8. Abstract

The present study aims to evaluate the hepatoprotective and antioxidant effects of gold nanoparticles (GNaP) biosynthesized through the mediation of *Azolla microphylla* and *Azolla microphylla* extract on acetaminophen-induced hepatocyte damage in common carp fish (*Cyprinus carpio* L.). The gold nanoparticles (100, 150, 200 µg/ml) and *Azolla microphylla* extract powder (100, 200, 400 µg/ml) were added to the primary hepatocytes in different conditions; treatment-I (before 12 mM acetaminophen), treatment-II (after 12 mM acetaminophen) and treatment-III (both before-and after-12 mM acetaminophen) and incubated. Among these, control group treated with 12 mM acetaminophen produced significantly elevated levels (50-80%) of lactate dehydrogenase (LDH), catalase (CAT), glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT), malondialdehyde (MDA) and significantly decreased the levels (60-75%) of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Treatment with methanol extract of *Azolla microphylla* phytochemically biosynthesized gold nanoparticles (100, 150, 200 µg/ml) and *Azolla microphylla* methanol extract powder (100, 200, 400 µg/ml) significantly improved the viability of cells in a culture medium. It also significantly reduced the levels of lactate dehydrogenase (LDH), catalase (CAT), glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT), malondialdehyde (MDA), and significantly increased the levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

In conclusion, gold nanoparticles biosynthesized through *Azolla microphylla* demonstrated effective hepatoprotective and antioxidant effects than methanol extract of *Azolla microphylla*.

8.1. Introduction

Liver is the vital organ which regulates many metabolic functions and plays the major role in clearance and transformation of chemicals exposes it to toxic injury [1]. Liver injury or liver dysfunction is the most serious ailment and are mainly caused by excess consumption of alcohol, high doses of acetaminophen, chemotherapeutic agents, hepatitis viral infection, dantrolene sodium, valporic acid, peroxidised oil and isonicotinic acid hydrazide, etc [20]. Recently, liver injury has become one of the major problems in aquaculture; many farms...
have been suffering from “liver and gall syndrome”, with the symptoms of liver enlargement (up to two fold of original size) and colour change. The exact causes of this disease are not clear; pathogenic bacteria or viruses have not been identified. Xenobiotic challenge due to drug abuse and environmental pollution may be one of the most important causes of the disease. To prevent and control fish diseases, a large quantity of antibiotics and chemicals have been added into the water environment and feeds, which may, in turn, cause problems to fish. However, no effective methods have been reported for the treatment of “liver and gall syndrome”. Hence much attention has been focused on the use of medicinal
plants and plant based drugs to prevent and control this disease [3]. It has been reported that, among the variety of drugs, acetaminophen (APAP or N-acetyl-p-aminophenol or Paracetamol or 4-hydroxyacetanilide) is the most common cause of drug-induced liver injury. APAP is a non-steroidal anti-inflammatory drug (NSAID), most widely used over-the-counter analgesic and antipyretic drug with few side effects when taken in therapeutic doses. At higher doses (3 h following 500 mg paracetamol/kg body wt), APAP causes centrilobular hepatic necrosis or massive hepatic necrosis and nephrotoxicity in both humans and animals [4-6]. As a result, the U.S. Food and Drug Administration (FDA) has recently recommended a stronger warning on the acetaminophen label. However, nephrotoxicity is less common than hepatotoxicity in acetaminophen overdose. The occurrence of nephrotoxicity causes renal tubular damage and acute renal failure in more severely poisoned patients and is often observed in those who developed major liver injury [7]. This hepatotoxicity depends on the extent of acetaminophen bio-activation to the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI) by CYP450 and it is efficiently detoxified by glutathione (GSH), which is an important cellular non-enzymatic antioxidant for detoxification of drugs and foreign chemicals [8-10]. After an overdose of acetaminophen, increased amount of toxic NAPQI metabolites are generated, which extensively deplete hepatocellular GSH and covalently binds to cysteine groups on protein, forms acetaminophen-protein adducts which results in hepatocytes lysis or necrosis [11, 12]. In addition serum levels of many biochemical parameters like lactate dehydrogenase (LDH), catalase (CAT), glutamate oxalate transaminase (GOT), glutamate pyruvate transaminase (GPT), bilirubin, and malondialdehyde (MDA) were elevated. Several animal experiments have been used to evaluate hepatoprotective and antioxidant effects against the classical hepatotoxicants, namely carbon tetrachloride (CCL4), acetaminophen (APAP), and D-galactosamine [13]. Hepatotoxicants metabolized within hepatic cells via CYP450 enzyme system to generate toxic intermediates results in hepatic necrosis. By means of in vivo studies, a large number of samples cannot be evaluated easily, for which isolated liver cells are the system of choice.
Cell culture system provides opportunity to study toxic mechanisms in isolation from the multiple physiological systems that regulate normal *in vivo* activity [14, 15]. Furthermore, *in vitro* models such as cell culture system have the potential to significantly reduce the number of animals sacrificed for toxicological research [16]. The use of fish hepatocyte culture allows the evaluation of hepatoprotective and antioxidant potential of biosynthesized gold nanoparticles using methanol extract of *Azolla microphylla*. Azolla is a genus of small aquatic fern that is found in the temperate and tropical regions of the world. *Azolla microphylla* is one of the species from the genus Azolla, it is a pteridophyte plantae belonging to the Salvinacea family [17]. The phytochemical investigation on the *Azolla microphylla* showed the presence of tannins, polyphenols, sugar, anthroquinone glycosides and steroids [18]. Polyphenols has long been known to exhibit strong antiproliferative, hepatoprotective and free radical scavenger [19]. Recently this research group has isolated and purified rutin and quercetin from *Azolla microphylla* for the first time [20]. Therefore *Azolla microphylla* is one of the important sources for various antioxidants and hepatoprotective agents. Researchers in the field of nanotechnology are gaining new insights into its versatile applications in the treatment of various diseases, including hepatotoxicity. Research into the preparation and biological applicability of noble metal nanoparticles with a nearly monodispersed size distribution and arbitrarily variable size and geometry has attracted considerable research interest. It is reported that metal nanoparticles, especially gold nanoparticles have drawn more attention of scientists because of their unique nature of stability, oxidation resistance and biocompatibility [21, 22]. Gold nanoparticles widely applied in the field of medicine such as chemical biosensing, imaging, drug delivery and therapeutic labeling [23]. Gold nanoparticles have a growing role in medical biotechnology. Production of nanoparticles can be achieved mainly through chemical, physical, and biological methods. Biological methods for nanoparticles synthesis using microorganisms, enzymes and plants or plant extracts have been suggested as possible ecofriendly alternatives to chemical and physical methods. However, major drawback of using microbes such as bacteria, fungi and yeast are the requirement of
manipulation of reaction parameters such as pH, temperature and incubation time for the synthesis of nanoparticles [24]. On the other hand, due to high rate of generation of nanoparticles, plant based synthesis is comparatively simpler and more cost-effective [25]. Plant phytochemicals such as polyphenols, flavonoids, triterpenes, tannins, glycosides, vitamins, proteins and steroids are capable of reducing the gold ions (Au$^{3+}$ or Au$^{1+}$) to neutral gold nanoparticles (Au$^{0}$) and ensures good control over size distribution and crystallinity of the nanoparticles [26]. Therefore, there is a growing interest in the field of plant mediated synthesis of nanoparticles. Biologically synthesized and functionalized, gold nanoparticles provide many desirable attributes for use as carriers in drug delivery systems as the functionalized gold nanoparticles core is essentially inert and nontoxic. Another essential aspect while working with gold nanoparticles is safety and biocompatibility (Gold nanoparticles is already approved by the US Food and Drug Administration). Roy at al. (2012) evaluated the Andrographolide (bioactive component of the medicinal plant Andrographis paniculata) loaded nanoparticles for the paracetamol induced hepatotoxicity in mice [27]. Various extracts of Swertia species 2001 [28], Azadirachta indica leaf extract [29], Trianthema portulacastrum L extract [30] and Capparis spinosa aqueous extract [31] were shown to have antioxidant and hepatoprotective effects in animals and monolayer cultures of hepatocytes with experimentally induced hepatotoxicity. From the results of the different investigations mentioned above, it is evident that plants acts as a natural reservoir for medicinal agents and avoid the common side effects of synthetic chemicals. Therefore, the present study aims to evaluate the effects of Azolla microphylla methanol extract gold nanoparticles on hepatoprotective and antioxidant effects of acetaminophen-induced hepatocytes damage in common carp (Cyprinus carpio L.) fish.
8.2. Materials and Methods

8.2.1. Materials

Acetaminophen, gentamycin sulfate, streptomycin and penicillin were received as gift samples from Pharma-fabricon pharmaceuticals Ltd, Madurai, India. Leibovitz’s-15 medium (L-15), ethylenediaminetetra acetic acid (EDTA), hydrogen tetracholoroaureate (III) hydrate H\textsubscript{Au}Cl\textsubscript{4}.3H\textsubscript{2}O (99.9%), tryphan blue, N-2-hydroxyethylpiperazine-N’-2’ethane sulfonic acid (HEPES), insulin, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) and fetal bovine serum (FBS) were purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India. 0.25% Trypsin and 24-well, cell culture plates were procured from Gibco Company (Canada). 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA) obtained from Sigma-Aldrich, MO, USA. LDH, CAT, GPT, GOT and GSH-Px spectrophotometric assay kits were purchased from Crest biosystems (Div of Coral Clinical systems), Goa, India. SOD, MDA spectrophotometric assay kits were obtained from Qualigens diagnostics, Pvt. Ltd, Mumbai, India. All other chemicals and solvents used in this study were of analytical grade and obtained from Merck, Mumbai, India.

8.2.2. Plant collection and preparation of Azolla microphylla extract

\textit{Azolla microphylla} fern were received from Vivekananda Kendra-NARDEP (Natural Resources Development Project), Vivekanandapuram, Kanyakumari, India. The whole parts of the plant were washed with tap water, rinsed with distilled water and air-dried under shade with good ventilation at room temperature (≈37°C) for a week. The fine powder (≈60 mesh size) was prepared from dried plants using kitchen blender (Bajaj
electronics Ltd, India) and was sterilized at 121°C at 15 psi pressure for 15 min. About 5 g of *Azolla microphylla* powder was weighed and macerated with 50 ml of 99.9% methanol in a 250 ml conical flask and kept at room temperature for 72 h. After 72 h of maceration, the methanol extract of *Azolla microphylla* was filtered with Whatman No: 1 filter paper. The filtered extract was centrifuged at 10000 rpm for 10 min at 4°C, supernatant was collected in a brown bottle and was stored at 4°C for further studies.

### 8.2.3. Green synthesis of gold nanoparticles

1 mM solution of hydrogen tetracholoroaureate (III) hydrate (HAuCl₄·3H₂O) was prepared using de-ionized water. 5 ml (5% v/v with water) of methanolic extract of *Azolla microphylla* was mixed with 25 ml of aqueous solution of HAuCl₄·3H₂O (1 mM). The mixture was left on constant magnetic stirring at room temperature (≈35°C) for 30 min and observed for change in colour. The bio-reduction of gold ions (Au¹⁺ or Au³⁺) to neutral ions (Au⁰) in the solution was monitored and the methanol extract was adjusted using de-ionized water.

### 8.2.4. Characterization of nanoparticles

Characterization of the biosynthesized gold nanoparticles was carried out before starting the experiment for their hepatoprotective and antioxidative effects in primary hepatocytes. Hydrogen tetracholoroaureate (III) hydrate (aqueous) treated methanol extract of *Azolla microphylla* was centrifuged at 10000 rpm for 15 min at 4°C, supernatant solution was collected and maintained at -80°C for 24 h and then freeze dried in a lyophilizer (Christ Gefriertoncknungsanlagen GmbH Model 1-4) for 48 h. Molecular size and shape of the lyophilized gold nanoparticles were characterized using FESEMEDX and HRTEM analyses. The field emission scanning electron microscopy (FESEM) analysis was performed using JEOL JSM-6700 electron microscope. For SEM imaging study, sample of a solution (gold nanoparticles re-dispersed with 1ml of de-ionized water) was placed on a carbon strip attached to SEM brass, extra solution was wiped using blotting paper and then allowed to dry by putting it under a mercury lamp for 5 min.
Morphological details of biosynthesized gold nanoparticles were revealed under transmission electron microscope (TEM-JEOL model 2100 instrument operated at an acceleration voltage of 200 kV). For TEM analysis, gold nanoparticles were re-dispersed with 1ml de-ionized water. Few drops of dispersed gold nanoparticles were placed over the carbon coated copper grid and air-dried at 60°C for 5 min. The elemental composition of gold nanoparticles were obtained using Dispersive X-ray analysis (JEOL JSM-6700) at variable pressure scanning electron microscope equipped with INCA X-sight Oxford instrument facility, at an acceleration voltage of 20 keV.

8.2.5. Sample processing for analyses

The obtained methanol extract was concentrated under reduced pressure and freeze dried using lyophilizer (Christ Gefriertrocknungsanlagen GmbH Model 1–4). The freeze dried extract obtained (200 mg) was then diluted with Leibovitz’s-15 culture medium (L-15) to obtain solutions equivalent to 100, 200 and 400 µg/ml that were used for experimental study. The lyophilized powder of the gold nanoparticles was diluted with Leibovitz’s-15 culture medium (L-15) to obtain solutions equivalents to 100, 150 and 200 µg/ml which were used for experimental study.

8.2.6. Experimental animals and maintenance

Fresh water common carp (Cyprinus carpio L.) were obtained from commercial hatcheries, Jadavpur, Kolkata, India. Fish of uniform size length (12 ± 2 cm) and weight (145.46 ± 1.24 g) were segregated from the stock and acclimatized to laboratory conditions for 7 days in an aquarium (60 × 30 × 40 cm) with 50 L of dechlorinated tap water (8 individuals /aquarium). The total mortality of fish was less than 1 %. During the experiment, water temperature was 25±1°C, pH was 7.1 ± 0.25, dissolved oxygen was 8.015 ± 0.5 mg/l, hardness was 425±3.5, turbidity was 2.5 NTU and total solids were 14.05±0.1 mg/L. Fish were reared at 25°C in a recirculation system with 12 h light-dark photoperiod and fed with commercial diets (Tokyu baby pellet, Floating type, Japan) twice a day. Feeding was withheld 1day prior to cell isolation, with an average weight of 146 ± 2.5 g at the start of the experiment.
8.2.7. Isolation of hepatocytes
Carp hepatocytes were isolated according to previously reported methods [3, 32-34] with slight modifications. Fish weighing 146 ± 2.5 g were netted and anesthetized by 3-aminobenzoic acid ethyl ester (Sigma, USA) and sanitized with 70% alcohol. An incision was made ventrally from the anus to the pectoral girdle to expose the liver; blood was cleared from the caudal vein. Liver was removed and the hepatic portal vein was quickly cannulated. The liver was washed with sterilized water and the tissues were transferred to Ca$^{2+}$-and Mg$^{2+}$-free buffer, consisting of 160.8 mM NaCl, 3.15 mM KCl, 0.7mM NaHPO$_4$, 5mM EDTA and 33mM HEPES, pH 7.65. The tissue samples were then minced with sterile dissecting blade and scissor and washed four times with phosphate buffered saline containing 1000 µg streptomycin, 1000 µg penicillin and 25 µg gentamycin sulfate. Tissue fragments (1-2 mm$^3$) were digested in a solution of 0.25% trypsin (1:20 w/v) for 40 min at 25°C. The resultant cell slurry was filtered through 70 mesh sieve screen to remove large fragments. Cells were further trypsinized and concentrated by centrifuging at 100× g for 4 min in a bench-top clinical centrifuge at 4°C. The cells were resuspended in solution containing L-15 medium with 2% v/v fetal bovine serum (FBS), 20 mM HEPES, and 14.3 mM NaHCO$_3$, 50 µg/100 ml gentamycin sulfate, 1 mM insulin and centrifuged at 1000 rpm for 2 min. Finally the cells were suspended in 10 to 15 ml L-15 culture medium and cell viability was assessed with trypan blue (0.05%) exclusion method. The number of cells in the suspension was determined in a Neubauer counting chamber. When viability was >90%, cells were evaluated for experiment.

8.2.8. Cell plating and culture conditions

The final cell density of hepatocytes was adjusted to 1.5×10$^6$ cells/ml and cells were seeded into 24-well micro plates with 400 µl cell suspension per well (6×10$^5$ cells/well). Culture media used during the present work was Leibovitz’s L-15 medium and supplemented with 72 mg/L penicillin, 50 mg/L gentamicin, 100 mg/L streptomycin and 10% fetal bovine serum (FBS). The plates were incubated either at 17 or 23°C or both for 24 h, before the following experiments were conducted.
8.2.9. Determination of hepatoprotective and antioxidant effects of biosynthesized gold nanoparticles and methanol extract of *Azolla microphylla*

The hepatoprotective and antioxidant effects of biosynthesized gold nanoparticles and *Azolla microphylla* extract were investigated using an *in vitro* model of acetaminophen-induced hepatocellular damage under three separate conditions of treatment according to the method described by Jia et al. (2012) and Yin et al. (2011) [35, 3]

(a) **Control:** Primary hepatocytes without adding acetaminophen or gold nanoparticles or *Azolla microphylla* extract.

(b) **Acetaminophen treatment:** A 24 h primary hepatocytes treated with 12 mM acetaminophen and incubated for 4 h.

(c) **Treatment-I:** After 24 h incubation of hepatocytes, the cells were pre-incubated with 100, 150, 200 µg/ml of gold nanoparticles or 100, 200, 400 µg/ml methanol extract powder of *Azolla microphylla* for 4 h, and then cells were washed with phosphate buffered saline and incubated with 12 mM acetaminophen for another 4 h.

(d) **Treatment-II:** After 24 h incubation of hepatocytes, cells were first treated with acetaminophen at a concentration of 12 mM for 4 h, and then, cells were washed and incubated with 100, 150, 200 µg/ml of gold nanoparticles or 100, 200, 400 µg/ml methanol extract powder of *Azolla microphylla* for another 4 h.

(e) **Treatment-III:** First, cells were pre-incubated with 100, 150, 200 µg/ml of gold nanoparticles or 100, 200, 400 µg/ml methanol extract powder of *Azolla microphylla* for 4 h, and then, acetaminophen was added at a final concentration of 12 mM; after 4 h of incubation with acetaminophen, the cells were further treated with 100, 150, 200 µg/ml of gold nanoparticles or 100, 200, 400 µg/ml methanol extract powder of *Azolla microphylla* for another 4 h.

For analysis of each set of conditions, five experiments including control and acetaminophen treated hepatocytes were evaluated. Control (without adding acetaminophen and gold nanoparticles or *Azolla microphylla* extract), acetaminophen treatment and 3 concentrations of each biosynthesized gold nanoparticles or *Azolla microphylla* extract treatment were set, and each treatment was performed in triplicate. Before gold nanoparticles or *Azolla microphylla* extract or acetaminophen was added, the old medium was completely removed and replaced with fresh medium containing gold nanoparticles or *Azolla microphylla* extract or acetaminophen. At the end of each set of experiment, 0.5 ml aliquot of supernatant from each individual well was collected in a 2 ml
centrifuge tube, centrifuged at 200×g and stored at -20°C for various spectrophotometric biochemical analysis mentioned below.
8.2.10. Cell viability assay

Viability of hepatocytes treated with GNaP and *Azolla microphylla* extract was measured by MTT assay method. Briefly, Cells (6×10⁵ cells/well) were cultured in a 24-well plate, and after treatment-I, treatment-II and treatment-III of the cells with GNaP or *Azolla microphylla*, added 20 μL (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) to each well and incubated for 4 h at 37˚C in a 5% CO₂. After 4h of incubation, the medium was removed and 100 µL of MTT fixative solution (isopropanol with 0.04 M HCl) was added. Viable cells were determined by measuring the absorbance at 570 nm using UV-visible spectrophotometer. Wells containing cells not exposed with GNaP or *Azolla microphylla* were taken as blanks. The cell viability was calculated using the following equation: Viability (%) = At/Ac × 100

Where At, Ac are mean absorbance of gold nanoparticles for treated and control cells respectively, (n = 5; where n is the no. of independent experiments).

8.2.11. ROS and biochemical analysis

The supernatants were collected and assayed for levels of ROS (reactive oxygen species) generation, lactate dehydrogenase (LDH, E.C. 1.1.1.27), catalase (CAT, E.C.1.11.1.6), glutamate oxalate transaminase (GOT, E.C.2.6.1.1), glutamate pyruvate transaminase (GPT, E.C.2.6.1.2), glutathione peroxidase (GSH-Px, E.C.1.11.1.9), superoxide dismutase (SOD, E.C.1.15.1.1) and malondialdehyde (MDA) by spectrophotometric method (Varian Cary 50 UV-spec) using commercially procured diagnostic kits. Briefly, Lactate dehydrogenase (LDH) was expressed as U/L, glutamate pyruvate transaminase (GPT) and glutamate oxalate transaminase (GOT) were expressed as U/ml, using spectrophotometric diagnostic kits were obtained from Crest biosystems (Div of Coral Clinical systems), Goa, India. SOD activity was assayed by the inhibition of nicotinamide adenine dinucleotide (reduced) phenazine methosulphate nitrobluetetrazolium reaction system as adapted, and the result was expressed as U/ml, using commercial diagnostic kit was obtained from Qualigens diagnostics, Pvt. Ltd, Mumbai, India. CAT and GSH-Px activities were assayed by measuring the amount of substrate consumed (hydrogen peroxide and glutathione,
respectively) after carrying out the reactions for a specified period of time, and the results have been expressed as U/ml, using diagnostic kit were obtained from Crest biosystems (Div of Coral Clinical systems), Goa, India. MDA level was evaluated by the thiobarbituric acid-reactive substances method (TBARS) using commercial diagnostic kit was received from Qualigens diagnostics, Pvt. Ltd, Mumbai, India and the final level of MDA was expressed as U/ml.
8.2.12. Measurement of ROS generation

Intracellular ROS production in the primary hepatocytes was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation [36]. After completion (control, acetaminophen, treatment-I, treatment-II and treatment-III) of the experiments, 20 μM DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate) was added and incubated for another 45 min. The DCFH-DA was removed by washing the cells with PBS (Phosphate buffered saline). Finally, 100 μM H₂O₂ was added into the cells and incubated for 45 min. The change in the fluorescence was monitored by fluorescence spectrophotometer at λ<sub>ex</sub> = 475 nm, λ<sub>em</sub> = 525 nm.

8.2.13. Statistical analysis

Values are expressed as mean ± standard deviation. Data within the groups are analyzed using one-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons test. Values were considered statistically significant when p<0.05. Statistical analyses were carried out using SPSS statistics version 20 software.

7.4. Results 7.4.1. Characterization of biosynthesized gold nanoparticles
Figure 8.1(a & b) showed the field emission scanning electron microscope images of the biosynthesized gold nanoparticles. The overall morphological shapes of the gold nanoparticles are spherical and rectangular at higher magnification. The average sizes of the gold nanoparticles were 17-40 nm.

Fig.8.1.FESEM image measured at 5% v/v Azolla microphylla extract mediated synthesis of gold nanoparticles

Energy Dispersive X-ray analysis (EDX) of gold nanoparticles (Fig.8.2) showed strong signal for elemental gold. The gold nanocrystallites displayed an optical absorption band peak at 2.15 keV. EDX analysis suggested that phytochemicals were absorbed on the surface of the gold nanoparticles and are strongly responsible for the stability of the biosynthesized gold nanoparticles.
Fig. 8.2. EDX pattern of gold nanoparticles synthesized with 5% v/v Azolla microphylla extract in aqueous 1mM HAuCl₄ solution.

In Fig. 8.3 (a, b & c), the HRTEM images clearly proved the size and shape of the gold nanoparticles as a function of concentration of the phenolic compounds present in the plant extract. The shapes of the gold nanoparticles were spherical, triangular, hexagonal and rod.

Gold nanoparticles corresponding to HRTEM image exhibited the variation in the particle size ranging from 3 to 20 nm with the average of 8.3 nm. The obtained each shape of the nanoparticles was quite uniform in size and up to 4-15 nm.

Fig. 8.3. HRTEM images of biosynthesized gold nanoparticles

7.4.2. Effects of Azolla microphylla methanol extract powder comparison with biosynthesized gold nanoparticles against acetaminophen-induced hepatocytes damage

Cultured primary hepatocytes treated with 12 mM acetaminophen alone showed a significant reduction in cell viability compared to the control (Fig. 8.4). Significant increase in cell viability was observed in treatment-I and treatment-III of the hepatocytes with biosynthesized GNaP and Azolla microphylla extract at all the three tested concentrations when compared to the group treated with APAP alone. In the case of treatment-II, the cells treated with biosynthesized GNaP at 150 and 200 µg/ml showed slight increase in cell viability while no effect was observed when the cells were treated-II with 100 µg/ml of biosynthesized GNaP as well as at all the three concentrations (100, 200 and 400 µg/ml) of
Azolla microphylla extract.
Fig. 8.4. Effects of methanol extract of Azolla microphylla mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m. ext) on cell viability (percentage of control value) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6). *p<0.05, compared to control; #p<0.05 as compared to acetaminophen treated hepatocytes.

7.4.2.1. LDH and CAT

Figures 8.5 and 8.6 showed, sub-cultured primary hepatocytes treated with 12 mM acetaminophen, 30% and 40% increased the amount of LDH and CAT respectively, in the culture medium as compared with control (without adding acetaminophen or Azolla microphylla). Methanol extract of Azolla microphylla mediated biosynthesized gold nanoparticles (100, 150, 200 µg/ml) significantly reduced the levels of LDH and CAT than A. microphylla methanol extract (100, 200, 400 µg/ml) as compared with 12 mM acetaminophen treatment. Dose-dependent effects were observed, 200 µg/ml gold nanoparticles exhibited more potent action than other concentrations of both tested gold nanoparticles and Azolla microphylla extract. Furthermore, treatment-I with 12 mM acetaminophen, all concentrations of tested gold nanoparticles and Azolla microphylla extract slightly reduced the level of LDH and CAT, at same time no significant effects on treatment-II with 12 mM acetaminophen was observed.
Fig. 8.5. Effects of methanol extract of Azolla microphylla mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m. ext) on lactate dehydrogenase (LDH) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6).

*p<0.05, compared to control; #p<0.05 as compared to acetaminophen treated hepatocytes.
Fig. 8.6. Effects of methanol extract of Azolla microphylla mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m ext) on catalase (CAT) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6). *p<0.05, compared to control; #p<0.05 as compared to acetaminophen treated hepatocytes.

7.4.2.2. GOT and GPT

Figures 8.7 and 8.8, showed, treatment of the 24 h cultured primary hepatocytes with 12 mM acetaminophen drastically increased the levels of GOT and GPT by 34% and 60% respectively as compared with control hepatocytes. Treatment-III of methanol extract of Azolla microphylla mediated biosynthesized gold nanoparticles (100, 150, 200 µg/ml) and Azolla microphylla extract (100, 200, 400 µg/ml) significantly reduced the levels of GOT and GPT by 50% and 10% respectively as compared with 12 mM acetaminophen treated hepatocytes. 200µg/ml of gold nanoparticles showed significant hepatoprotective effect compared to all other concentrations of both gold nanoparticles and Azolla microphylla extract. However, treatment-II of primary hepatocytes with all concentrations of both Azolla microphylla extract and gold nanoparticles showed no significant effect on GOT and GPT (GOT and GPT levels reduced less than 5%) as compared with 12 mM acetaminophen treated hepatocytes.
Fig. 8.7: Effects of methanol extract of *Azolla microphylla* mediated synthesized gold nanoparticles (GNaP)/*Azolla microphylla* extract (A. m ext) on glutamate oxalate transaminase (GOT) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6).

*Fig. 8.7: Effects of methanol extract of *Azolla microphylla* mediated synthesized gold nanoparticles (GNaP)/*Azolla microphylla* extract (A. m ext) on glutamate oxalate transaminase (GOT) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6).*

*Fig. 8.7: Effects of methanol extract of *Azolla microphylla* mediated synthesized gold nanoparticles (GNaP)/*Azolla microphylla* extract (A. m ext) on glutamate oxalate transaminase (GOT) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6).*

\[ p < 0.05, \text{ compared to control; } \# p < 0.05 \text{ as compared to acetaminophen treated hepatocytes.} \]

7.4.2.3. GSH-Px and SOD

Treatment of the hepatocytes primary culture with 12 mM acetaminophen drastically reduced the GSH-Px and SOD contents by about 50% and 75% respectively as compared with the control hepatocytes. Treatment-III of methanol extract of *A microphylla* mediated biosynthesized gold nanoparticles (100, 150, 200 µg/ml) and *Azolla microphylla* extract (100,
200, 400 µg/ml) significantly increased the levels of GSH-Px and SOD contents by 60-70% as compared with 12 mM acetaminophen treated control hepatocytes. 200 µg/ml of gold nanoparticles effectively increased the contents of GSH-Px and SOD as compared with all other concentrations of *Azolla microphylla* extract and gold nanoparticles. Figure 8.9 showed, all the concentrations of *Azolla microphylla* extract and gold nanoparticles showed no significant effects on treatment-II with 12 mM acetaminophen. On the other hand, treatment-I with 12mM acetaminophen of hepatocytes with all the concentrations of *Azolla microphylla* extract and gold nanoparticles slightly increased (almost 20-30%) the levels of GSH-Px and SOD. Furthermore, treatment-II with 12 mM acetaminophen with gold nanoparticles and *A. microphylla* extract had some positive effect on increasing the content of SOD (Fig.8.10).
Fig. 8.9. Effects of methanol extract of Azolla microphylla mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m ext) on glutathione peroxidase (GSH-Px) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6). *p<0.05, compared to control; #p<0.05 as compared to acetaminophen treated hepatocytes.
Fig. 8.10. Effects of methanol extract of *Azolla microphylla* mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m ext) on superoxide dismutase (SOD) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6).

* p<0.05, compared to control; # p<0.05 as compared to acetaminophen treated hepatocytes.

### 7.4.2.4. MDA

24 h cultured hepatocytes treated with 12 mM acetaminophen, increased the formation of MDA up to 33% as compared with control hepatocytes (Fig.8.11). Treatment-III with all concentrations of *A microphylla* (100,200,400 µg/ml) and gold nanoparticles (100, 150, 200 µg/ml), drastically reduced the formation of MDA almost 60%. On hepatocytes, treatment-I with all concentrations of *A microphylla* and gold nanoparticles slightly reduced the formation of MDA compared with 12 mM acetaminophen treated hepatocytes. Furthermore, the primary hepatocytes under treatment-II (12 mM acetaminophen) with *Azolla microphylla* and gold nanoparticles did not showed any positive effects in the inhibition of MDA.
Fig. 8.11. Effects of methanol extract of Azolla microphylla mediated synthesized gold nanoparticles (GNaP)/Azolla microphylla extract (A. m ext) on melondialdehyde (MDA) in Acetaminophen-induced hepatocytes. Results expressed as mean ± Standard Deviation (n=6). *p<0.05, compared to control; #p<0.05 as compared to acetaminophen treated hepatocytes.

7.4.2.5. ROS generation

Significant reduction of intracellular ROS formation was found in hepatocytes of the common carp treated with 12 mM acetaminophen alone compared to control (Fig. 8.12). Significant reduction of intracellular ROS was observed in treatment-I and treatment-III of the hepatocytes with biosynthesized GNaP and Azolla microphylla extract at all the three concentrations when compared to the group treated with 12 mM acetaminophen. While in the case of treatment-II, biosynthesized GNaP and Azolla microphylla extract all the three concentrations no effect was observed.
The ability of conventional drugs and chemicals often inadequately treated liver disease and produced serious adverse effects. Since previous reports suggested that, many medicinal plant and their extracts possess hepatoprotective and antioxidant properties. Hence, it is logical to search these medicinal plants and their extracts for use in the treatment of liver disease in order to replace currently used drugs with doubtful efficacy and safety [37]. The present study was to evaluate and compare the hepatoprotective and antioxidant effects of methanol extract of *Azolla microphylla* mediated biosynthesized gold nanoparticles and methanol extract of *Azolla microphylla* against acetaminophen– induced hepatocytes damage in common carp fish (*Cyprinus carpio* L.). The result suggested the highest hepatoprotective and antioxidant effects of *Azolla microphylla* mediated biosynthesized gold nanoparticles (200 µg/ml); and this effect is attributed due to possibly the highest content of polyphenols of *Azolla microphylla* extract got adsorbed on the surface.
of the gold nanoparticles which may significantly improve the viability of hepatocytes in culture medium. Hepatoprotective effect is connected to antioxidant activity, since it is free radical induced damage. Polyphenols present in the methanol extract of Azolla microphylla have free radical scavenging ability might be the possible reason for its antioxidant property. In the assessment of hepatocyte damage, biosynthesized gold nanoparticles were used as anti-hepatotoxicants due to their unique biocompatible properties. Pure gold has been used in complementary medicine for many decades. Besides its many beneficial effects, some toxic side effects have been reported to be associated with the use of gold compounds, but not pure gold. On the other hand, no evidence indicates that pure colloidal gold causes toxicity at the clinical, histological, cellular and molecular levels [38]. The general aspect of nanoparticles is that the smaller size provides a larger surface area for the particle and hence increases the effect. The nano size of the particles also increases the penetration potential of the gold particles, hence again aiding in better utilization of the metal properties [39, 40]. In this study, we utilized freshly isolated carp fish hepatocytes to determine the hepatoprotective and antioxidant effects of green synthesized gold nanoparticles and Azolla microphylla methanol extract against paracetamol induced hepatotoxicity. Paracetamol induced hepatocyte and liver injury is frequently used to screen hepatoprotective agents. The exact mechanism of paracetamol induced liver toxicity is not clear. However, it is known that paracetamol is mainly metabolized in the liver by cytochrome P450 system via the enzyme CYP2E1, to N-acetyl-p-benzoquinoneimine (NAPQI), a highly reactive, electrophilic molecule that causes harm by formation of covalent bonds with other intracellular proteins [41]. This reaction also inhibits the rate of glutathione synthesis and increases toxicity. If glutathione is not replenished, toxic NAPQI will begin to bind with hepatocytes and finally leads to cell apoptosis and necrosis. Furthermore, paracetamol is also shown to directly inhibit cellular proliferation, to induce oxidative stress, deplete adenosine triphosphate level and alter Ca$^{2+}$ homeostasis [42]. Non-functional plasma enzymes play a key role for hepatocyte damage/hepatotoxicity. Diagnosis of hepatotoxicity is analyzed by monitoring the number of such nonfunctional
plasma enzymes. The increase in the level of non-functional plasma enzymes depends on the degree of cellular damage, cellular leakage and loss of functional integrity of cell membrane [43]. Present study demonstrates a significant increase in the levels of LDH, CAT, GOT, GPT, MDA and reduced levels of GSH-Px and SOD indicates increased permeability, severe damage to plasma membrane and necrosis of hepatocytes, when added higher dose of acetaminophen (12 mM). In treatment-I and treatment-III with administration at different doses of methanol extract of A. microphylla mediated biosynthesized gold nanoparticles (100,150, 200 µg/ml) decreased the levels of LDH, CAT, GOT, GPT, MDA and increased the levels of GSH-Px and SOD. It indicates a possible stabilization of plasma membrane as well as repair of primary hepatocytes damages caused by higher dose of acetaminophen exposure. Lactate dehydrogenase (LDH) is a non-specific, highly sensitive, cytoplasmatic enzyme present in all major organs. The extracellular appearance of LDH is used to detect cell damage or cell death. It is released into the peripheral blood after cell death caused by,
e.g. ischemia, excess heat or cold, starvation, dehydration, injury, exposure to bacterial toxins, after ingestion of certain drugs and from chemical poisonings [44, 45]. Our findings also similar with those of Reen et al. (2001) who treated with various Swertia species extracts in primary monolayer cultures of rat hepatocytes against APAP-induced toxicity [28]. Acetaminophen treated hepatocytes released higher level of LDH in the culture medium due to damage of the plasma membrane. In treatment-I and treatment-III gold nanoparticles and Azolla microphylla extract replenished the ruptured plasma membrane and significantly reduced the amount of LDH. GOT and GPT are enzymes mainly found in the liver. Although not specific for liver disease, it can be used in combination with other enzymes to monitor various liver disorders. Biochemical markers such as GOT and GPT are known to elevate (above the international normalized ratio (INR)) in response to hepatotoxicants such as acetaminophen [46]. Our results (treatment-I and treatment-III) indicated that, significant increase in GOT and GPT with 4h exposure to 12 mM acetaminophen confirmed the hepatocyte damage. The metabolism of APAP catalyzed by liver microsomal cytochrome P450 rapidly overproduces free radicals that deplete hepatic glutathione (GSH) and initiate a chain lipid peroxidation of the hepatocyte membrane. It is well known that GSH, the most important non-enzymatic biological antioxidant protecting against chemically induced cytotoxicity. This free radical overproduces ultimately results in the increased levels of intracellular reactive oxygen species (ROS) and cytotoxicity. The overproduction of ROS mainly form superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), peroxyl radicals (ROO$^\cdot$) and hydroxyl radicals (OH$^\cdot$). The excessive ROS generation depletes the endogenous antioxidant enzymes and triggers hepatocyte damage. Cells containing large number of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) to prevent or repair the damage caused by ROS, as well as to regulate signaling pathways. Our results showed that, significant decrease of GSH-Px in APAP-treated hepatocyte and 3-fold elevation of CAT, while treatment-I or treatment-III with gold nanoparticles and Azolla microphylla extract significantly increased the levels of GSH-Px, suggesting the antioxidant effect of Azolla microphylla mediated synthesis gold
nanoparticles played an important protective role against APAP-mediated toxicity. MDA is a well-known end product of lipid peroxidation. The level of MDA is the direct evidence of toxic processes caused by free radicals [47, 48]. Present study demonstrated a level of MDA in the acetaminophen treated hepatocytes was significantly increased. The increase in MDA level suggested the enhanced lipid peroxidation leads to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. In treatment-I and treatment-III with gold nanoparticles and Azolla microphylla extract powder significantly reduced the level of MDA. These results indicated the gold nanoparticles and Azolla microphylla extract restored its activity, which may also contribute to the suppressed lipid peroxidation. Although the hepatoprotective and antioxidant effect of Azolla microphylla mediated biosynthesized gold nanoparticles is very significant. In treatment-I and III with methanol extract of Azolla microphylla mediated synthesized gold nanoparticles and methanol extract of A. microphylla powder had significant effect of hepatoprotective activity, while treatment-II did not showed any effect. Green synthesized gold nanoparticles having excellent hepatoprotective and antioxidant effect as compared with Azolla microphylla extract powder. This suggests that, the treatment of efficient flavonoids rich Azolla microphylla and mediated biosynthesized gold nanoparticles could act in different ways such as hepatoprotective, scavenging free radicals, and maintenance of normal lipid metabolism.
References


