio Marker enzymes

Creatine Kinase(CK):

Simple, direct and automation-ready procedures for measuring CK activity are very desirable. Creatine Kinase Assay is based on enzyme coupled reactions in which creatine phosphate and ADP is converted to creatine and ATP by CK, the generated ATP is used to phosphorylate glucose by hexokinase to generate glucose-6-phosphate, which is then oxidized by NADP in the presence of glucose-6-phosphate dehydrogenase. The produced NADPH, measured at 340 nm.

Aspartate aminotransferase (AST):

This method was based on the use of glutamate dehydrogenase for the enzymatic estimation of the glutamate formed. The dehydrogenation of the glutamate gave rise to the reduction of a diazonium salt, and it was possible to perform a photometric reading of the coloured compound at 520 nm

Lactate Dehydrogenase Assay (LDH):

LDH is an enzymatic assay of viability. 500µl of PBS/lysis buffer solution was prepared (450 µl of 1X PBS and 50 µl of lysis buffer for each well on the 24 well plate) and 40 µl for each blank well on the 96 well plate.Cells were washed 2 times with 1XPBS using 1 ml of PBS per well.Finally lysis buffer was added to each well and incubated for 45 minutes at 37ºC.

Histopathology:

Histological processing was done by adopting the procedure described by Humason [26]. Briefly the tissues were removed from fixative, washed in running tap water and processed for dehydration in an increasing percentage of ethyl alcohol. After this the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Paraffin blocks were cut with a Leica manual rotary

microtome at thickness of 5µ and were stained with Harris Hematoxylin[27] and counter stained with eosin. Photomicrographs were taken using Magnus (MLX) equipment.

RESULTS

The effects of various treatments on antioxidant status:

Fig 1 shows the effects of various treatment on antioxidant activity in heart tissue of the zebrafish. When compared with negative control, Catalase activity was significantly decreased when treated with indomethacin alone whereas it achieved its maximum level during SN and *chlorella vulgaris* treatment alone and in combination with Indomethacin. SOD was analysed in heart tissue of the zebra fish to verify the presence of oxidative stress. Compared with the control, the activity of superoxide dismutase is increased in indomethacin treated fishes,whereas SOD activity is comparable with control between other groups.

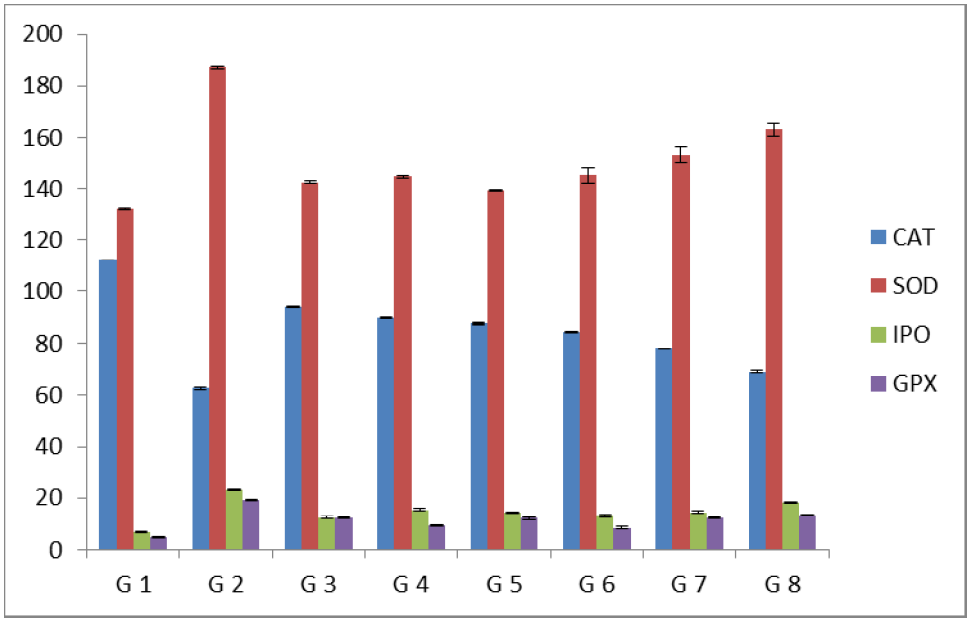

Fig 1: The effects on activity of various antioxidant enzymes in heart tissue of the zebrafish

The effect of *chlorella vulgaris* on lipid peroxidation (LPO) in the zebrafish showed significant changes at different concentrations and exposure periods. When exposed to treatment of indomethacin alone, the LPO was increased to higher level as compared to control. After exposure of indomethacin and cotreatment with silymarin and various concentration of *chlorella vulgaris,* LPO was decreased considerably. Similar results were noted for the activity of Glutathione peroxidase. The above mentionedfindingssuggested that oxidative stress induced by any drug is an important issue in aquatic ecosystems. The present observations on the induction of oxidative stress and antioxidant system would make it clear that indomethacin has a high degree of impact on antioxidant system in heart of zebrafish particularly during the exposures to IM that would normally affect and

alter the fish health. However, the conditions were restored by methanolic extract of *chlorella vulgaris*when comparedwith silymarin as positive control.

Cardio marker enzymes:

The effect of various treatment on cardiac marker enzymes were given in Fig 2. The treatment has its effect more on the activity of Lactate dehydrogenase when compared to other enzymes under study.

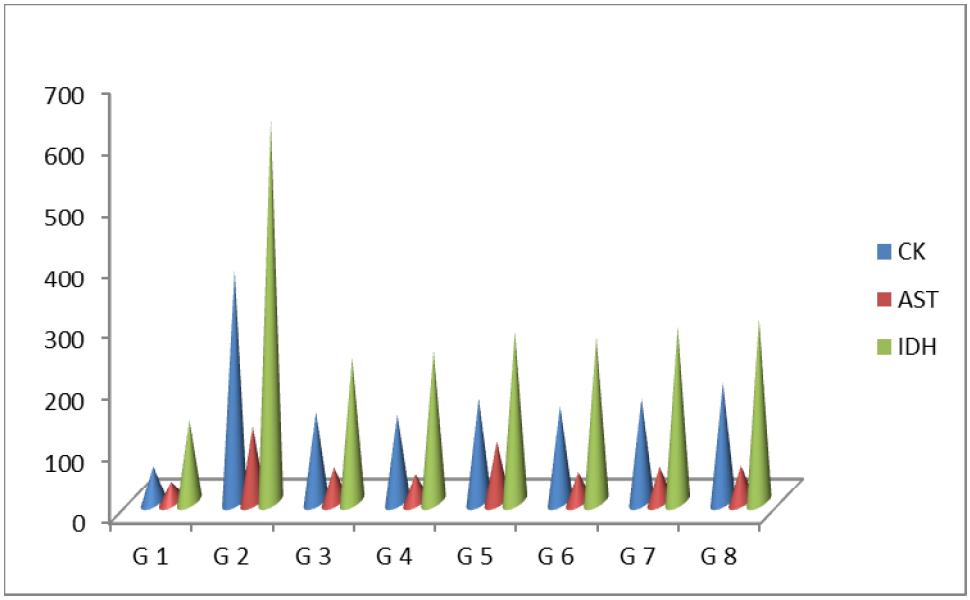

Fig2: Effect of various treatment on Cardio marker enzymes of zebrafish

Creatine kinase activity was much elevated in indomethacin treated groups when compared to that of the control, whereas silymarinand *Chlorella vulgaris* treatment alone and upon coadministration with indomethacin lowers the activity of creatine kinase.

Indomethacin and silymarincoadministered with indomethacin groups showed higher AST activity when compared with the control, whereas in silymarin and *Chlorella vulgaris* treatment alone and *Chlorella vulgaris* coadministerd with indomethacin groups the activity of AST is much reduced.

When compared with control groups, indomethacin treatment showed increase in the activity of LDH. However, Lactate dehydrogenase activity is considerably reduced in silymarinand *Chlorella vulgaris*treatment alone and upon coadministration with indomethacin.

Cardiac tissue pathology of the Zebra fish:

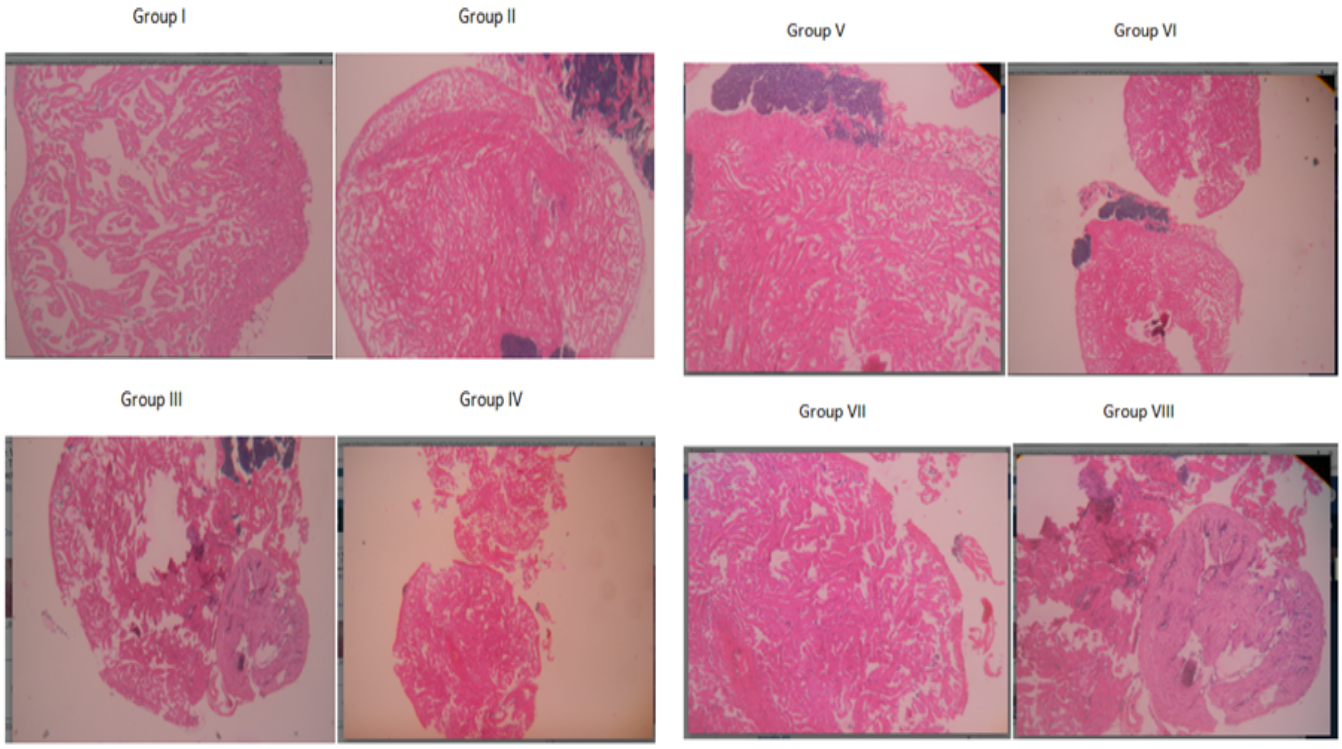

Fig 3: Microscopic appearance of heart tissue of Zebra fish.

In Fig 3, Group I- represents the normal architecture of the cadiomyocytecells. Group II,V, VI,VII,VIII represents the mild shrunken cells, necrotic cells and distortion. The staining reveals deposition of connective tissue and fibrotic areas in black and healthy myocardial tissue in red. There is still some trabeculated myocardium in the injured area (IA). Erythrocytes accumulate at the IA. Extensive fibrosis is visible. The myocardium has been degraded by this stage. Infiltration of IA along with inflammatory cells was observed. Black indicated the thickened epicardial layer compared with the control situation fibrotic tissue accumulation is seen.It is well observed that the cardiac tissue was not damaged and remains unaltered in the histological sections upon treatment with *chlorella vulgaris.*

DISCUSSION

Oxidative stress is a key factor in cardiovascular complications and study of antioxidant enzyme system is considered a vital parameter for defense mechanism against oxidative damage to tissues. During the present investigation a significant alterations were observed in the CAT activity, Gpx level, LPO and cardiac marker enzymes in heart tissue of zebrafish exposed to indomethacin which is being restored upon treated with *chlorella vulgaris*at different concentrations and exposure periods. This indicates the cardioprotective property of *chlorella vulgaris*which may be due to the antioxidant, free radical scavenging and membrane stabilising potential of *chlorella*

vulgaris[28]. The protective role is also attributed due to the presence of polyphenolicconpounds and pigments in *chlorella vulgaris*[29,30].

High concentration of metals in fish tissues can lead to redox reactions, generating free radicals, especially reactive oxygen species e.g. oxygen superoxides, peroxides, hydroxyl radical and hydrogen peroxides[31]. The highly reactive compounds,molecules or ions formed by the incomplete reduction of oxygen may induce alteration and change some physiological responses of fish [32,33,34], LPO and plasma membrane alternations [35]. Arsenic and iron induced toxic effects on *Tilapia mosambica*[36], Oxidative stress- induced apoptosis [37]were reported as a possible mechanism of arsenic toxicity in zebrafish Daniorerio liver cell line.

Heart failure is a progressive condition in which the heart muscle is unable to pump enough blood to meet the body's needs for blood and oxygen. Azebrafish-dilated cardiomyopathy modelwas developed using short-term Indomethacin treatment. Indomethacin will induce QT prolongation and cardiac arrhythmia in zebrafish. Indomethacin treatment inzebrafishhas showed enlarged atrial and ventricular size and venous congestion. Terfenadine-treated cardiomyocytes showed apoptotic change and reduced ventricular contraction.Thus, Zebrafish has been a model for human cardiac disease for a much shorter time period than most other animal models, which helps explain the smaller number of total proteomic and cardiac related publications[38]. Zebrafish have been used successfully in drug discovery and chemical screening processes[39].

CONCLUSION

More recently, the zebrafish has been used to study mechanisms leading to human cardiac diseases and to model human congenital and acquired cardiac diseases. Predictably, this field will grow rapidly in the coming years owing to the increase in sequencing efforts, the growing interest in cardiac diseases, and the improved availability of the zebrafish model for clinical and basic researchers interested in studying cardiac diseases.Our study revealed that Indomethacin induced a distinct oxidative stress in the heart of zebrafish connected with the production of ROS (reactive oxygen species). *Chlorella vulgaris* has a high degree of impact on antioxidant system and cardiac marker enzymes in heart tissue of zebrafish particularly during the exposures to Indomethacin thereby protecting the animal without altering the fish health. Our data indicated that methanolic extract of *Chlorella vulgaris*has protective effects against Indomethacin-induced cardiotoxicity in zebrafish through attenuating lipid peroxidation, increasing Gpx content, renewing the activities of antioxidant enzymes. Thus, microalgal extracts can be explored as better nutraceutical supplements towards attenuating oxidative stress and tissue damage in human.

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