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Research Article

Synergistic Antioxidant Capacity of Chitosan Nanoparticles and Lycopene Against Aging Hepatotoxicity Induced by D-galactose in Male Rats

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Abstract

Background and Objective: Ageing is frequently accompanied by the occurrence of several diseases, such as Alzheimer's and disturbance of vital organs like liver, the combination between some compounds with antioxidant properties are considered a new hope for treatment of aging consequences, so this paper aimed to evaluate the synergistic effect between chitosan nanoparticles and lycopene in ameliorating liver functions and alleviate hepatotoxicity and oxygen free radicals which are a reactive species that are permanently liberated in living cells and increased greatly in aging and causes health disturbances. This study aimed to synthesis chitosan nanoparticles (CHNPs), to determine their characteristics and to estimate the synergistic role of CHNPs incorporation with lycopene (Ly) against oxidative stress of aging and hepatotoxicity. **Materials and Methods:** Male wistar rats were divided into 9 groups (10 rats/group) as follow: Control group, D-galactose group(100 mg kg^{-1}), CHNPs either (low dose) or (high dose) (140 or 280 mg kg^{-1}), Ly group (20 mg kg^{-1}), CHNPs either low dose or high dose with Ly and D-gal plus Ly and/or CHNPs with Low and high dose treated groups. The CHNPs were characterized by TEM, Zeta potential and size distribution of particles. At the end of the experiment, some biochemical parameters were measured as lipid profile, tumor marker TNF-α and IL-6, markers of inflammation and tissue damage LDH and CRP with histological, comet assay and TEM examination of liver tissues. **Results:** Chitosan showed size distribution (pdi) 0.370 nm. D-galactose induced hepatic biochemical alterations and cellular changes. CHNPs in two doses either low or high dose alone or combined with Ly significantly elicited remarkable amelioration in liver enzymes biomarkers, improved lipid profile, decreased tumor necrosis factor-α and IL-6, enhanced antioxidant enzymes as SOD, CAT and GPx with decreasing marker of lipid peroxidation and proved by structural alterations in TEM, histological and comet assay of liver tissues. **Conclusion:** It could be proved that CHNPs and Ly could synergistically afforded protection against liver injury and oxidative stress as a result of aging. Consequently, CHNPs was an effective agent in the drug delivery in liver diseases medications. CHNP-s and Ly enhanced liver enzymes and improved their antioxidant capacities in liver.

Key words: Chitosan nanoparticles, lycopene, antioxidant enzymes, oxidative biomarkers, hepatotoxicity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ageing is a complex natural phenomenon that is frequently accompanied by the occurrence of several diseases, such as schizophrenia, cognitive impairment, Alzheimer's and Parkinson's diseases and others. There is a major remedy of proof that suggests that oxidative damage and damage play a sensitive role in the process of biological ageing[1].

Oxygen free radicals are a reactive species that are permanently liberated in living cells as a portion of ordinary metabolism. Excess production of reactive oxygen species (ROS) is deleterious to cells and is probably have toxic influences in cells implicated in the pathogenesis of some ageing diseases[2].

D-galactose (D-gal) is a physiological nourishing agent, but over provide of D-gal can cause osmotic stress due to the production of ROS and result in abnormal metabolism. Literature suggests that administration of D-gal can lead to senescent syndrome in experimental animals analogous to ageing in humans[3]. It was shown that abnormal aggregation of galactitol from overflow of D-gal, through the activity of aldose reductase in cells, caused osmotic stress and generation of free radicals[4]. In addition, D-gal is a reducing sugar and causing the activation of the advanced glycation end product receptor and stimulates free radical production[5].

Mice injected with D-gal for 6-10 weeks exhibit cumulative impairment of memory and learning ability and elevated manufacturing of ROS in the brain and impairment of neurogenesis in the hippocampus[6]. Accumulating evidence suggests that chronic injection of D-gal could accelerate the metabolic rate, induce neurological impairment and decrease activity of antioxidant enzymes and further cause oxidative damage and accelerate the ageing process[4].

Chitosan is an innate polysaccharide containing different amounts of (1-4)-glycosidic bonds[7]. Chitosan has catched much awareness as a biomedical material, as it prompts a broad diversity of biological activities, such as antitumor activities[8], immunostimulating effects[9], antiallergic effects[10], hemostatic agent[11], anticoagulant effects[12] hypocholesterolemic effects[13], anti-inflammatory activities[14] and free radical scavenging activities[15]. However, because of its insolubility in water and highly molecular weight, the applications of chitosan are roughly with limits. Nanoparticle formulation supplies a sensible pharmaceutical principle for promoting bio availability orally and therapeutic effectiveness of chitosan particles[16].

Chitosan nanoparticles (CHNPs) display more outstanding actions and there is report that chitosan nanoparticles have multiplied immune-improving effect, antimicrobial and anticancer activity than those of particles of chitosan. In addition, nanoparticles retains a powerful surface warp, comparable to large particles, this manufactures more decay pressure with an identical elevation in saturation solubility[17]. The elevated saturation solubility, promotes an increment in concentration between cells in the intestine and the mesenteric circulation beneath.

Lycopene (Ly), a phytochemical pertinence to carotenoid family, is a pigment with red-colored, acyclic and a polar carotenoid[18]. It is amply found in vegetables and fruits with red colour, such as tomatoes, pink grape-fruit, pink guava, watermelon and carrots with 9-42 mg kg^{-1} range concentrations relayed on the diversity[19]. Ly offers a range of exclusive biological characteristics possessing to its acyclic structure and major rate of double bonds.

Varieties of reports have been issued that lycopene extends strong antioxidant capability against the proteins oxidation, also has the prospect of irrigation singlet oxygen 100 times more effectively than vitamin E and more than 100 times more than glutathione (GSH). Moreover, it can also scavenge peroxyl radicals, prohibiting the process of lipid peroxidation at low surface tension[20,21].

It is the most quencher effectively of singlet oxygen amongst all carotenoids and newly it has been in essential request as a food additive and an antioxidant naturally. Lycopene also displayed powerful neurocurative agent, anti-proliferative and anti-inflammatory with preservation of normal cell metabolism, awareness improving characteristics, adjusting lipid metabolism in the blood. So, purpose of study was to explore the potential advantageous influences and the synergistic effect between CHNPs and Lycopene on D-gal using a rat model experiment.

MATERIALS AND METHODS

Chemicals: Pure chitosan (CH), lycopene (Ly) and D-galactose (D-gal) were brought from Sigma Aldrich Chemicals, while, the other chemical from Sisco Laboratory, India.

Preparation of chitosan nanoparticles (CHNPs): About 20 mg of chitosan was added to 40 mL of acetic acid. Then, 20 mL of 0.75 mg mL^{-1} sodium tripolyphosphate was quietly dropped drop by drop and continuous stirring. CHNPs were stored in water deionized. Supernatant was isolated and CHNPs was dried for forthcoming analysis[22].

Experimental location and duration: Experimental study was carried out at Biology Department, Faculty of Science,

Taif University, Saudi Arabia. The experimental duration (Treatment duration) was for 8 weeks at the end of July till the end of September, 2017.

Animal model: About 40 animals were obtained from King Fahad of Animal Research, King Abdul-Aziz University. Experiments were conducted by the NIH ethics for the Care and Handling of Laboratory Animals 8th edition. The experimental animals were healthy and adult male albino rats, weighing 200-250 g. They were kept under standard conditions with food *ad libitum*.

Experimental design: The animals were divided into nine groups (n = 10/group) as following: 1st control group was administrated distilled water. 2nd group was given D-gal at a dose 100 mg kg^{-1} for 8 weeks[23] as previous studies reported that chronic administration of D-galactose (D-gal) induces changes that mimic natural aging in animals[24]. The daily dose of D-galactose and route of exposure have been well known for induction of aging[25] and 3rd, 4th groups were treated with chitosan nanoparticles either low (CHNPs-LD) or high doses (CHNPs-HD) (140 and 280 mg kg^{-1}) respectively according to El-Denshary *et al.*[26] as they tested LD50 of chitosan nanoparticles and then determined these doses which didn't cause higher mortality in male rats. 5th group was administrated Lycopene (LY) (20 mg kg^{-1})[27]. 6th and 7th groups were administrated CHNPs-LD+Ly and CHNPs-HD+Ly, respectively. 8th and 9th groups were administrated D-gal plus CHNPs-LD plus Ly and D-gal plus CHNPs-HD plus Ly. All the groups were treated i.p for 60 successive days.

Determination of particles size and morphology of CNPs: Size and morphology of CHNPs was analyzed using Nanotrac analyzer 6H×4W×15D, Model-Nanotrac. CHNPs were slashed into various sizes pieces and eliminated with a thin gold palladium layer and the morphology of nanoparticles was constructed with Malvern Instruments Ltd.

Preparation of tissue homogenates for measurement of redox state: Liver tissues (0.25 g) were used for the oxidative stress analysis. Tissues were perfused with a 50 mM of sodium phosphate buffer saline (pH 7.4), 0.25 M sucrose and 0.1 mM (EDTA) and then added a protease inhibitor solution to protect enzymes which are sensitive to oxidation .

Oxidative and antioxidant biomarkers activity: The LPO level was estimated by using the method of Ohkawa *et al.*[28]. Superoxide dismutase (SOD) activity was estimated according to the method described by Marklund and Marklund[29] (Kits from Human Co., Germany). Catalase (CAT) was estimated according to the method of Aebi[30]. CAT is expressed as mmol mg^{-1}.

Glutathione (GSH) activity was estimated by Couri and Abdel-Rahman[31] (Kits from Human Co., Germany). Glutathione peroxidase (GPx) activity assayed by Hafeman *et al.*[32] (Kits from Human Co., Germany).

Myeloperoxidase (MPO) activity was estimated by Suzuki *et al.*[33]. Xanthine oxidase (XO) activity was determined by Litwack *et al.*[34]. Total thiol's level was assayed by Hu[35].

TNF-α and IL-6 activity in liver homogenates: The levels of cytokines (IL-6 and TNF-α) were estimated in tissue homogenates by using (ELISA) assay were obtained from Immuno-Biological Laboratories, USA.

Hepatorenal biomarkers determination: The total cholesterol (TC) and triglycerides (TG) levels were determined by the method of Carr *et al.*[36]. HDL-c will be determined according to the methods of Warnick *et al.*[37]. LDL-c level was calculated according to Friedewald[38]. The protein level was estimated by Bradford[39].

Liver function biomarkers: Serum AST and ALT were estimated according to the methods of Reitman and Frankel[40] ALP according to Choi *et al.*[41]. LDH as an indicator of damage will be determined by Vassault[42]. γ-GGT levels were determined with kits from Human Co., Germany and data is expressed as U L^{-1}.

Inflammation marker activity: CRP was estimated as by Wener *et al.*[43] using SEA821- ELISA Kit For C-reactive protein (CRP).

Histological and transmission electron microscopy (TEM) evaluation: Liver portion was fixed in 10% neutral buffer formalin and other histological processing Gabe[44]. For ultrastructural, the tissues were fixed in 2.5% glutaraldehyde for 48 h and processing was according to Weakley[45].

Single cell gel electrophoresis (SCGE) (Comet assay): (Liver) pieces of animal groups were placed into a small Petri dish with a solution ice-cold (Ca^{2+} and Mg^{2+} and 10% DMSO 20 mM and EDTA). The cell viability was examined by constructing the comet images after electrophoresis Endoh *et al.*[46]. The comet assay as previously described by Collins and Dunsinka[47].

Statistical analysis: Data are expressed as mean values±SE. Statistical analysis was performed using two-way analysis of variance (ANOVA) to assess significant differences among treatment groups. For each significant effect of treatment, the *post hoc* Tukey's test was used for comparisons. The criterion for statistical significance was set at $p<0.05$. All statistical analyses were performed using SPSS statistical version 20 software package (SPSS Inc., USA).

RESULTS

TEM of chitosan nanoparticles (CHNPs): The observed micrograph showed synthesized chitosan nanoparticles (CHNPs) in the spherical form (Fig. 1).

Hepatorenal biomarkers: ALT, AST, ALP, LDH and γ-GT were significantly increased in D-gal treated group. Administration of Ly and/or CHNPs-(LD), CHNPs-(HD) mitigated hepatic function increment in D-gal group. All these groups significantly decreased liver enzyme biomarkers as compared to D-gal treated group. Ly group elicited non-significant decrease in liver enzyme markers as compared to control group and this confirm Ly antioxidant capacities to be about normal values. The group treated with CHNP-s (HD) and Ly elicited the highly significant decrease in liver enzyme as compared to D-gal group and this confirmed the best synergistic effect between CHNP-s(HD) and Ly in alleviating D-gal hepatotoxicity (Table 1).

A remarkable decrease of protein levels was recorded in D-gal treated group as compared to control group. Concurrent administration of Ly with CHNPs-(LD) and/or CHNPs-(HD)to the rats ameliorated total protein levels and significantly increased its level as compared to D-gal-group. The group treated with CHNP-s (HD) and Ly afforded significant increase in total protein as compared to D-gal group with slight decrease when compared to control group, these finding confirmed the capacities of CHNPs and Ly in enhancing liver abilities of protein formation which means elevating liver whole viability (Table 2).

Triglycerides and total cholesterol levels were remarkably and significantly elevated in D-gal group, groups treated with CHNP-s(HD) and CHNP-s(LD) and Ly afforded non-significant decrease in total cholesterol and triglycerides levels when compared with control group. The best significant decrement in total cholesterol and triglycerides were recorded in group treated with D-gal and followed by CHNP-s(HD) and Ly as this group elicited significant decrease in total cholesterol and triglycerides when compared with D-gal group with significant increase when compared with control group. Meanwhile, there was significant decrease in HDl-c level with significant increase in LDL-c and v-LDL-c in D-gal group while these values were ameliorated greatly in group treated with combination of D-gal, CHNPs-(HD) and Ly as compared to D-gal treated group only. These values were non-significantly increased in groups treated with combination of either CHNPs-(LD), CHNPs- (HD), Ly and combined group of CHNPs-(LD) and Ly. (Table 2) This confirmed the abilities of chitosan nanoparticles and Ly in enhancing liver capacities in lipid metabolism and motivate breakdown of high cholesterol level and reducing triglycerides level and increasing HDL-c with reducing LDL-c levels.

Tumor necrosis marker and interleukin-6 level: There was elevation in TNF-α and IL-6 levels in D-gal treated group as compared to control group while there was significant decrease in their values in either CHNPs-(LD), CHNPs-(HD)

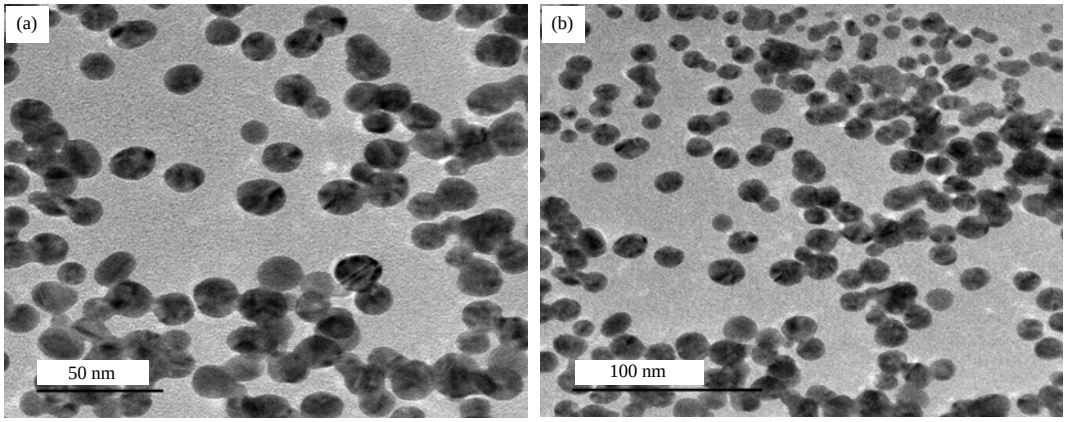

Fig. 1(a-b): TEM images of biosynthesized CHNPs

Table 1: Changes in hepatic and renal functions in male rats treated with D-galactose, chitosan (low and high dose) and/or lycopene on male rats

Parameters	Control	D-gal	CHNPs-LD	CHNPs-HD	Ly	CHNPs (LD)+Ly	CHNPs (HD)+Ly	D-gal+CHNPs (LD)+Ly	D-gal+CHNPs (HD)+Ly
ALT (U L^{-1})	14.33 ± 0.32^{e}	148.15 ± 6.62^{a}	16.36 ± 2.64^{d}	14.88 ± 10.39^{e}	13.25 ± 0.52^{fg}	12.36 ± 0.65^{g}	12.01 ± 1.36^{g}	45.65 ± 2.698^{b}	29.88 ± 2.39^{c}
AST (U L^{-1})	16.36 ± 1.36^{d}	213.95 ± 1.36^{a}	16.36 ± 4.69^{d}	14.69 ± 11.69^{e}	14.35 ± 1.32^{e}	13.02 ± 1.04^{f}	12.35 ± 0.25^{g}	48.39 ± 3.25^{b}	31.39 ± 4.25^{c}
ALP (U L^{-1})	33.59 ± 3.68^{e}	90.36 ± 4.23^{a}	35.98 ± 5.68^{d}	35.29 ± 8.69^{d}	33.35 ± 3.02^{e}	31.66 ± 3.02^{fg}	30.54 ± 2.05^{g}	50.36 ± 4.36^{b}	44.36 ± 5.36^{c}
LDH (U L^{-1})	103.56 ± 7.69^{e}	687.95 ± 9.36^{a}	112.69 ± 8.69^{d}	110.69 ± 9.65^{d}	103.25 ± 6.25^{e}	100.02 ± 3.65^{f}	102.03 ± 4.02^{e}	282.69 ± 6.39^{b}	220.98 ± 5.36^{c}
γ-GT (U L^{-1})	5.36 ± 1.02^{e}	12.20 ± 1.06^{a}	5.69 ± 2.36^{e}	6.02 ± 2.36^{d}	5.02 ± 0.59^{f}	4.36 ± 0.98^{g}	4.02 ± 0.56^{g}	8.98 ± 2.36^{b}	7.25 ± 1.69^{c}
Total proteins (g dL^{-1})	8.43 ± 1.36^{c}	4.68 ± 1.69^{f}	8.69 ± 1.63^{c}	8.32 ± 1.02^{c}	8.11 ± 1.69^{c}	9.02 ± 1.69^{b}	9.35 ± 0.45^{ab}	6.39 ± 1.69^{e}	7.35 ± 2.02^{d}

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, γ-GT: Gamma glutamyl transferase. D-gal: D-galactose, CHNPs (LD): Chitosan nanoparticles (Low dose), CHNPs (HD): Chitosan nanoparticles (High dose), LY: Lycopene. Values are expressed as Mean±SE, n = 10 for each treatment group. Symbols are arranged alphabetically indicate significance comparison as compared to control group and other treated groups from higher to lower ($p<0.05$)

Table 2: Changes in lipid profile in male rats treated with with D-galactose, chitosan (low and high dose) and/or lycopene on male rats

Parameters (mg dL^{-1})	Control	D-gal	CHNPs-LD	CHNPs-HD	Ly	CHNPs (LD)+Ly	CHNPs (HD)+Ly	D-gal+CHNPs (LD)+Ly	D-gal+CHNPs (HD)+Ly
Triglycerides (mg dL^{-1})	74.35 ± 4.36^{e}	169.28 ± 4.22^{a}	72.02 ± 4.25^{g}	77.32 ± 5.25^{d}	72.36 ± 4.36^{g}	73.03 ± 5.35^{f}	74.09 ± 5.03^{e}	128.06 ± 6.06^{c}	130.09 ± 9.03^{bc}
Total cholesterol (mg dL^{-1})	102.25 ± 7.03^{e}	365.69 ± 3.65^{a}	101.36 ± 3.65^{f}	104.36 ± 4.65^{de}	102.36 ± 5.66^{e}	98.96 ± 8.03^{gh}	92.96 ± 9.35^{h}	140.06 ± 6.03^{b}	120.03 ± 3.06^{c}
HDL-c	40.95 ± 5.36^{b}	29.03 ± 4.98^{d}	40.32 ± 4.36^{b}	40.65 ± 3.65^{b}	41.03 ± 2.65^{a}	28.06 ± 3.65^{d}	26.06 ± 2.69^{e}	37.06 ± 4.23^{c}	36.98 ± 3.02^{c}
LDL-c	31.06 ± 1.66^{e}	44.18 ± 2.36^{a}	32.02 ± 2.02^{de}	33.05 ± 2.14^{de}	31.02 ± 3.65^{e}	31.25 ± 3.65^{e}	33.95 ± 3.28^{c}	36.69 ± 3.25^{b}	35.15 ± 3.02^{b}
vLDL-c	14.80 ± 2.03^{f}	28.13 ± 2.06^{a}	14.40 ± 1.36^{f}	15.46 ± 1.65^{f}	14.47 ± 1.25^{f}	21.40 ± 4.36^{e}	22.61 ± 5.03^{de}	24.62 ± 3.06^{c}	25.01 ± 4.35^{bc}

HDL-c: High density lipoprotein, LDL-c: Low density lipoprotein, vLDL-c: Very low density lipoprotein, D-gal: D-galactose, CHNPs (LD): Chitosan nanoparticles (low dose), CHNPs (HD): Chitosan nanoparticles (High dose), LY: Lycopene. Values are expressed as Mean±SE, n = 10 for each treatment group. Symbols are arranged alphabetically indicate significance comparison as compared to control group and other treated groups from higher to lower ($p<0.05$)

Table 3: Changes in serum TNF-α and Il-6 level in male rats treated with D-galactose, chitosan (low and high dose) and/or lycopene on male rats

Groups	Tumor necrosis factor alpha (TNF-α) (Pg g^{-1}) tissue	Interleukin-6 (IL-6) (Pg g^{-1}) tissue
Control	5.08 ± 0.68^{c}	4.36 ± 1.02^{d}
D-gal	29.03 ± 2.03^{a}	23.06 ± 3.02^{a}
CHNPs-LD	4.35 ± 1.69^{d}	3.69 ± 0.28^{e}
CHNPs-HD	4.79 ± 2.15^{d}	3.02 ± 0.87^{e}
Ly	3.65 ± 1.02^{e}	3.03 ± 0.89^{e}
CHNPs (LD)+Ly	3.06 ± 0.36^{e}	3.02 ± 0.65^{e}
CHNPs (HD)+Ly	2.68 ± 0.32^{f}	2.65 ± 0.78^{f}
D-gal+CHNPs (LD)+Ly	8.03 ± 1.36^{b}	11.68 ± 2.12^{bc}
D-gal+CHNPs (HD)+Ly	7.32 ± 0.69^{b}	9.03 ± 1.36^{c}

TNF-α: Tumour necrosis factor Alpha IL-6:Interleukin-6. D-gal: D-galactose, CHNPs (LD): Chitosan nanoparticles (Low dose), CHNPs (HD): Chitosan nanoparticles (High dose), LY: Lycopene. Values are expressed as Mean±SE, n = 10 for each treatment group. Symbols are arranged alphabetically indicate significance comparison as compared to control group and other treated groups from higher to lower

and/or lycopene treated groups alone or combined with D-gal in comparable to control group and compared to D-gal group alone. Group treated with combination of D-gal, CHNP-s(HD) and Ly elicited highly significant decrease in both TNF-α and IL-6 levels as compared to D-gal treated group with slight significant increase when compared with control group (Table 3).

Oxidative stress biomarkers: SOD, CAT and Gpx levels were significantly decreased in D-gal treated group as compared to control group, while MDA level was significantly increased as compared to control group. SOD, GPx activity were non-significantly increased in CHNP-s (LD) treated group as compared to control group while groups treated with either CHNPs(HD), Ly, combined groups between CHNPs (LD and HD) and Ly afforded slight significant increase in SOD as compared to control group. The groups treated with D-gal and then followed by either CHNP-s(LD) and Ly and CHNP-s(HD) and Ly elicited significant decrease in SOD and GPx as compared to control group while induced significant increase as compared to D-gal treated group. The best treated group was CHNP-s(HD) combined with Ly after treatment with D-gal (Table 4).

CAT activity was significantly decreased in CHNP-s(LD) as compared to control group. While, CHNP-s (HD), Ly and combined groups of CHNP-s in two doses with Ly elicited non-significant increase in CAT activity as compared to control group. The group treated with D-gal and then followed by CHNP-s (HD) and Ly elicited slight decrease in CAT activity as compared to control group (Table 4).

MDA level induced highly significant increase in D-gal treated group as compared to control group. Meanwhile, this value was significantly decreased in CHNP-s (LD),CHNP-s (HD), Ly and combined groups between Ly and CHNP-s either LD or HD. The best treatment was recorded in group treated with

Table 4: Effect of D-galactose, chitosan nanoparticles, Lycopene each alone and their combination on antioxidant markers in liver homogenates in male rats

Groups	Super oxide dismutase (SOD) (U g^{-1})	Malondialdehyde (MDA) (U g^{-1})	Glutathione peroxidase (Gpx) (U g^{-1})	Catalase (CAT) (U g^{-1})
Control	20.13±1.17^{d}	5.39±0.05^{c}	14.13±0.36^{b}	8.05±1.03^{b}
D-gal	9.52±1.25^{g}	30.52±0.22^{a}	6.52±2.41^{g}	1.36±0.05^{f}
CHNPs-LD	20.74±1.07^{d}	4.11±0.24^{d}	14.25±1.47^{b}	7.65±1.36^{cd}
CHNPs-HD	21.03±1.23^{c}	3.53±0.12^{e}	13.47±0.36^{c}	8.23±1.54^{b}
Ly	21.85±1.05^{bc}	4.52±0.35^{d}	15.48±0.85^{ab}	8.22±2.03^{b}
CHNPs (LD)+Ly	22.75±1.35^{ab}	3.15±0.39^{e}	12.32±0.40^{de}	8.32±2.03^{b}
CHNPs (HD)+Ly	21.75±1.15^{bc}	3.01±0.52^{e}	13.65±1.36^{c}	9.21±2.36^{a}
D-gal+CHNPs (LD) +Ly	15.75±1.15^{f}	9.32±1.32^{b}	9.65±1.02^{f}	5.03±1.36^{e}
D-gal+CHNPs (HD)+Ly	18.52±2.98^{e}	5.23±1.36^{c}	11.03±0.68^{e}	7.03±1.36^{d}

SOD: Superoxide dismutase, MDA: Malondialdehyde, GPx: Glutathione peroxidase, CAT: Catalase, D-gal: D-galactose, CHNPs (LD): Chitosan nanoparticles (Low dose), CHNPs (HD): Chitosan nanoparticles (High dose), LY: Lycopene. Values are expressed as Mean±SE, n = 10 for each treatment group, symbols are arranged alphabetically indicate significance comparison as compared to control group and other treated groups from higher to lower ($p<0.05$)

Table 5: Changes in serum hepatic antioxidant enzymes and thiol level in male rats treated with D-galactose, chitosan (low and high dose) and/or lycopene on male rats

Parameters	Control	D-gal	CHNPs (LD)	CHNPs (HD)	Ly	CHNPs (LD)+Ly	CHNPs (HD)+Ly	D-gal+CHNPs (LD)+Ly	D-gal+CHNPs (HD)+Ly
Myeloperoxidase (MPO) (nmol min^{-1} mL^{-1})	14.36±1.03^{e}	30.66±3.62^{a}	15.06±1.36^{d}	14.36±2.30^{e}	15.06±2.36^{d}	14.03±1.25^{e}	14.85±1.01^{e}	20.85±2.36^{bc}	19.08±3.02^{c}
Xanthine oxidase (XO) (U g^{-1})	15.36±1.69^{f}	36.06±4.36^{a}	16.03±2.36^{e}	16.20±1.36^{e}	15.69±3.03^{f}	16.35±2.02^{e}	17.36±2.03^{d}	27.32±2.05^{bc}	25.89±3.08^{c}
Hepatic thiol (µmol g^{-1})	8.25±1.36^{b}	4.03±1.03^{e}	7.39±2.03^{c}	7.33±1.36^{c}	8.14±1.56^{b}	8.69±1.96^{a}	8.58±1.69^{a}	6.25±1.3^{d}	6.90±1.87^{d}

MPO: Myeloperoxidase, XO: Xanthine oxidase, D-gal: D-galactose, CHNPs (LD): Chitosan nanoparticles (Low dose), CHNPs (HD): Chitosan nanoparticles (High dose), LY: Lycopene. Values are expressed as Mean±SE, n = 10 for each treatment group. Symbols are arranged alphabetically indicate significance comparison as compared to control group and other treated groups from higher to lower ($p<0.05$)

D-gal and CHNP-s (HD) and Ly as it afforded non-significant decrease in MDA activity as compared to control group (Table 4).

The hepatocellular oxidative markers (MPO and XO) levels were significantly increased by D-gal after 30 days of treatment while thiol level was significantly decreased as compared to control group (Table 5). CHNP-s(LD) and Ly treated groups afforded slight increase in MPO and XO with slight decrease in thiol level as compared to control group. The groups treated with D-gal and followed by CHNP-s either low or high dose and Ly elicited significant increase in MPO and Xo with significant decrease in thiol level as compared to control group but afforded significant decrease as compared to D-gal treated group (Table 5).

Transmission electron microscope evaluation: Electron micrograph of liver section, CHNPs-(LD), CHNPs- (HD) and Ly groups. Control group showing the normal hepatocytes with euchromatic rounded nuclei (N) and the cytoplasm contained mitochondria with dense matrices, well-developed rough endoplasmic reticulum (R) and glycogen granules (G) and normal mitochondria with normal crista (M) (Fig. 2a). D-galactose treated group showed proved large vacuoles and areas of cytoplasmic organelles in their cytoplasm with severely pyknotic nuclei (green arrow) with irregular nuclear boundaries, with condensed endoplasmic reticulum, large vacuoles (G) with fatty changes (F) and appearance of fat droplets as well as mitochondria showed condensed matrix and inconspicuous cristae and appearance of red blood corpuscles as a sort of hemorrhage (black arrow) (Fig. 2b). Ly and/or CHNPs-(LD) and/or CHNPs-(HD) treated groups had few vacuoles in their cytoplasm with normal nuclei (N) and normal mitochondria (M) with normal cristae. CHNPs-(HD) in combination with Ly showed restoration of normal nuclei(N) with regular boundaries and restoration of endoplasmic reticulum (ER) and normal mitochondria (M) (Fig. 2c-g). D-gal combined with either CHNPs-(LD) and Ly or CHNPs-(HD) and Ly groups showed condensed red blood cells with appearance of condensed area of hemorrhage (H) and large vacuole (V) and showing restoration of nuclear membrane and nucleus (N) with partial pyknosis (PK) and mitochondria (M) (Fig. 2h, i).

The best treated group was the last group in which rats treated with D-gal and then treated with combination of CHNPs-(HD) and Ly and the lowest effect was recorded treatment of D-gal group with CHNPS-(LD) only without combination.

Histopathology evaluation: Representative light micrographs of Fig. 3a, the histology of the hepatic tissues of the control group was normal in appearance of central vein (CV) and

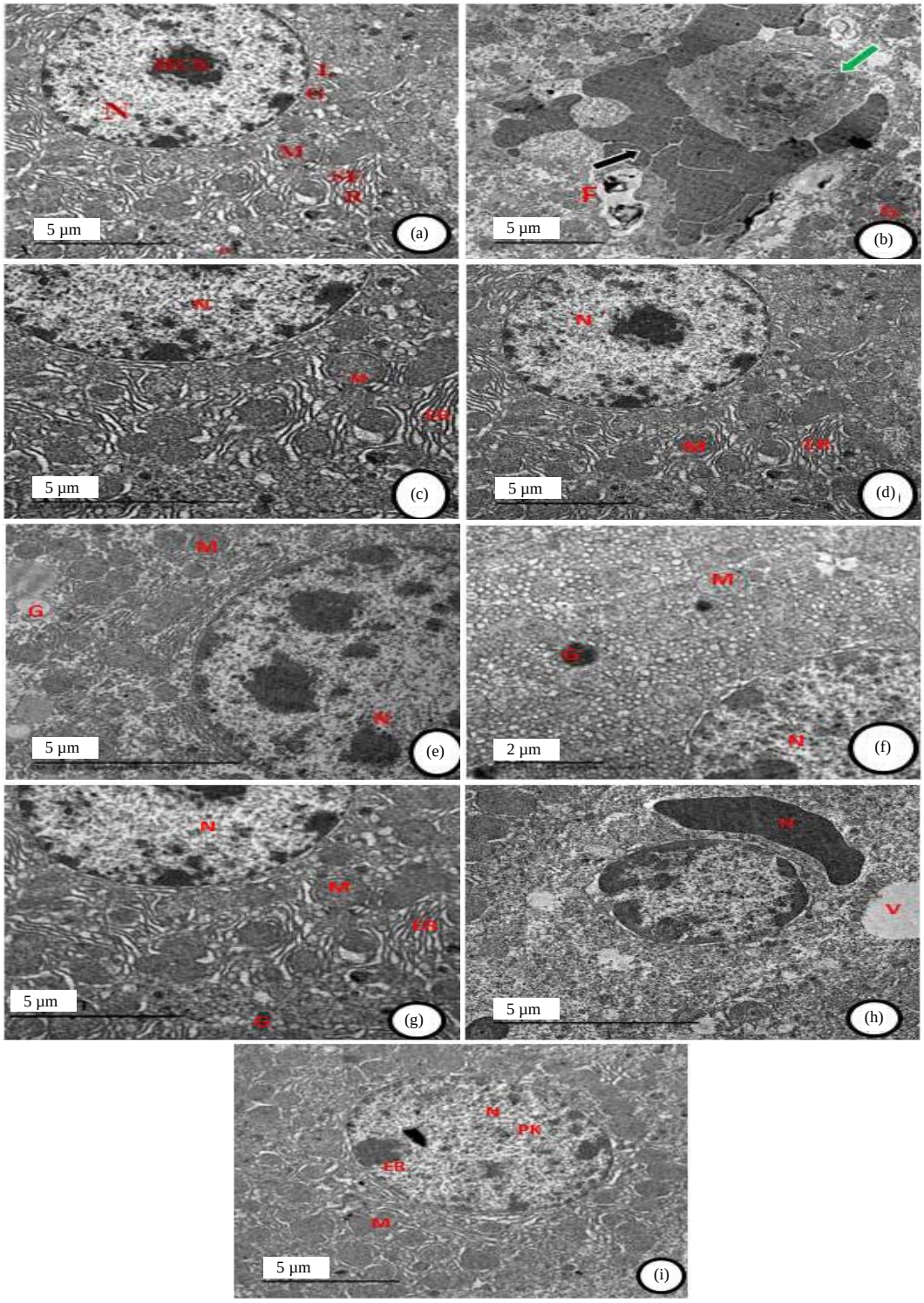

Fig. 2(a-i): Electron micrograph of liver section, CHNPs-(LD), CHNPs-(HD) and Ly groups. (a) Control group showing the normal hepatocytes (5 µm). (b) D-galactose treated group showed proved large vacuoles with severely pyknotic nuclei (5 µm). (c-g) Ly and/or CHNPs-(LD) and/or CHNPs-(HD) treated groups had few vacuoles in their cytoplasm with normal nuclei. CHNPs-(HD) in combination with Ly showed restoration of normal nuclei with regular boundaries (5 and 2 µm). (h and i) D-gal combined with either CHNPs-(LD) and Ly or CHNPs-(HD) and Ly groups showed condensed red blood cells with appearance of condensed area of hemorrhage (5 µm)

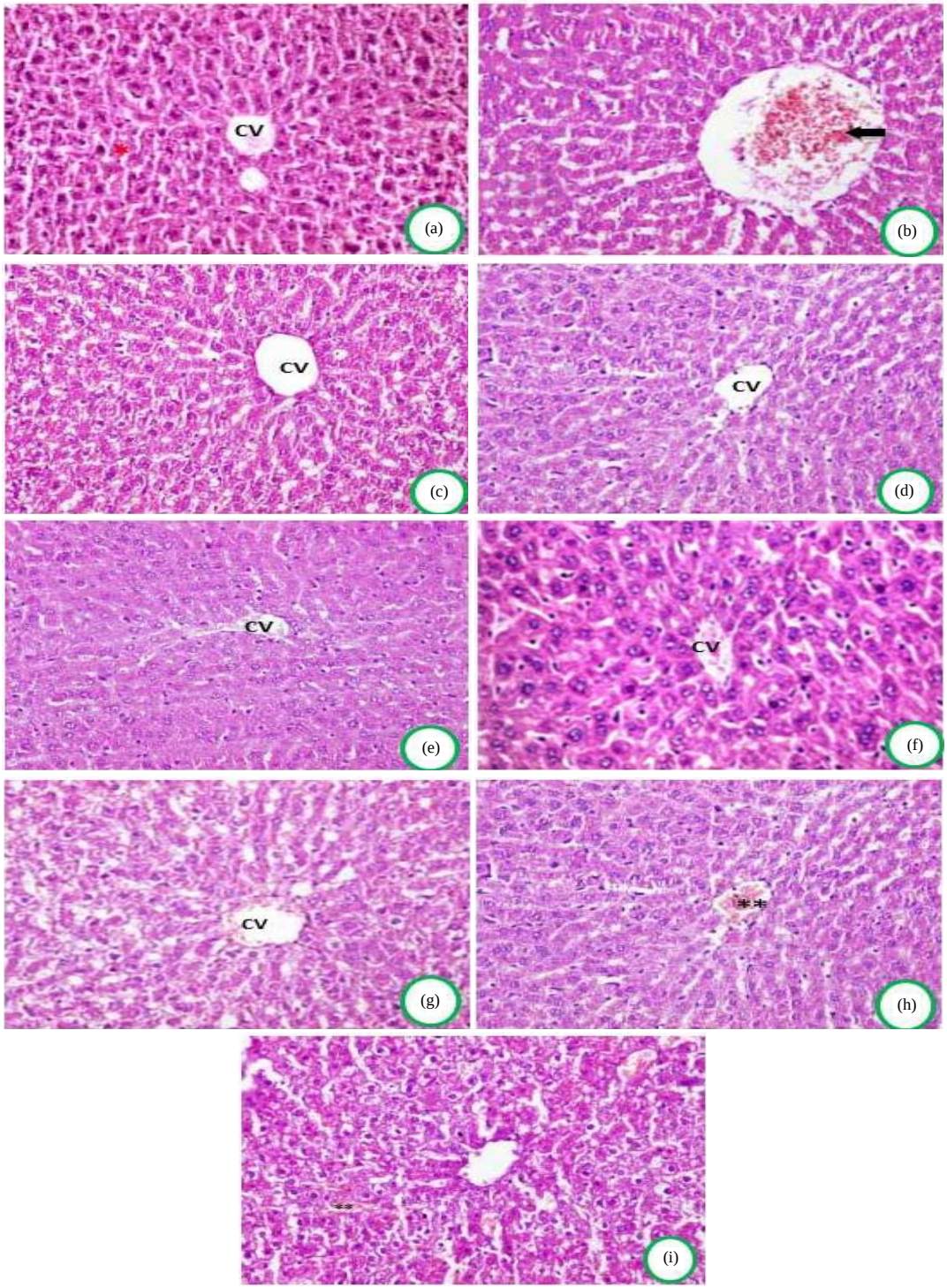

Fig. 3(a-i): Representative light micrographs of (a) The histology of the hepatic tissues of the control group was normal in appearance (400X). (b) D-gal treated group showed hepatic tissues with large congested central vein (400X). (c-f) Groups treated with either Ly CHNPs-(LD), CHNPs-(HD) and/or CHNPs-(LD) or CHNPs-(HD) combined with Ly, all these groups showed normal hepatic tissues(400X). (g) Group treated with combinations of either CHNPs-(HD) and Ly showing normal hepatic structures. (h) Group treated with CHNPs- (LD) and Ly with D-gal showed restoration of almost normal histological structures (400X) and (i) Group treated with D-gal combined with CHNPs-(HD) showing the best restoration of normal structures, CHNPs-(HD) and Lycopene except very small congested hepatocytes (400X)

hepatic lobule (*) (400X) (Fig. 3a). D-gal treated group showed hepatic tissues with large congested central vein (black arrow) with appearance of hydropic degeneration (Fatty change) (400X) (Fig. 3b). Groups treated with either Ly CHNPs-(LD), CHNPs-(HD) and/or CHNPs-(LD) or CHNPs-(HD) combined with Ly, all these groups showed normal hepatic tissues with normal cords of hepatocytes and normal central vein (CV) (400X) (Fig. 3c-f). Group treated with combinations of either CHNPs-(HD) and Ly showing normal hepatic structures with normal central vein (CV) (Fig. 3g). Group treated with CHNPs- (LD) and Ly with D-gal showed restoration of almost normal histological structures with appearance of small congested central vein with appearance of small congested central vein (**) (400X) (Fig. 3h). Group treated with D-gal combined with CHNPs-(HD) showing the best restoration of normal structures was recorded in the group treated with combination of D-gal, CHNPs-(HD) and Lycopene except very small congested hepatocytes (**) (400X) (Fig. 3i). The best histological picture was recorded in the last group in which rats treated with D-gal and then treated with combination of CHNPs-(HD) and Ly and the lowest effect was recorded treatment of D-gal group with CHNPS-(LD) only without combination.

Comet assay: Comet images of cells derived from liver of control group which showed intact nuclei and normal round cell without a tail (Fig. 4a). D-gal group showed a high degree of damage with the appearance of more than one apoptotic cells with a large tail and small head and the relaxed loops of damaged DNA extend to the anode to form a comet-shaped structure (Fig. 4b). The Lycopene, CHNPs-(LD),CHNPs-(HD) each alone showed more percent of intact cells with undamaged DNA and fewer numbers of comet cell (Fig. 4c). Combined groups CHNPs-(LD) with Ly and CHNPs-(HD) with Ly treated groups showed intact nuclei with undamaged DNA in a supercoiled state (Fig. 4d-g). D-gal combined with CHNPs-(LD) and D-gal+CHNPs-(HD) and/or lycopene showed amelioration of the hepatic cells as recorded fewer parameters in the tail length and % of damaged DNA and tail (Fig. 4h and i).

DISCUSSION

Liver injury was motivated by using of D-galactose at for 8 weeks. The results showed an elevation in the oxidative damage and antioxidant status decrement in D-galactose treated rats. Chitosan nanoparticles in two doses either high or low dose (CHNPs-LD and HD) and lycopene (Ly) treatment resulted in declined levels of all markers of oxidative status, interleukins, TNF-α while the enzymatic/non-enzymatic antioxidant markers were remarkably elevated. Thus, the study revealed that chitosan nanoparticles in two doses either high or low dose (CHNPs-LD and HD) and lycopene (Ly) displays the protection against the hepatic injury caused by D-galactose which is the main principle of inducing ageing in experimental animals.

The chitosan characterization revealed that it is with lower molecular weight. This resulted in facilitation of the coupling process with other molecules such as lycopene. The *in vivo* study proved that the protective role of chitosan nanoparticle alone or in combination with lycopene against oxidative stress and hepatotoxicity induced by D-galactose in rat model was evaluated.

The treatment with D-galactose afforded severe hepatic damage and oxidative injury as well as histological changes as those reported in literature. Animals treated with D-gal showed a remarkable increment in liver function enzymes (AST, ALT and ALP) and tumor markers (TNF-α) and oxidative markers (MDA) joined by a remarkable decline in enzymes of antioxidant capacities (GPx, SOD and catalase) in liver tissue.

Elevation of ALT and AST reflects hepatocyte injuries while elevation of ALP reflects cholestasis[48] which is in agreement with the obtained results. The increased levels of ALT, AST, ALP reported in the present study in D-gal group indicated severe hepatic cells.

The increase in hepatic MDA level showed in the present study in D-gal treated group proved the elevation in LP resulting in tissue injury and impairment of the antioxidant defense mechanisms to prohibit the excessive free radicals formation[49]. The results of the present study were further confirmed by the histopathological and transmission electron microscopy studies which revealed that liver tissue more or less have remarkable alterations in the tissue.

There is a problem in the water-insolubility property of chitosan, nanoparticle formulation supplies a responsible pharmaceutical basis for improving oral bioavailability and therapeutic efficiency of chitosan and other drugs that are poorly soluble[16]. Additionally, nanoparticles own a stronger curvature of the surface and this triggers more dissolution pressure[17]. The increased saturation solubility, eventually, chitosan nanoparticles (CHNPs) could manifest more superior activities than chitosan large particles due to their small size. Not wonderfully, CHNPs have enhanced immune capacity and anticancer activity than those of CS[50].

Other biological activities such as the antioxidant capacity of CHNPs received the less attention. D-galactose plus CHNPs groups at the two tested doses revealed remarkable amelioration in the liver toxicity biomarkers, antioxidant

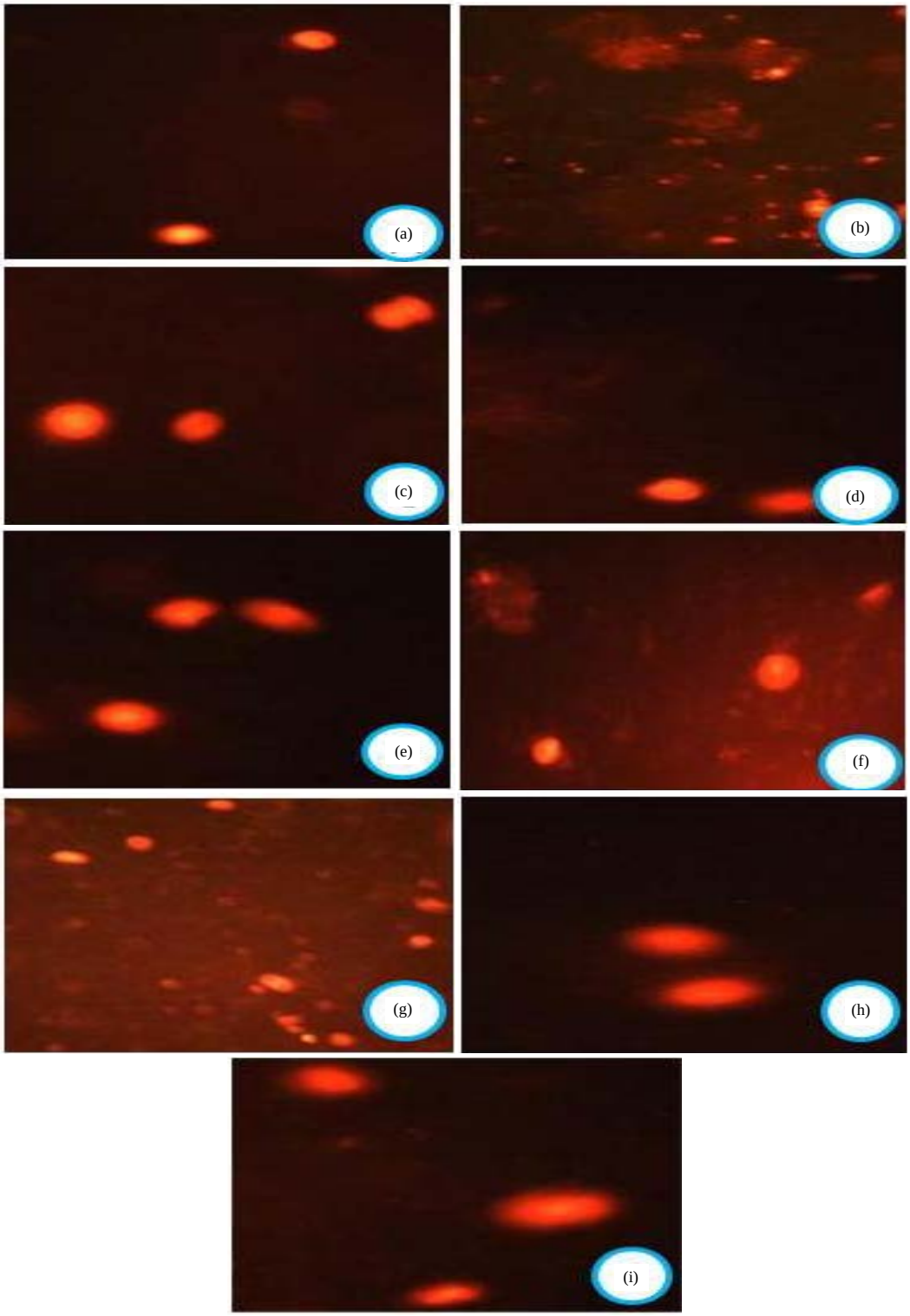

Fig. 4(a-i): Comet images of (a) Cells derived from liver of control group which showed intact nuclei, (b) D-gal group showed a high degree of damage, (c) The Lycopene, CHNPs-(LD),CHNPs-(HD) each alone showed more percent of intact, (d-g) Combined groups CHNPs-(LD) with Ly and CHNPs-(HD) with Ly treated groups showed intact nuclei and (h and i) D-gal combined with CHNPs-(LD) and D-gal +CHNPs- (HD) and/or lycopene showed amelioration of the hepatic cells

enzymes and markers of tumor as well improvement in the liver histology as well as TEM and comet assay sections. These findings are in harmony with the findings of Wen *et al.*[51], who revealed a CHNPs protective role with a diameter of 83.66 nm against H_2O_2-induced RAW-264.7 cell injury through returning the endogenous antioxidants activities (SOD, GPx and CAT), along with improvement of their gene expression.

The obtained results confirmed the antioxidant capacity of CHNPs as it reduced MDA level which is the marker of lipid peroxidation, this finding is differed from results obtained by Wen *et al.*[51], who used chitosan nanoparticles to pretreat cells before hydrogen peroxide treatment and hydrogen peroxide was used to induce oxidative injury, the uptake of chitosan nanoparticles and hydrogen peroxide were not simultaneous and chitosan nanoparticles did not affect the uptake of hydrogen peroxide.

Recently, there is no scientific report has been assayed to describe the protective role of CHNPs alone or combined with Lycopene (Ly) against D-galactose (D-gal) elicited hepatotoxicity and liver tissues oxidative stress, the hepatoprotective effect of chitosan has been authenticated in several reports, for instance, Jeon *et al.*[52] reported the chitosan antioxidant effect on carbon tetra chloride-induced liver damage in rats and showed that chitosan has great antioxidative effects, which decrease lipid peroxides production and elevate antioxidant enzyme activities as SOD and CAT during lipid peroxidation by CCl_4.

Using of lycopene in a dose (20 mg kg^{-1}) reduced oxidative stress and enhanced antioxidant enzymes activity and this is reinforced by previous studies by Gao *et al.*[53], who confirmed that purified lycopene has been shown to exert beneficial effects on biomarkers of oxidative stress, particularly in decreasing DNA oxidative damage. Their rationale for dose selection (6.5, 15, or 30 mg lycopene) is based on previous studies, which have shown that lycopene supplementation at these doses, present in tomato products, is bioavailable, as well as protective against oxidative stress[54,55].

Oxidative stress-induced apoptosis also plays a crucial role in D-galactose-induced brain neurotoxicity in rodents. The expression of caspase-3 increased and Bcl-2 expression decreased in D-galactose-treated rat brains[56].

The obtained data demonstrated that D-galactose partially damaged normal hepatocytes morphological features and D-galactose-induced aging. However, CHNPs and Ly treatment restored cell morphology and architecture and reduced hepatic apoptosis. These data suggest that CHNPs and lycopene protected the liver against D-galactose-induced apoptosis damage.

In the same pattern, Subhapradha *et al.*[57] reported the hepatoprotective effect of β-Chitosan against carbon chloride-induced oxidative damage in rats. They showed that, additionally to normalizing the oxidative injury markers, which is assigned to the antioxidant characteristics of chitosan and restoration of plasma AST and ALT indicates that chitosan may preserve the cell membrane and prevent an infiltration of hepatic enzymes into the blood. Thus, the hepatoprotective effect of chitosan is due to a avoidance of free radicals by its antioxidant capacity and its capability to inhibit lipid accumulation by its antilipidemic characters[58]. Santhosh *et al.*[59] showed that co-treatment with chitosan may prevent hepatotoxicity induced by some drugs in rats.

Moreover, chitosan was demonstrated to be hepatoprotective against oxidative injury elicited by radiotherapy. Treatment with lycopene and CHNPs at two doses was effective to enhance the antioxidant property of the body and decline the oxidative stress by the elevation of antioxidant enzymes (CAT, SOD and GPx) and decrement of MDA level. Treatment with lycopene plus CHNPs to rats treated with D-galactose (D-gal) could markedly suppress the high serum level of liver biochemical parameters (ALT, AST and ALP) as well as decrease the elevated tumor markers levels (AFP and CEA). Moreover Mohamed[60] reported that administration of chitosan to rats prior and post gamma radiation improved the tested parameters so Mohamed[60] proved the same action of CHNP-s in the current study as it is a therapeutic alternative for oxidative stress, hyperlipidaemia and hormonal changes. Parallel to obtained results, chitosan may be contributed to the prevention of oxidative stress consequences.

The obtained results are greatly reinforced by Jiang *et al.*[27], who reported that the levels of liver antioxidant activities of SOD and GSH due to the oxidative stress exhibited in the NAFLD. SOD and GSH are able to scavenge the lipid hydroperoxides, lipid peroxide radicals and other products which are toxic metabolites of NAFLD. They were greatly increased in lycopene treated group as compared to NAFLD treated group[61].

Lycopene is a natural pigment, synthesized by plants and microorganisms. Red fruits and vegetables are the most common sources of lycopene, which exhibits the highest antioxidant activity among all dietary carotenoids. Therefore, nowadays, the essential role of Lycopene is beginning to be recognized and the most important health benefits through their ability to protect against oxidative damage and this supported with the current results[62].

Recent studies have reported that lycopene is an effective antioxidant and radical scavenger. Lycopene is the most potent aggregator of singlet oxygen among natural carotenoids, due to its conjugated dienes high number and recently, they have shown that lycopene is active two times as β-carotene in protecting lymphocytes for NO_2. Radical-induced membrane damage, which indicates that lycopene is the most scavenger of ROS among other essential carotenoids. Additionally, lycopene was shown to protect human against oxidative injury thus, based on the benefits of lycopene[63].

In agreement with the current results, it was reported that the liver biomarkers ALT, AST, TG and TC were significantly elevated in NAFLD groups, the levels of LDL-C in liver were elevated significantly and HDL-C was significantly declined in rats with high fatty liver. Pretreatment with Lycopene showed that Lycopene is able to prohibit the increased changes in ALT and AST, to decline the TG, TC and LDL-C levels and to elevate the HDL-C level. In addition, the histopathological changes correlated with the examination of liver viability[27].

Reinforcing the obtained results, the hepatic necrosis, degeneration, infiltrating lymphocytes and fatty change were spotted in NAFLD group. Treatment with lycopene alleviated these histopathological changes induced with HFD. Thus, these results suggested that the suppression of the liver function markers elevations, liver damage and lipid-lowering may depend on the protective impact of lycopene against HFD. Moreover, lycopene improved SOD level, increased GSH activities and declined MDA level against the high fatty liver, suggesting that the activity of antioxidants may have a part in the hepatoprotective effects mechanism[27].

TNF-α is a proinflammatory cytokine, which is related to a pathological and physiological circumstances, including cytotoxicity and immune-modulation activity. TNF-α is triggered by the monocyte lineage in liver. Furthermore, the current studies have reported that TNF-α inhibition decrease the hepatic fatty storage content[64].

Histological investigation showed that D-gal treated group showed hepatic tissues with large congested central vein with appearance of hydropic degeneration and these changes were reduced after treatment with chitosan nanoparticles alone or combined with lycopene, the best treatment group was shown in group treated with combination of chitosan nanoparticles (high dose) with lycopene as hepatic tissues showed restoration of normal structures. The same finding was obtained in TEM examination as combination of chitosan nanoparticles in high dose with lycopene alleviated hepatotoxicity of D-gal with condensed area of hemorrhage and appearance of normal nuclear membrane, this confirmed the capacities of chitosan nanoparticles and lycopene combined in reducing oxidative stress markers and previous studies[65-67] confirmed that antioxidant capacities of some active compounds could alleviates toxicity of compound in hepatic tissues.

CONCLUSION

The obtained findings revealed that D-galactose induced highly oxidative stress in hepatic tissues and alter its biochemical markers with elevating level of tumour necrosis factor and changed hepatic lobules structures and using of chitosan nanoparticles either in low or high dose with lycopene have highly synergistic effect against oxidative stress induced by D-galactose and act as hepatoprotective and antioxidant agents. Also they enhanced the hepatic antioxidant enzymes and ameliorated liver biomarkers and protect DNA against any damage and thus diminishing genotoxicity.

SIGNIFICANCE STATEMENT

This study discovers the hepatoprotective effects of chitosan nanoparticles and lycopene against oxidative stress and hepatotoxicity induced by D-galactose and found that Chitosan nanoparticles combined with lycopene alleviated hepatotoxicity of D-galactose and restored hepatic tissues structure to a great extent. This would help the researchers in evaluating the detail mechanism of chitosan nanoparticle and lycopene against aging. Also using this combination in drug will protect against ageing in people and improve their hepatic structure and biomarkers with enhancing antioxidant capacities.

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