Original Article

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Hepatoprotective effects of *Artemia salina* L. extract against carbon tetrachloride-induced toxicity

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Abstract

The protective effects of *Artemia salina* L. extract was examined against carbon tetrachloride (CCl_4)-induced cell toxicity. In the *in vitro* model of study, markers such as cell viability, cellular reduced and oxidized glutathione, and lipid peroxidation in $HepG_2$ cells was evaluated. Human liver cancer cell line $HepG_2$ was treated with CCl_4 and *Artemia salina* extract, and markers of hepatotoxicity were investigated. *Artemia salina* extract showed significant dose-dependent protective effects against the cytotoxicity of CCl_4. This extract was able to normalize the levels of GSH, and thiobarbituric acid-reactive (TBARs), which were altered due to CCl_4 intoxication in $HepG_2$ cells. As the oxidative stress markers were ameliorated, it might be concluded that *Artemia salina* extract possesses protective effects probably due to its antioxidant constituents

Keywords: Artemia salina L., CCl_4, Hepatotoxicity, $HepG_2$ cells, Oxidative stress.

1. Introduction

Artemia salina L. (*A. salina* L.), the brine shrimp, is an invertebrate belonging to saline aquatic and marine ecosystems (1). In Iran, there are only two habitats for them, *Artemia urmiana* in Urmia and *A. salina* in Maharloo Lake, in Fars province (2). It plays an important role in the energy flow of the food chain in ecosystems (1). The resilience of these organisms makes them ideal test samples in laboratory experiments for evaluation of toxicity of a series of toxins and plant extracts (1). These features are due to the readily availablability of eggs and the small size of organisms, which are enough to allow statistically valid numbers to be maintained under laboratory circumstances (3).

It has been reported that *A. salina* contains carotenoids (4). This carotenoid content is solely acquired from food uptake. In this regards, *A. salina* stores ingested carotenoids as intact or transforms them into other carotenoids (5). Most of the carotenoid content can be acquired from microalgae, yeast, or egg yolk during feeding processes (6).

Carotenoids are among the most potent

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antioxidants in plants and animals (7). A diverse variety of carotenoid derivatives with antioxidant properties, such as β-carotene, canthaxanthin, echinenone, and zeaxanthin can be found in Artemia body mass (8). In this regard, *A. salina* has shown various carotenoid contents in its life cycle. For instants, the only carotenoids existent in the eggs and freshly-hatched nauplii of *A. salina* are echinenone and canthaxanthin (9). These carotenoids are an intermediate in the conversion of β-carotene into canthaxanthin. Moreover, echinenone can be converted into canthaxanthin (10). Therefore, canthaxanthin is the final product after carotenoid transformations in Artemia (10). On the other hand, zeaxanthin can be easily absorbed by the gut, but is not metabolized to produce any other intermediates (11). Hence, brine shrimps are new potent antioxidants in order to combat with oxidation, lipid peroxidation, and free radical damages (12).

The liver is the most important and the first barrier between ingested toxicants and blood (13). So protection of hepatocytes from hazardous metabolites and drugs is necessary. Carbon tetrachloride is a standard substance for determination of antioxidant and hepatoprotective properties of different compounds (13). It can be metabolize to trichloromethyl and then to peroxy-radical by cytochrome P_{450} enzymes in the liver (13). These free radicals can harm cells by lipid peroxidation (14).

In this study, we aimed to examine the antioxidant and cell protective properties of *A. salina* extract along with its cytotoxicity on $HepG_2$ cell lines. According to our best knowledge, this is the first study that evaluates the antioxidant and cell protective activity of *A. salina*, which makes it suitable as a hepatoprotective agent for preventing hepatic diseases.

2. Material and Methods

2.1. Chemicals

Human liver cancer cell line $HepG_2$ was obtained from Pasteur Institute (Tehran, Iran). RPMI-1640 and FBS were from GibCo (United States). Trypan blue, methylthiazolydiphenyl-tetrazolium bromide (MTT), Dithio-bis-nitro benzoic acid (DTNB), CCl_4, and Na_2HPO_4 were purchased from Merck (Darmstadt, Germany), and thiobarbituric acid (TBA) was from Sigma Chemical Company (Germany). All other used chemicals were of the highest quality available in the market.

2.2. Preparation of Extract

A. salina was cultured in a 50 liter pound and after growing; the medium was filtered through fine gauzes to separate Artemia body mass from the medium and their food. Then, Artemia was freezed in -20 and -70 °C and lyophilized to achieve a dry powder. 5 g of lyophilized Artemia body mass was extracted with 80% methanolic solvent three times with 200 ml solvent in three days. Then cell bodies were filtered by Whatman papers, and the solvent was poured in a rotary to obtain 0.8 g dry extract.

2.3. In vitro hepatoprotective activity study

Human hepatoma cell lines ($HepG_2$) were cultured and maintained in RPMI-1640, pH 7.3, containing 0.37% $NaHCO_3$ supplemented with 10% FCS (fetal bovine serum albumin), 1% penicillin, and streptomycin (100 IU/mL penicillin and 100 IU/mL streptomycin) in a humidified 5% CO_2-95% air mixture at 37 °C. Cells were seeded in 96-well microplates (30000 cells/well/90 µL) and routinely cultured in a humidified incubator for 24 hours. The cells were then treated with different concentrations (from 10 µg/mL to 200 µg/mL) of the Artemia extract 1 hour before CCl_4 100 mM (CCl_4/ethanol; 1/10) exposure. The appropriate, safe, and non-toxic dosage of extract was determined (Table 1) for using in other experiments on $HepG_2$ cell line.

After 24 hours of incubation, 10 µL of MTT solution was added to every well, cells were re incubated for an additional 4 h. The cell culture media and MTT solution were removed and the cells remained in the bottom of the wells. Then, 100 µL of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of the converted dye was measured at a wavelength of 570 nm. Five wells were used for each concentration of extract, and three independent experiments were performed for each assay.

Table 1. Effect of different concentrations of Artemia extract on $HepG_2$ cells viability, as assessed by MTT test (% viability).

Concentration	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL
Control	100±7.6	100±8.4	100±7.8	100±8.9
AS	97.26±5.6	97.3±5.2	92±6.3	88±4.6

2.4. Lipid peroxidation in $HepG_2$ cell

As a biomarker for lipid peroxidation, concentration of thiobarbituric acid-reactive (TBARs) agents was measured. $HepG_2$ cells (3x106 cells/flask) were pre-incubated in flasks for 24 h at 5% CO_2-95% air at 37 °C. The control cultures were prepared by adding RPMI-1640 without any addition. After incubation with or without extract, the culture medium was removed. After rinsing with 0.5 mL free PBS twice, cells were collected by trypsinization. After determining the viability of the detached cells, 250 µL of 70% (w/v) trichloroacetic acid containing 1 mL of 0.8% (w/v) thiobarbituric acid with 750 mL deionized water was added to cells and was shaken with vortex. The suspensions were transferred into glass tubes and boiled for 30 min. After cooling to room temperature and centrifugation for 10 min at 5000 rpm, the absorbance of the supernatant was determined at 532 nm.

2.5. Glutathione content in $HepG_2$ cells

$HepG_2$ cells (3x106 cells/flask) were pre-incubated in flasks for 24 hours at 5% CO_2-95% air at 37 °C. Cells were rinsed with PBS and collected by trypsinization. After determining the viability of detached cells, 200 µL of 20% trichloroacetic acid with 1800 µL of PBS were added to the cell suspension. After shaking with vortex and centrifuging, the supernatant was divided to two even parts (each 1 mL).

For measurement of reduced glutathione (GSH), two mL of Na2HPO4 (0.3 M) and 0.5 mL of DTNB (0.01 M) were added to 1 mL of the supernatant and was shaken with vortex. The absorbance was then measured at 412 nm. For measuring oxidized glutathione (GSSG), 1 mL of 5% sodium borohydride was added to 1 mL of the upernatant, followed by a 1 hour incubatation in 45 °C. Then 0.5 mL of Na_2HPO_4 (0.3 M) was added to each tube. After neutralization with HCl (2.7 N), 0.5 mL of DTNB (0.01 M) was also added and was shaken with vortex. The absorbance was then measured at 412 nm.

2.6. β-carotene determination

β-carotene determination was performed spectrophotometrically at 450 nm. *A. salina* was extracted with 3 mL hexane/ethanol (1:2) solution. After vortexing the solution was centrifuged (3000 rpm, 10 min) and the supernatant was divided into two distinct phases. The upper phase was hexane phase including β-carotene compounds. According to the below equation, the amount of *β*-carotene was calculated in µg/mL:

β-carotene content (µg/mL)=25.2×A450

The obtained values for *β*-carotene content in Artemia were then converted and expressed as mg g^{-1}.

2.7. Statistical analysis

All of the values in at least three performed experiments refer to Mean±SD. Statistically significant differences found between control and experimental groups using one way ANOVA test. The GraphPad Instat and Microsoft Office Excel were used for Statistical analysis. The minimal level of significance was chosen as $p<0.05$.

3. Results

Carbon tetrachloride was toxic towards $HepG_2$ cells in a dose-dependent manner. It caused loss of cell viability for 24 h with and LC_{50} of about 100 mM (data not shown).

Different concentrations of extract were tested on $HepG_2$ cells to obtain a non-toxic appropriate dose for further experiments (Table 1). The results presented in Figure 1, revealed that the pre-incubation of $HepG_2$ cells with 10 to 200 µg/mL of Artemia extract 1 h before CCl_4 (100 mM), led to reduction of cytotoxicity.

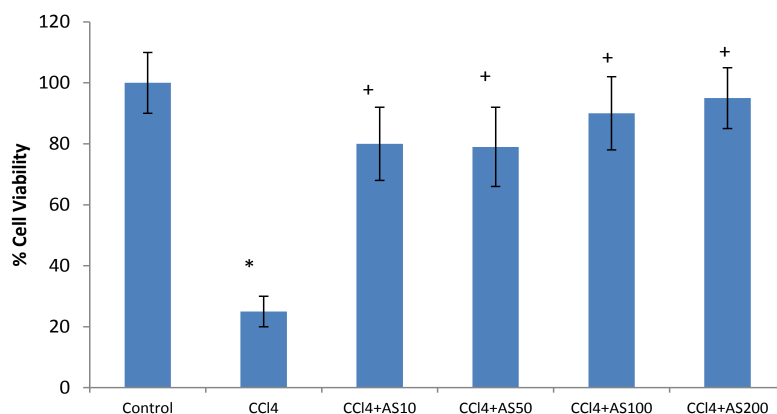

Figure 1. Effect of *Artemia salina* extract on cytotoxicity in $HepG_2$ cell.

Data are given as Mean±SD for at least three independent experiments.
*Significant difference as compared to control cells ($P<0.05$).
+Significant difference as compared to CCl_4-treated $HepG_2$ cell ($P<0.05$).

Incubation of the cells with 100 mM CCl_4 for 24 h decreased the GSH content and TBARs level of the cells significantly (Figure 2&3). Pre-incubation of the cells with Artemia extract also significantly affected TBARs level and the GSH content of the cells. The obtained value for *β*-carotene content in Artemia extract was 7.67 µg/mL.

4. Discussion

Methanolic extract of Artemia has shown protective properties against CCl_4 in *in vitro* as revealed by a decrease in cell death. Furthermore, $HepG_2$ cells treated by Artemia extract showed higher levels of glutathione (GSH) and lower levels of TBARs.

For studying the hepatoprotective effects of medicinal plants, extract or newly discovered drugs, the hepatic injury induced by CCl_4 is usually used as an experimental method (15). CCl_4 can be metabolized by microsomal CYP_{450} in the liver of animals and human to a highly reactive trichloromethyl free radical ($CCl_3^•$) which can cause peroxidation of lipids and damage to proteins and other components of the cell due to beginning of

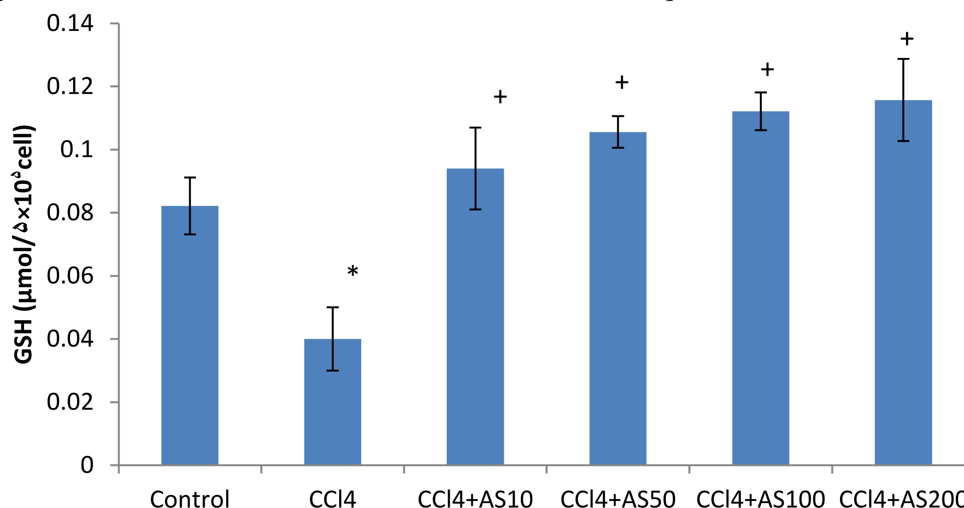

Figure 2. Effect of *Artemia salina* extract on GSH content in $HepG_2$ cell.

Data are given as Mean±SD for at least three independent experiments.
*Significant difference as compared to control cells ($P<0.05$).
+Significant difference as compared to CCl_4-treated $HepG_2$ cell ($P<0.05$).

a chain of reactive free radical formation that finally can result in cell lysis (16). Deactivation of cellular defense mechanisms and induction of oxidative stress are two main factors involved in CCl_4-induced hepatotoxicity. Oxidative stress affects a wide range of intra and intercellular targets, including DNA, lipids, proteins, and cellular defense mechanisms, namely glutathione (GSH) reservoirs. Lipid peroxidation is a common result of ROS formation and oxidative stress in the liver (17).

Artemia extract decreased the lipid peroxidation induced by CCl_4. N hexane extract administration also prevented from cellular GSH consumption (Figure 2&3). The role of Artemia extract in preventing the lipid peroxidation and its effects on cellular glutathione compounds may have been due to its antioxidant effects and attenuation of oxidative stress.

It has been found that carotenoids and their derivatives possess a potent antioxidant activity (18). Hsu and hester, showed that β-carotene can be converted into echinenone and canthaxanthin by metabolic enzymes of *A. salina* (19). The antioxidant and protective effects of carotenoids and their derivatives have been specified (20). β-carotene compounds has been detected in Artemia. These compounds are good examples of antioxidant agents, with different characteristics in biological systems through which they show their protective properties. Hence, protective properties of Artemia extract (methanol extract in this study) could be attributed to the polyphenolic and β-carotene compounds in this fraction. Its cytoprotective and hepatoprotective effects against CCl_4 could be by different mechanisms such as: inhibition of liver enzyme cytochrome P_{450}, which is responsible for metabolism of CCl_4 to reactive free radicals; antioxidant effects; free radical scavenging, which is responsible for cell damage; or induction or regeneration of the liver cells. However, lack of an appropriate positive control might serve as a limitation for the current investigation.

5. Conclusion

Extract of Artemia possesses protective effects against CCl_4-induced cytotoxicity probably due to its antioxidant ingredients. However, more future studies on this extract are needed to clarify the exact component(s) responsible for hepatoprotection.

Acknowledgment

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Conflict of interest

None declared.

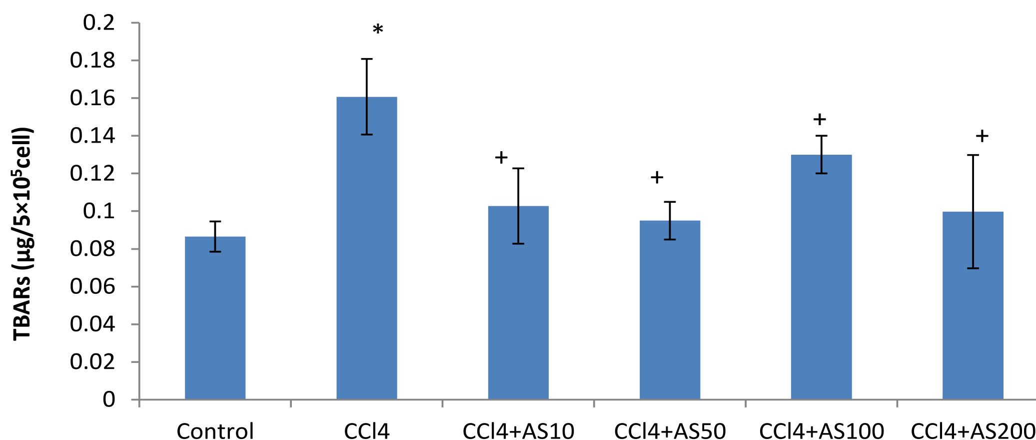

Figure 3. Effect of *Artemia salina* extract on lipid peroxidation (TBARs) in $HepG_2$ cell.

Data are given as Mean±SD for at least three independent experiments.
*Significant difference as compared to control cells (P<0.05).
+Significant difference as compared to CCl_4-treated $HepG_2$ cell (P<0.05).

6. References

1. Nguta J, Mbaria J, Gakuya D, Gathumbi P, Kabasa J, Kiama S, editors. Evaluation of acute toxicity of crude plant extracts from Kenyan biodiversity using brine shrimp, *Artemia salina* L. (Artemiidae). *Open Conf Proc J.* 2012;3:30-4
2. Hajirostamloo M. Genetic differentiation of *Artemia parthenogenetica* from Various ecological population of Iran. *Int J Biol Life Sci.* 2009;5:3-9.
3. Gambardella C, Mesarič T, Milivojević T, Sepčić K, Gallus L, Carbone S, *et al.* Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemical responses. *Environ Monit Assess.* 2014;186:4249-59.
4. Anuevo GM, Nomura N. Improvement of total polyphenol content, total carotenoid and antioxidant activities of *Artemia salina* nauplii bioencapsulated with green tea extract. *J Curr Res Sci.* 2014;2(2):203-8.
5. Leite AdS, Dantas AF, Oliveira GLdS, Gomes Júnior AL, de Lima SG, Citó AMdGL, *et al.* Evaluation of toxic, cytotoxic, mutagenic, and antimutagenic activities of natural and technical cashew nut shell liquids using the *Allium cepa* and *Artemia salina* bioassays. *Biomed Res Int.* 2015;2015:1-7.
6. Devi NK, Kumar AT, Balasubramanian T. Pigment Deficiency Correction in Captive Clown Fish, *Amphiprion ocellaris* Using Different Carotenoid Sources. *J Fishscicom*. 2016;10(1):4-11.
7. Carocho M, Ferreira IC. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food Chem Toxicol*. 2013;51:15-25.
8. Wade NM, Gabaudan J, Glencross BD. A review of carotenoid utilisation and function in crustacean aquaculture. *Rev Aquacul*. 2015:1-16.
9. Minyuk G, Chelebieva E, Chubchikova I. Secondary carotenogenesis of the green microalga *Bracteacoccus minor* (Chodat) Petrova (Chlorophyta) in a two-stage culture. *Int J Algae*. 2014;16:354-68.
10. Schöpf L, Mautz J, Sandmann G. Multiple ketolases involved in light regulation of canthaxanthin biosynthesis in Nostoc punctiforme PCC 73102. *Planta.* 2013;237:1279-85.
11. Yan F, Dibner J, Knight C, Vazquez-Anon M. Effect of carbohydrase and protease on growth performance and gut health of young broilers fed diets containing rye, wheat, and feather meal. *Poult Sci.* 2016: pew300.
12. Lokhmatikov AV, Voskoboynikova N, Cherepanov DA, Skulachev MV, Steinhoff H-J, Skulachev VP, *et al.* Impact of Antioxidants on Cardiolipin Oxidation in Liposomes: Why Mitochondrial Cardiolipin Serves as an Apoptotic Signal? *Oxid Med Cell Longev*. 2016;2016:1-19.
13. Sohn D-H, Kim Y-C, Oh S-H, Park E-J, Li X, Lee B-H. Hepatoprotective and free radical scavenging effects of *Nelumbo nucifera*. *Phytomedicine*. 2003;10:165-9.
14. Mustafa SA, Karieb SS, Davies SJ, Jha AN. Assessment of oxidative damage to DNA, transcriptional expression of key genes, lipid peroxidation and histopathological changes in carp *Cyprinus carpio* L. following exposure to chronic hypoxic and subsequent recovery in normoxic conditions. *Mutagenesis*. 2015;30:107-16.
15. Wu J-G, Kan Y-J, Wu Y-B, Yi J, Chen T-Q, Wu J-Z. Hepatoprotective effect of ganoderma triterpenoids against oxidative damage induced by tert-butyl hydroperoxide in human hepatic $HepG_2$ cells. *Pharm Biol.* 2016;54:919-29.
16. Yu Z, Sun W, Peng W, Yu R, Li G, Jiang T. Pharmacokinetics *in vitro* and *in vivo* of two novel prodrugs of oleanolic acid in rats and its hepatoprotective effects against liver injury induced by CCl_4 . *Mol Pharm.* 2016;13:1699-710.
17. Liu L, Zhang K, Sandoval H, Yamamoto S, Jaiswal M, Sanz E, *et al*. Glial lipid droplets and ROS induced by mitochondrial defects promote neurodegeneration. *Cell*. 2015;160:177-90.
18. Zarei M, Mobasher MA, Morowvat MH, Mousavi P, Montazeri-Najafabady N, Hajighahramani N, *et al*. Effects of menthone and piperitone on growth, chlorophyll a and β-carotene production in *Dunaliella salina*. *J App Pharm Sci.* 2016;6:215-9.
19. Hsu W-J, Chichester C, Davies B. The metabolism of β-carotene and other carotenoids in the brine shrimp, *Artemia salina* L.(Crustacea: Branchiopoda). *Comp Biochem Physiol.* 1970;32:69-79.
20. Murador DC, Mercadante AZ, de Rosso VV. Cooking techniques improve the levels of bioactive compounds and antioxidant activity in kale and red cabbage. *Food Chem.* 2016;196:1101-7.