

# Hepatoprotective Activity of Chitosan Nanocarriers Loaded with the Ethyl Acetate Extract of a *Stenotrophomonas* sp. Bacteria Associated with the Red Sea Sponge *Amphimedon ochracea* in CCl<sub>4</sub> Induced Hepatotoxicity in Rats

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## Abstract

The present study investigated the protective role of chitosannano-carriers loaded with the ethyl acetate extract of a *Stenotrophomonas* sp. bacteria associated with the Red Sea Sponge *Amphimedon ochracea* CCl<sub>4</sub> induced hepatic damage in rats using the hepatological parameters. Healthy 80 male Sprague-Dawley rats weighed 50 - 80 gm were used. The animals were randomly divided into eight groups. Serum ALT and AST, albumin, direct bilirubin and histopathological examinations were investigated. Liver targeting of the drugs has been achieved after administration of drug loaded spherical particles. This mode of administration of drugs enhanced its overall delivery to the liver via passive targeting. The present study revealed that the treatment with ethyl acetate bacterial extract (10 mg/Kg body weight) protected rats from hepatotoxic action of CCl<sub>4</sub>. Usage of chitosan nanoparticles loaded with the bacterial extract as a drug delivery system has greatly improved the curative effect of bacterial extract. Moreover, histopathological studies showed marked reduction in fatty degeneration and centrilobular necrosis in animals receiving the nano-chitosan loaded with the bacterial extract along with ethanol compared to the control group.

## Keywords

Hepatoprotective Activity, Chitosan Nanocarriers, Extract of *Stenotrophomonas* sp. Bacteria, Red Sea Sponge

## 1. Introduction

Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. So it has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and folk reproduction [1]. The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. A healthy liver is a crucial factor for overall health and well-being. But it is continuously and variedly exposed to environmental toxins and abused by poor drug habits, as well as alcohol, prescribed and over-the-counter drug which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease [2] [3]. Thus, liver diseases are some of the fatal diseases in the world today. Marine sponges for the past decades have been considered as a very fertile field for the discovery of bioactive natural chemical substances with respect to the diversity of their primary and secondary chemical components and metabolites. As a significant portion of the bioactive metabolites, thought originally to be products of the source animal often synthesized by their symbiotic microbiota, researchers have paid more attention to microorganisms as a renewable source of bioactive natural products. More specifically, bacteria from the marine environment have shown great potential as suggested by the diversity of secondary metabolites [4] [5]. In contrast to the terrestrial environment where plants are the most prolific sources of natural products, this leading position in the sea is taken by invertebrates such as sponges, tunicates, bryozoans, or mollusks, with sponges being the most prolific sources of natural products [6]. The wealth of their secondary metabolites has been related to the role of chemical defense played by these constituents; such ecological function is believed to be crucial for the survival of the producer organisms, which are soft-bodied, sessile or slow-moving animals, lacking, in most cases, morphological defense structures such as shells or spines [7]. Sponges have been the focus of much recent interest due to the following two main (and often interrelated) factors: (i) they form close associations with a wide variety of microorganisms, and (ii) they are a rich source of biologically active secondary metabolites. This increasing research interest has greatly improved our knowledge of sponge-microbe interactions [7]. With the development of the phylogenetic analysis of 16S rRNA gene sequences, putative identification of bacteria by comparison with sequences from known closely related organisms can be easily achieved. The efficiency of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases, only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biological properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of drug and reducing its toxic side effects *in vivo* is a challenging risk. One of the approaches is the use of colloidal drug carriers that can provide site specific or targeted drug delivery combined with optimal drug release profiles.

Among these carriers, liposomes and nanoparticles have been the most extensively investigated. Liposomes present some technological limitations including poor reproducibility and stability as well as low drug entrapment efficiency. Polymeric nanoparticles, which possess a better reproducibility and stability profiles than liposomes have been proposed as alternative drug carriers that overcome many of these problems. Nanoparticles are solid colloidal particles with diameters ranging from 1 - 1000 nm. They consist of drug carriers in which the active ingredient is dissolved, dispersed, entrapped, encapsulated, adsorbed or chemically attached. Polymers used to form nanoparticles can be both synthetic and natural polymers. Most of the polymers prepared from water insoluble polymers are involved heat, organic solvent or harsh conditions that can be harmful to the drug stability. Moreover, some preparation methods such as emulsion polymerization and solvent evaporation are complex and require a number of preparation steps that are more time and energy consuming. In contrast, water soluble polymers offer mild and simple preparation methods without the use of organic solvent and high shear force. Among water soluble polymers available chitosan is one of the most extensively studied. This is because chitosan possess some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic and inexpensive. Furthermore, it possesses positive charge and exhibits absorption enhancing effect. These properties render chitosan a very attractive material as a drug delivery carrier. In the last two decades, chitosan nanoparticles have been extensively developed and explored for pharmaceutical application [8] [9] [10] [11]. Thus, the objective of the present study was designed to test the hepatoprotective activity of the ethyl acetate extract of the sponge associated bacterial extract as well as its nano chitosan loaded form as a drug delivery system.

## 2. Material and Methods

### 2.1. Sampling of the Sponge Sample

The Red Sea represents a lot of different environmental ecosystem with more than 10 thousands sea organisms. However, it started to suffer from several major threats such as urbanization and coastal development, industries including power and desalination plants and refineries, recreation and tourism, waste water treatment facilities, power plants, coastal mining and quarrying activities, oil bunkering and habitat modification such as the filling and conversion of wetlands [12]-[27]. Recently, several biologically active compounds were isolated from the Red Sea especially from marine sponges and their associated microorganisms [28] [29] [30]. Sponge sample was collected by SCUBA diving at depth 1.5 m in the Red Sea (El-Gona station; GPS: N: 27°22'39.98", E: 33°40'58.95") in May 2010 (Figure 1). The sponge sample was taxonomically identified as Amphimedonochrace [31]. Order Haplosclerida: Family Niphatidae (Figure 2). Taxonomic identification was performed by Prof. Rob. W. M. VanSoest (University of Amsterdam, Netherlands). A voucher fragment is incorporated in the collections of the Zoological Museum of Amsterdam, now part of the Netherlands



**Figure 1.** Egypt map showing the sponge sampling site (El-Gouna, Egypt) on Red Sea.



**Figure 2.** Sponge sample (*Amphimedon ochracea*).

Centre for Biodiversity Naturalis NCB Naturalis) at Leiden, The Netherlands, under registration number (ZMA Por. 22534). Another sponge sample was also deposited in the Marine Biotechnology and Natural Products Lab. (MBNP) at the national Institute of Oceanography and Fisheries (NIOF) under registration number (MBNP/Sp-1).

## **2.2. Processing of the Sponge Sample**

Sponge was transferred to plastic bags containing seawater and transported to

the laboratory. Sponge specimen were rinsed in sterile seawater, cut into pieces with sterile scalpels of ca. 1 cm and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. The supernatant was diluted in ten-fold series ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ) using autoclaved environmental water. Fifty microliter aliquots of each dilution were placed on various agar plates' solid media.

### 2.3. Isolation of Sponge Associated Bacteria

Two growth media were prepared to isolate a wide range of the sponge associated bacteria; Marine Agar [32] and ISP Medium 2 [33] served as general rich media to grow many heterotrophic marine bacteria. All media were supplemented with 0.2  $\mu$ m pore size filtered cycloheximide (100  $\mu$ g/ml), nystatin (25  $\mu$ g/ml) and nalidixic acid (25  $\mu$ g/ml). Cycloheximide and nystatin inhibit fungal growth, while nalidixic acid inhibits many fast-growing Gram-negative bacteria [34]. All media contained DifcoBacto agar (18 g/l) and were prepared in 1 lartificial seawater (NaCl, 234.7 g,  $MgCl_2 \cdot 6H_2O$ , 106.4 g,  $Na_2SO_4$ , 39.2 g,  $CaCl_2$ , 11.0 g,  $NaHCO_3$ , 1.92 g, KCl, 6.64 g, KBr, 0.96 g,  $H_3BO_3$ , 0.26 g,  $SrCl_2$ , 0.24 g, NaF, 0.03 g and  $ddH_2O$  to 10.0 l) [35]. The inoculated plates were incubated at 28°C for 5 - 10 days. Distinct colony morphotypes were picked and re-streaked until visually free of contaminants. Isolates were inoculated into liquid media ISP Medium 2. The isolates were maintained on plates for short-term storage and long-term strain collections were set up in medium supplemented with 30% glycerol at -80°C [36].

### 2.4. Molecular Identification and Phylogenetic Analysis

The isolated bacteria were first identified to the species level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database. Specifically, the 16S rRNA gene from the strain was amplified using universal primers 27f (5'-AGAGTTTGATCCTGGC-TCAG-3') and 1492r (5'-GTTACCTTGTTACGACTT-3'). The PCR conditions used were the same as those described previously [37]. The PCR products were purified and sequenced by the GATC-Biotech. Company (Tübingen, Germany). The sequences were compared with known sequences in the GenBank nucleotide database and the species level was identified as the nearest phylogenetic neighbor with 98% - 100% similarity [38]. Sequence alignment and phylogenetic analysis were performed using the Mega-5 software package [39]. Tree construction was conducted using neighbor joining algorithm (Jukes-Cantor correction) with bootstrap values based on 1000 replications. The 16S rRNA gene sequence of the isolate was deposited in GenBank under the accession Numbers indicated in brackets: HA-MS-52 [JX484799].

### 2.5. Crude Extract Preparation

The bacterial isolate was cultured in 500 ml Erlenmeyer flasks containing 200 ml of ISP Medium 2. The liquid cultures were grown for 7 - 14 days depending on their growth rate at 30°C while shaking at 150 rpm. Crude extracts were prepared from whole cultures containing cells and broths by ethyl acetate equal

volume/three times, evaporated and dried under reduced pressure using a rotary evaporator.

### **2.6. Gas Chromatography-Mass Spectrophotometer (GC-MS) Analysis**

Analysis was conducted using gas chromatography-mass spectrophotometer; GC-MS (Trace DSQII MS). MS conditions were as follows: Detector mass spectrometer voltage 70 eV and its source temperature was 300°C. The injector temperature was 240°C and the split less mode 0.5 µL injection. The HP 55% dimethyl-95% dimethyl tertiary-butyl non-polar column was performed with length 30 cm × 0.25 mm, coating thickness film 0.25 µm. The oven was adjusted at 80°C for 1 min and initial time 1.5 min with 40°C which ended by a final temperature of 300°C and 4 min hold time where the total run time was 15 min. The compounds were identified by comparison with the standards, and also matched with the in-built libraries. The components were identified by comparing their retention times with those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library [40]. Quantitative data were obtained by the peak normalization technique using integrated FID response.

### **2.7. Determination of Total Phenolic Contents**

Total phenolic compounds in the bacterial extracts were determined by the method of Taga, *et al.* (1984) [41]. One hundred microliters Folin-Ciocalteu reagent (50%, v/v) in distilled water and 2 ml sodium carbonate (2%, v/v) in distilled water were added and mixed well with 100 µl of bacterial extract. After 2 h incubation at RT, the absorbance of the resulting blue color solution was measured at 750 nm. Different concentrations (0 - 0.9 mg/ml) of standard gallic acid were used and processed similarly as sample. The concentration of total phenolic in bacterial extracts was determined as mg gallic acid equivalent in 1 ml of the extract using the standard curve of the gallic acid.

### **2.8. Determination of Total Flavonoid Contents**

The total flavonoid content was determined by a colorimetric method of Zhi-shen, *et al.* (1999) [42]. A 0.25 ml of the bacterial extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by the addition of 0.75 ml of 5% sodium nitrite solution. After 6 min, 0.15 ml of 10% aluminum chloride solution was added and the mixture was made up to 2.5 ml with distilled water and well mixed. The absorbance was measured immediately at 510 nm using a spectrophotometer. The results were expressed as mean ml of (+)-catechin equivalents.

### **2.9. Preparation of Chitosan Nanoparticles**

Chitosan nanoparticles were prepared according to the ionotropic gelation process [43]. Blank nanoparticles were obtained upon the addition of a triphosphate (TPP) aqueous solution (1 mg/ml) to a chitosan solution (2 mg/ml)

stirred at room temperature (rate of 400 rpm). The formation of nanoparticles was a result of the interaction between the negative groups of TPP and the positively charged aminogroups of chitosan. The ratio of chitosan/TPP was established according to the preliminary studies. Extract loaded nanoparticles were obtained according to the same procedure and the ratio of chitosan/TPP remained unchanged. Nanoparticles were collected by centrifugation at 10000 rpm for 40 min and supernatant were discarded.

### 2.10. Determination of Loading Capacity and Efficiency of Nanoparticles

Loading capacity of extract loaded nanoparticles was determined by ultra-centrifugation of samples at  $30000\times g$  and  $10^{\circ}C$  for 30 min. The amount of free extract was determined in clear supernatant by measuring the total phenolic compound of the extract supernatant of non-loaded nanoparticles as basic correction using HPLC semi-preparative. The extract loading capacity (LC) of nanoparticles was calculated from the equations indicated below:

$$LC = [(A - B)/A] \times 100$$

where A is the total amount of phenolic compounds in the extract; B is the free amount of phenolic compounds in the supernatant.

### 2.11. Animal and Experimental Design

Healthy 80 male Sprague-Dawley rats weighed 50 - 80 gm, purchased from the animal house of Faculty of Medicine, Alexandria University, and were used for the study. The animals were randomly divided into eight groups of ten animals each after an acclimatization period of two weeks. They were fed with standard diet, had free access to water, housed under standard conditions of humidity and controlled temperature ( $25^{\circ}C$ ) with 12 hours light and 12 hours dark exposure. The protocol was approved by the Institutional Animal Ethics Committee (623/02/b/CPCSEA).

**Group I (-ve control):** Rats received saline solution subcutaneously for one week.

**Group II (+ve control):** Rats received subcutaneously  $CCl_4$  (1 ml/kg body weight/day) for one week (induced toxicity group).

Group III and IV were received 10 mg/Kg b.w./day bacterial extract for one week, while group V received it for two weeks and they served as one-week-treated, control and two weeks-treated groups, respectively.

**Group III (one week extract-treated group):** Rats received subcutaneously  $CCl_4$  (1 ml/kg body weight/day) for one week and 10 mg/Kg body weight/day of extract.

**Group IV (control/extract-treated group):** Rats received saline solution subcutaneously and 10 mg/Kg body weight extract for one week.

**Group V (two weeks extract-treated group):** Rats received subcutaneously  $CCl_4$  (1 ml/kg body weight/day) for one week (as in group II) and 10 mg/Kg body weight/day of bacterial extract for two weeks.

At the same time, Groups VI, VII and VIII were received 10 mg/Kg b.w./day of bacterial extract-chitosan loaded nanoparticles and they served as one week, two weeks and control treated nano extract-chitosan groups, respectively.

**Group VI (one week nano extract-chitosan treated group):** Rats received subcutaneously CCl<sub>4</sub> (1 ml/kg body weight/day) for one week and 10 mg/Kg body weight/day of extract-chitosan loaded nanoparticles.

**Group VII (two weeks nano extract-chitosan treated group):** Rats received subcutaneously CCl<sub>4</sub> (1 ml/kg body weight/day) for one week and 10 mg/Kg body weight/day of extract-chitosan loaded nanoparticles for two weeks.

**Group VIII (control/nano-chitosan treated group):** Rats received saline solution subcutaneously and 10 mg/Kg body weight nano-extract for one week.

At the end of experiment (one week for one-week-treated rats and two weeks for two-weeks-treated rats), rats were sacrificed under diethyl ether anesthesia.

### 2.12. Blood Sample Collection

Unheparinized blood samples were collected through retro-orbital puncture, allowed to clot and centrifuged at 3000 rpm to separate clear serum. Kept for 15 min at RT, sera were separated by centrifugation at 3000 rpm for 20 min and stored at -20°C until used.

### 2.13. Histopathology Studies

The liver tissue was immediately removed after sacrifice and fixed in 10% formol saline. Paraffin sections of 5µ thickness were prepared, stained by Hematoxyline and Eosin (H & E) and examined microscopically.

### 2.14. Biochemical Measurements for the Assessment of Liver Function

#### a. Determination of liver function enzymes; Alanine Transaminase (ALT) and Aspartate Transaminase (AST)

Serum ALT and AST activities were determined colorimetrically using Diamond Diagnostic Kits (Egypt) according to Reitman and Frankel [44]. The absorbance of the sample (As) was read against blank at 540 nm after 5 min. the activity of the ALT and AST were expressed as U/ml and determined using standard curves.

#### b. Determination of serum albumin

Serum albumin was determined colorimetrically using Diamond Diagnostic Kits (Egypt) according to the method of Doumas, *et al.* (1971) [45]. Albumin level was calculated using the following equation:

$$\text{Albumin level in mg/dl} = \frac{As}{Ast} \times \text{concentration of standard}$$

where As = sample absorbance, Ast = standard absorbance.

#### c. Determination of direct bilirubin

Serum direct bilirubin was determined colorimetrically using Diamond Diagnostic Kits (Egypt), according to Hymans Van den Bergh and Mueller (1916)

[46]. Direct (conjugated) bilirubin couples directly with a diazonium salt of 3,5-dichloroaniline (DPD) in an acid medium to form azobilirubin. The direct bilirubin in serum is directly proportional to the color development of azobilirubin which is measured bichromatically at 540/600 nm.



### 2.15. Histopathological Study

The fixed liver tissues in formalin were dehydrated in ascending grades of alcohol, and then cleaned by immersing the tissues in xylene for 1 h (three times), followed by impregnation in melted paraffin, in wax, then in oven at 60°C for 1 h. The specimens were embedded in paraffin and were left to solidify at RT. Using a rotatory microtone, sections of 5 µm thick were cut and mounted on clean glass slides. Sections were stained with hematoxylin and eosin (H & E) and examined for any histopathological changes [47].

### Haematological Methods

Haemoglobin (Hb) concentration, packed cell volume (PCV), red blood cell count (RBC), mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC) were measured by the method of Schalm, *et al.* (1975) [48].

### 2.16. Statistical Analysis

Results were reported as Mean ± S.D. All biochemical analyses were carried out in triplicate measuring, and data were tested by one-way analysis of variance using SPSS program, version 11.5, \*p < 0.05 regarded as significant.

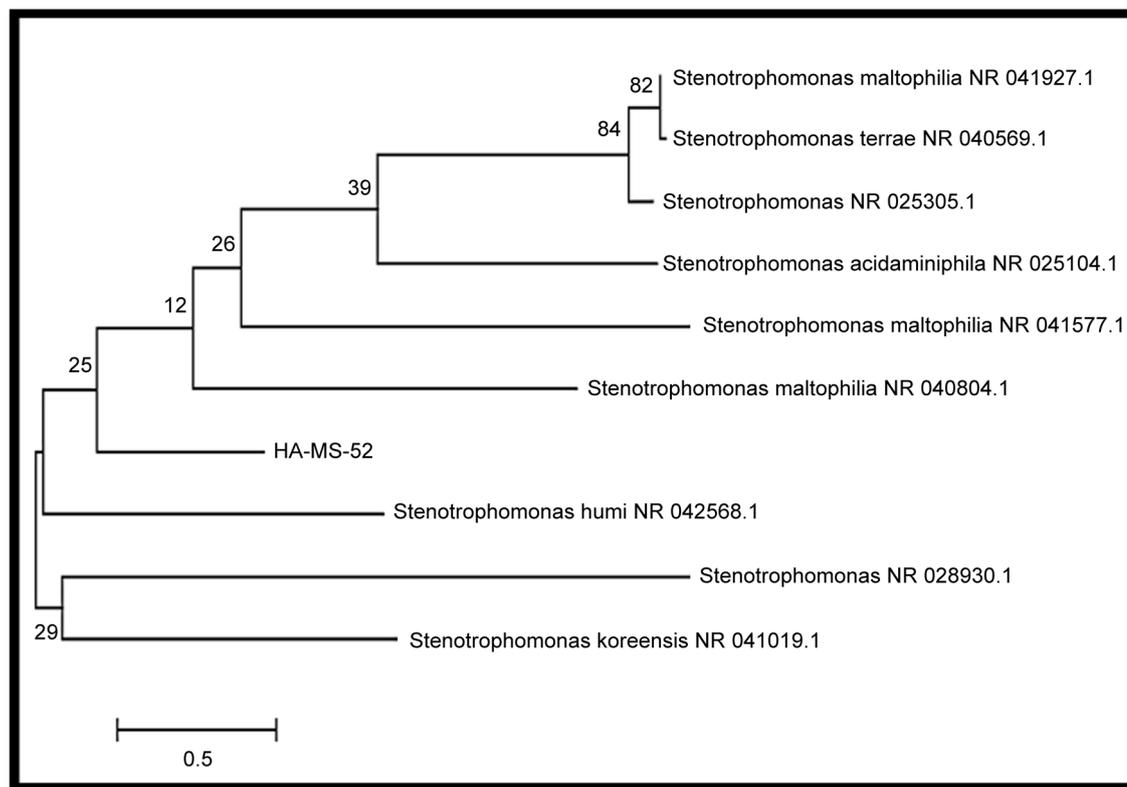
## 3. Results and Discussion

### 3.1. Phylogenetic Analysis of the Bacterial Strain

The bacterial isolate was identified using 16S ribosomal DNA sequences [49]. The tested isolates belong to the *Stenotrophomonas* sp. (Proteobacteria) (**Figure 3**). Interestingly, the Proteobacteria have been reported from diverse marine sponges irrespective of their taxonomic identity, geographic location, or natural products profile [50] [51]. The isolate HA-MS-52 revealed species-level similarity to *Stenotrophomonas maltophilia*.

### 3.2. GC-MS Analysis

Capillary GC in combination with mass spectrometry was found to be the method of choice for the analysis of complex mixtures of bioactive compounds. This technique allows much more precise alkaloidal identifications to be made than by comparative TLC and GLC methods, and enables even trace constituents to be analyzed [52]. The crude extracts of the bacterial isolate HA-MS-52 was subjected to GC/MS analysis, and it revealed 6 compounds (**Table 1, Figure 4**).

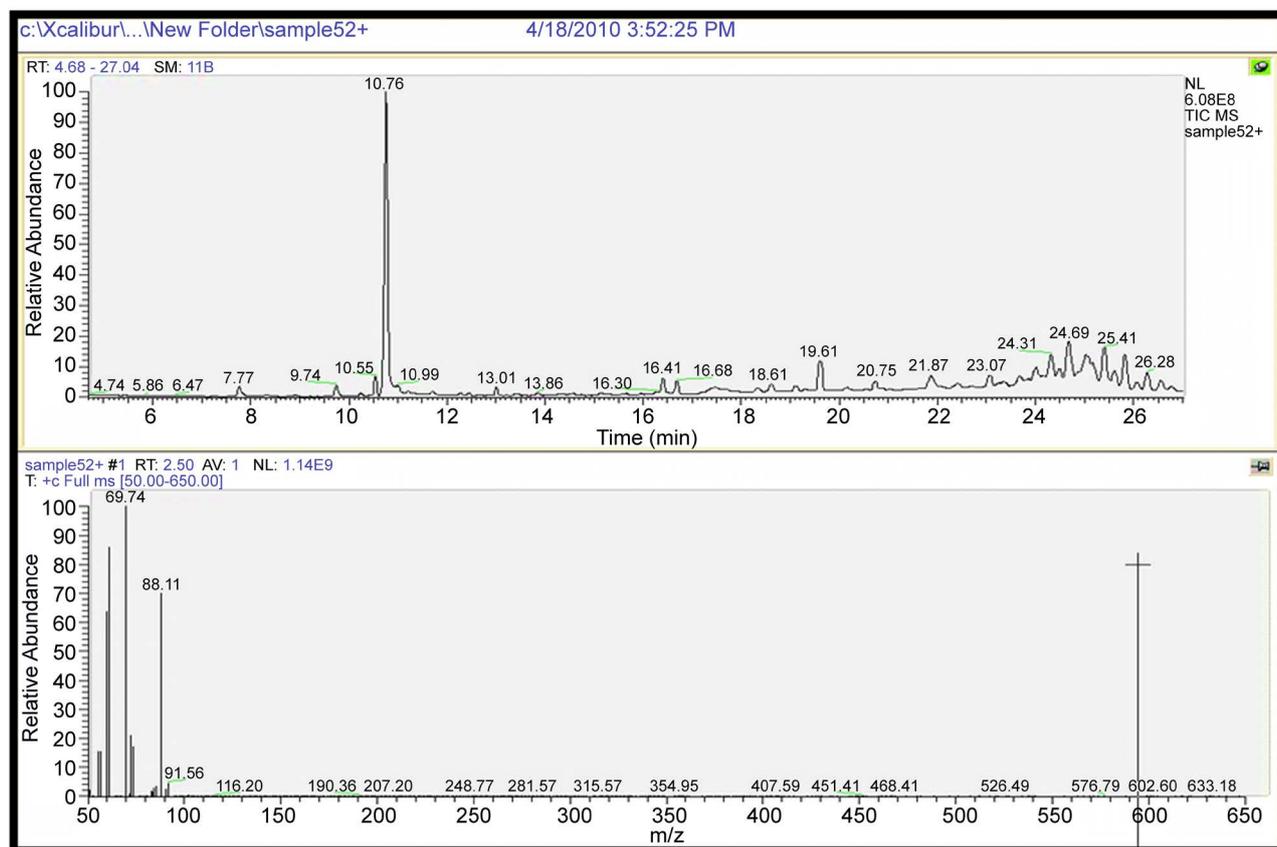


**Figure 3.** Molecular phylogenetic analysis of the bacterial isolate HA-MS-52 using Maximum Likelihood Method. The scale bar indicates 0.5 substitutions per nucleotide position.

**Table 1.** Bioactive compounds identified in the crude bacterial extract by Gas-Chromatography-Mass Spectrophotometer (GC-MS) Analysis.

Identified compounds*	RT (min)	Chromatogram % area
Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	11.92	2.22
Bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl-	13.40	1.46
Benzene, 1-methoxy-4-(1-propenyl)-	16.42	0.04
9,10-Secocholesta-5,7,10(19)-triene-1,3-diol, 25-[(trimethylsilyl)oxy]-, (3 $\acute{a}$ ,5Z,7E)-	25.36	0.03
Di-n-octyl phthalate	27.32	0.18
1,1-Dichloro-2-methyl-3-(4,4-diformyl-1,3-butadien-1-yl)cyclopropane	28.28	0.01

All the revealed compounds were identified regarding to the internal database which confirmed the presence of a variety of natural product families including mono- and di-terpenes, steroids, phenolics, flavonoids, carotenoids and fatty acids. The occurrence of different bioactive compounds suggests a wide range of biological application of the tested extracts. Sponges are important source of functional components for the development of new chemotherapeutic agents. Phytochemical investigation of the ethyl acetate extracts of the studied sponge-associated bacteria revealed the presence of various phytochemical such as phenolic compounds, flavonoids, saponins, steroids and tannins.



**Figure 4.** Gas Chromatography-Mass Spectrophotometer (GC-MS) chromatogram of ethyl acetate crude bacterial extract.

### 3.3. Total Phenolic Content and Total Flavonoid Contents

Highly significant quantities of phenolic contents were found in the crude bacterial extract measured as mg gallic acid equivalent/ml ( $2.7491 \pm 0.046$ ). The extract also shows highly significant quantities of flavonoid contents measured as equivalents of +catechin ( $12.6 \pm 0.634$  mg catechin equivalent/ml).

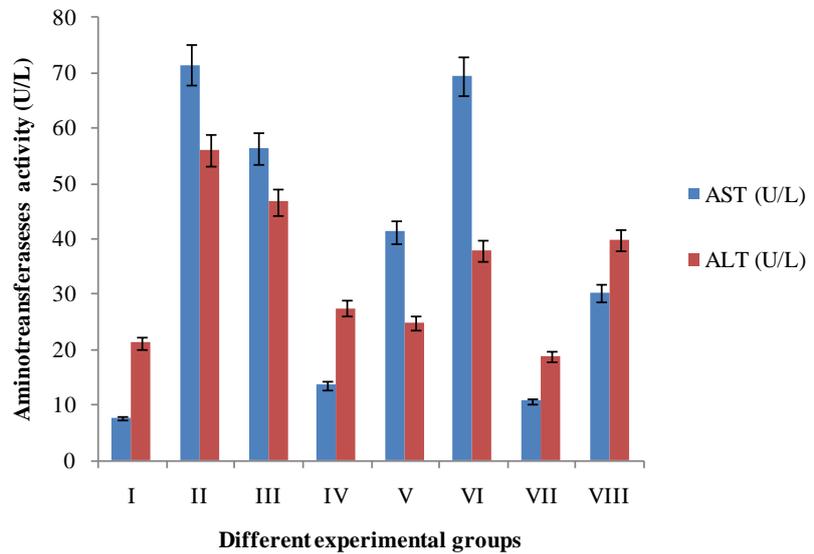
### 3.4. Loading of Bacterial Extract No. 5 on the Prepared Chitosan Nanoparticles Physical Characterization of Chitosan Nanoparticles

Stability of nanoparticles in suspension is directly proportional to zeta potential. The zeta potential of nanoparticles was found to be 30 mV. The observed positive zeta potential might be due to residual amine group which act as carrier of the bacterial extract.

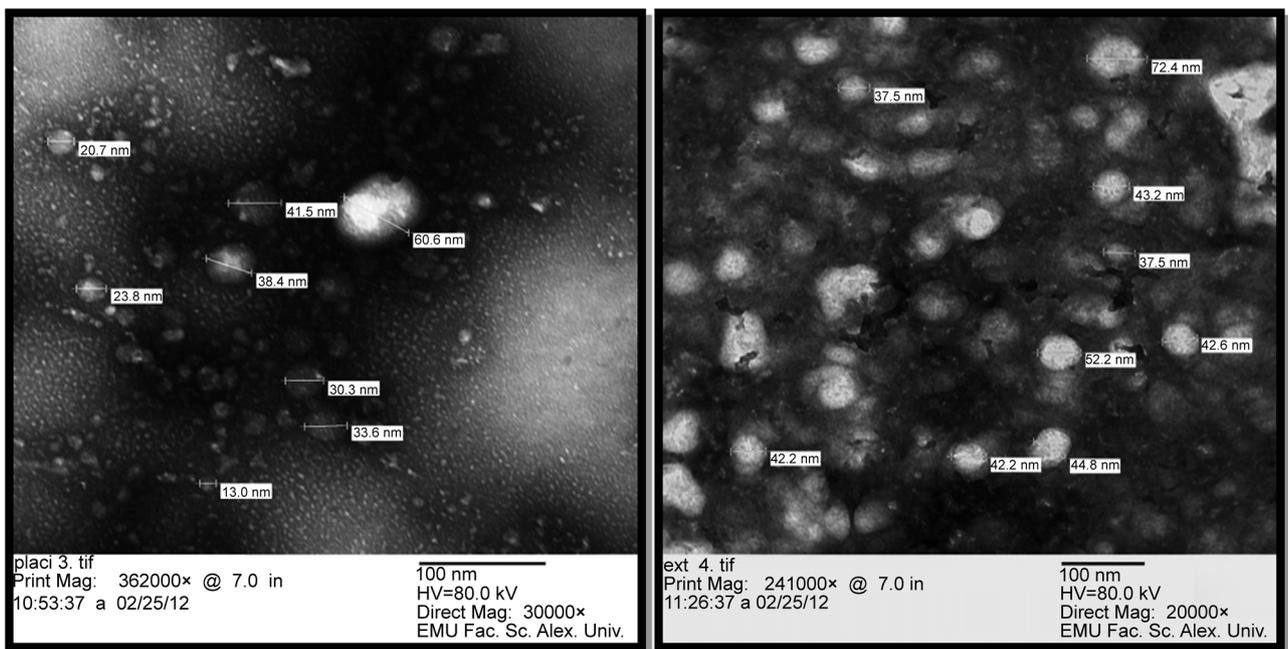
### 3.5. Assessment of Liver Function

#### a. Liver function enzymes; Alanine Transaminase (ALT) and Aspartate transaminase (AST)

**Figure 5** shows that the activity of both serum ALT and AST enzymes (markers for the liver function) were significantly ( $p = 0.002$ ) elevated for the rats in Group II *i.e.*, +ve control group (carbon tetrachloride treated group) comparing to rats in Group I, *i.e.*, -ve control one. Administration of 10 mg/Kg body weight



**Figure 5.** Levels of alanine aminotransferase (ALT) and aspartate transferase (AST) enzymes in rats era of the eight animal groups (Data are means of three measurements). \*ANOVA test is significant at  $p \leq 0.05$ .



**Figure 6.** Chitosan nanoparticles and Extract-loaded chitosan nanoparticles as shown under the transmission electron microscope (TEM). Numerical values are shown in white box represent the particle diameter size on nm. The particle size of the extract-loaded chitosan nanoparticles showed difference from those of placebo particles (extract-non-loaded nanoparticles). The prepared chitosan nanoparticles in the placebo group were in average size of  $443.95 \pm 90.580$  nm. Whereas, the extract-loaded nanoparticles was in average size of  $684 \pm 243.032$ nm (Figure 6).

of the ethyl acetate bacterial extract (bacterial isolate HA-MS-52) to rats in Group III (One week extract-treated group) and Group V (Two weeks extract-treated group) remarkably prevented the effect of carbon tetrachloride which induced elevation of serum ALT and AST as shown in Group II (+ve control group).

Effects of the bacterial extract on the activities of serum ALT and AST in rat of  $\text{CCl}_4$  treated groups either as plain extract (Groups III and V) or loaded in chitosan nanoparticles (Groups VI and VII) is shown in **Table 2** and **Figure 5**. Group II (+ve control group) showed marked elevation of serum ALT and AST levels ( $56.19 \pm 28.09$  U/l and  $71.51 \pm 24.94$  U/l, respectively) comparing to group I; -ve control ( $21.38 \pm 4.03$  U/l and  $7.72 \pm 2.16$  U/l, respectively). Group III (One week extract-treated group), showed marginal reversal of elevated ALT and AST levels in serum ( $46.76 \pm 14.16$  U/l and  $56.44 \pm 20.68$  U/l, respectively).

At the same time, the administration of the bacterial extract for one week more in Group V (two weeks extract-treated group) showed marked elevation of serum ALT and AST levels comparing to Group III (One week extract-treated group) ( $24.93 \pm 7.44$  U/l and  $41.37 \pm 19.69$  U/l, respectively). The serum AST and ALT levels of rats in Group IV (control extract-treated group) showed slight elevation on ( $27.56 \pm 7.11$  U/l and  $13.67 \pm 22.79$  U/l, respectively) comparing to Group I (-ve control group).

On the other hand, Group VI (One week nano extract-chitosan treated group) exhibited a higher reversal of elevated serum ALT and AST levels comparing to their levels in Group III, *i.e.*, one week extract-treated group as well as Group V, *i.e.*, two weeks extract-treated group ( $18.96 \pm 7.29$  U/l and  $31.85 \pm 7.35$  U/l, respectively). However, Group VI (one week nano extract-chitosan treated rats) showed lower reversal of elevated ALT and AST levels as compared to Group III (one week extract-treated group) ( $69.41 \pm 28.21$  U/l and  $56.44 \pm 20.68$  U/l, respectively). On the other hand, Group VII (one week nano extract-chitosan treated rats) showed the best reversal effect of ALT and AST levels among all induced groups (groups III, V, VI and VII), especially when compared to the two weeks extract-treated rats in Group V ( $10.85 \pm 7.35$  U/l and  $41.37 \pm 19.69$  U/l, respectively). Moreover, serum ALT and AST levels in control nano-chitosan treated rats (Group VIII) showed lower reversal effect of the elevated serum ALT and AST levels when compared to the two weeks nano extract-chitosan treated rats in Group VII ( $39.91 \pm 15.82$  U/l and  $30.38 \pm 9.77$  U/l, respectively).

**Table 2.** Serum levels of AST and ALT enzymes among the different rat groups.

Group	Treatment	AST (U/l) Mean $\pm$ S.D.	ALT (U/L) Mean $\pm$ S.D.
I	-ve Control	$7.72 \pm 2.16$	$21.38 \pm 4.03$
II	+ve Control ( $\text{CCl}_4$ administration)	$71.51 \pm 24.94$	$56.19 \pm 28.09$
III	One week extract	$56.44 \pm 20.68$	$46.76 \pm 14.16$
IV	Control extract	$13.67 \pm 22.79$	$27.56 \pm 7.11$
V	Two weeks extract	$41.37 \pm 19.69$	$24.93 \pm 7.44$
VI	One week nano extract-chitosan	$69.41 \pm 28.21$	$37.89 \pm 5.61$
VII	Two weeks nano extract-chitosan	$10.85 \pm 7.35$	$18.96 \pm 7.29$
VIII	Control nano-chitosan	$30.38 \pm 9.77$	$39.91 \pm 15.82$

\*ANOVA test is significant at  $p \leq 0.05$ .

**b. Levels of serum albumin**

Low significance observation ( $p = 0.1$ ) has been shown in serum albumin level among all experimental groups (Table 3). Group I values ranged from (1.748 - 2.636 g/dl), Group II (2.494 - 4.604 g/dl), Group III (2.352 to 3.417 g/dl), Group IV (1.734 - 3.162 g/dl), Group V (1.719 - 3.304 g/dl), Group VI (1.130 - 2.821 g/dl), Group VII (2.238 - 3.460 g/dl) and Group VIII (1.854 - 3.432 g/dl). Based on comparison between the mean values, the maximum albumin levels were recorded among animals in Group II and the lowest were among Group IV (Figure 7).

**c. Levels of serum direct bilirubin**

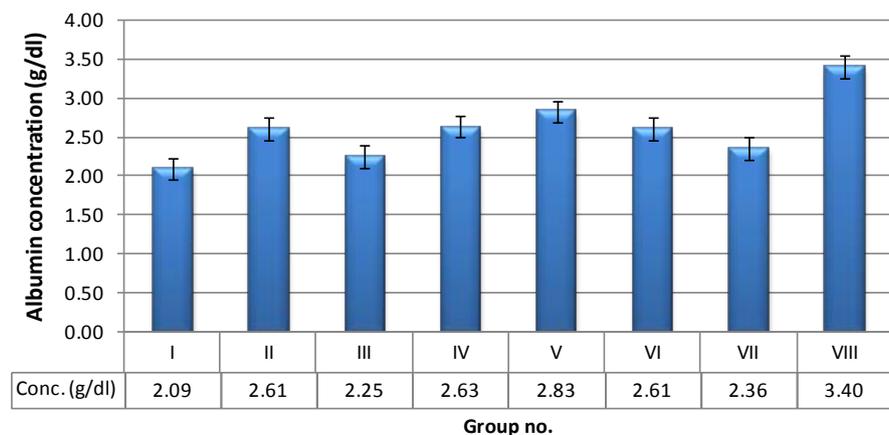
Serum direct bilirubin differs significantly ( $p = 0.02$ ) among all groups but with low significance (Table 4). Group I (the control group) values ranged from (1.008 - 1.224 mg/dl), Group II (2.491 - 2.678 mg/dl), Group III (1.037 - 2.736 mg/dl), Group IV (0.965 - 2.592 mg/dl), Group V (0.950 - 1.224 mg/dl), Group VI (0.965 - 1.138 mg/dl), Group VII (1.152 - 1.382 mg/dl) and Group VIII (0.994 - 1.152 mg/dl). Based on comparison between the mean values, the maximum

**Table 3.** Levels of albumin in rat sera of the different eight animal groups.

Group	Treatment	Concentration (g/dl) (Mean ± S.D.)
I	-ve Control	3.40 ± 0.13
II	+ve Control (CCl <sub>4</sub> administration)	2.10 ± 0.01
III	One week extract	2.61 ± 0.74
IV	Control extract	2.25 ± 0.07
V	Two weeks extract	2.63 ± 0.08
VI	One week nano extract-chitosan	2.36 ± 0.05
VII	Two weeks nano extract-chitosan	2.83 ± 0.05
VIII	Control nano-chitosan	2.61 ± 0.07

One way ANOVA test ( $p = 0.1$ )\*

\*ANOVA test is significant at  $p \leq 0.05$ .



**Figure 7.** Levels of albumin in rat sera of the different eight animal groups (data are means of three measurements). \*ANOVA test is significant at  $p \leq 0.05$ .

**Table 4.** Levels of direct bilirubin in rat sera of the eight animal groups.

Group	Treatment	Direct bilirubin conc. (mg/dl) (Mean $\pm$ S.D.)
I	-ve Control	1.08 $\pm$ 0.072
II	+ve Control (CCl <sub>4</sub> administration)	1.59 $\pm$ 0.76
III	One week extract	1.62 $\pm$ 0.77
IV	Control extract	1.03 $\pm$ 0.09
V	Two weeks extract	0.80 $\pm$ 0.07
VI	One week nano extract-chitosan	0.94 $\pm$ 0.09
VII	Two weeks nano extract-chitosan	1.06 $\pm$ 0.06
VIII	Control nano-chitosan	1.92 $\pm$ 0.08

One way ANOVA test (p = 0.02)\*

\*ANOVA test is significant at p  $\leq$  0.05.

albumin levels were observed among animals in Group II and the lowest were among Group IV (**Figure 8**). **Figure 8** shows all the *in vivo* values of the biochemical parameters measured in the eight animal groups.

### 3.6. Histopathology of the Liver Samples

The photomicrographs of the liver sections of all rat groups under investigation were given in **Figure 8**. Histopathological profile of the control animals showed normal hepatic architecture with discrete hepatic cells, well preserved cytoplasm and sinusoidal spaces (**Figure 8**). Disarrangement of normal hepatic cells with intense centrilobular necrosis was observed in **Figure 8** of Group II (+ve control group; induced group) CCl<sub>4</sub> intoxicated liver (**Figure 8**). Moderate accumulation of fatty lobules and cellular necrosis were observed in the animal treated with 10 mg bacterial extract suspension either as plain or loaded into chitosan nanoparticles (**Figure 8**). Conclusively, the areas of necrosis and ballooning degeneration of hepatocytes were observed in the toxic groups. The group of animals treated with the extract showed a marked protective effect with decreased necrotic zones and hepatocellular degeneration.

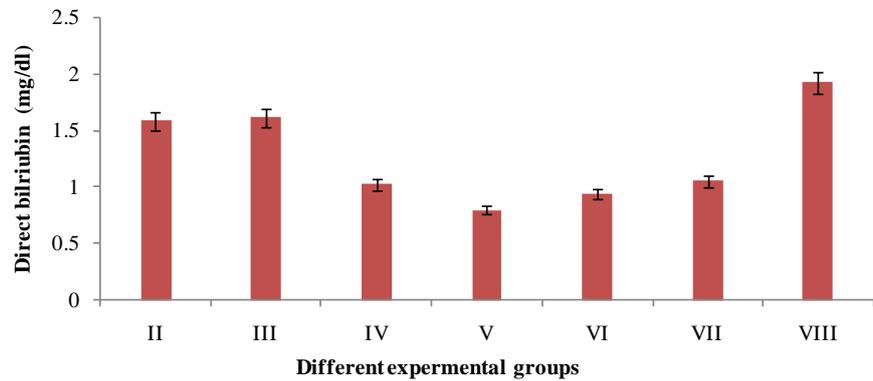
### 3.7. Complete Blood Analysis

#### a. Effect of the bacterial extract on RBCs and related indices

CCl<sub>4</sub> significantly decreased RBCs counts and Hb concentration. Administration of the bacterial extract significantly increased the RBCs count, Hb and haematocrite concentration when compared to control or carbon tetrachloride-intoxicated group as shown in (**Table 5**).

#### b. Effect of the bacterial extract on total and differential leucocytic counts

CCl<sub>4</sub> significantly affected total and differential leucocytes counts. There was a significant difference in the total WBCs count between all groups. Lymphocytes and eosinophiles percent were significantly increased by CCl<sub>4</sub> in (Group I). Monocyte percent was significantly increased in extract treated rats (Group III)



**Figure 8.** Levels of direct bilirubin in rat sera of the eight animal groups (data are means of three measurements). \*ANOVA test is significant at  $p \leq 0.05$ .

**Table 5.** Effect the bacterial extract (plain and chitosan-loaded nanoparticles) on some hematological indices among the eight rat groups.

Group	RBCs ( $\times 10^6/L$ )	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (Pg)	MCHC (g/dl)
I	6.47 $\pm$ 0.51	15.2 $\pm$ 0.43	40.1 $\pm$ 0.56	59.9 $\pm$ 0.33	23.7 $\pm$ 0.25	37.5 $\pm$ 0.63
II	4.41 $\pm$ 0.41	11.1 $\pm$ 0.66	30.5 $\pm$ 0.28	61.7 $\pm$ 0.38	21.2 $\pm$ 0.22	35.9 $\pm$ 0.52
III	4.89 $\pm$ 0.27	11.00 $\pm$ 0.23	30.2 $\pm$ 0.76	62.7 $\pm$ 0.07	20.1 $\pm$ 0.74	36.5 $\pm$ 0.11
IV	6.81 $\pm$ 0.33	13.1 $\pm$ 0.74	33.7 $\pm$ 0.64	58.4 $\pm$ 0.23	21.3 $\pm$ 0.12	37.4 $\pm$ 0.05
V	6.53 $\pm$ 0.48	13.8 $\pm$ 0.22	41.9 $\pm$ 0.80	59.5 $\pm$ 0.27	23.7 $\pm$ 0.71	37.8 $\pm$ 0.17
VI	4.15 $\pm$ 0.32	11.2 $\pm$ 0.04	34.1 $\pm$ 0.43	61.8 $\pm$ 0.22	22.4 $\pm$ 1.2	35.4 $\pm$ 0.70
VII	5.32 $\pm$ 0.64	13.8 $\pm$ 0.56	43.2 $\pm$ 0.33	59.7 $\pm$ 0.15	21.8 $\pm$ 0.65	37.5 $\pm$ 0.22
VIII	4.73 $\pm$ 0.22	15.3 $\pm$ 0.45	47.4 $\pm$ 0.41	59.3 $\pm$ 0.31	23.2 $\pm$ 0.46	37.5 $\pm$ 0.11

RBC: Red blood corpuscles, Hb: Hemoglobin, HCT: Haematocrite, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration. Values are Mean  $\pm$  SD. \*ANOVA test is significant at  $p \leq 0.05$ .

**(Table 6).**

Effect on blood platelets

Results indicated that platelet counts (PLT)  $\times 10^3 \mu l$  in control group was  $632.22 \pm 71.72$ . In  $CCl_4$ -intoxicated rats, platelet counts, platelet distribution width (PDW) and mean platelet volume (MPV) was significantly ( $p > 0.05$ ) decreased in comparison to normal control. Administration of bacterial extract either in the form of plain emulsion or carried into Thenano chitosan particles significantly increased platelets counts, platelet distribution width (PDW) and mean platelet volume (MPV) to levels nearly similar to that of normal control (Table 7).

The elevated levels of serum enzymes, ALT and AST of rats dosed with  $CCl_4$  to induce liver injury (+ve control group) are indicative of cellular leakage and loss of functional integrity of liver cell membrane (Table 2) [53] [54]. These values are significantly higher in Group II (+ve control group) comparing to Group I (-ve control group) indicating a possible induction of liver damage using  $CCl_4$  [55]. Although certain factors such as sex differences, haemolysis of red blood cells, presence of activators and inhibitors as well as presence of pyridoxine

**Table 6.** Effect of the bacterial extract (plain and chitosan-loaded nanoparticles) on total and differential leucocytes count among the eight rat groups.

Group	WBCs ( $\times 10^3/\mu\text{l}$ )	LYMPH (%)	NEUT (%)	EO (%)	MONO (%)	BASO (%)
I	6.83 $\pm$ 0.35	69.63 $\pm$ 0.37	25.20 $\pm$ 0.01	1.11 $\pm$ 0.12	5.21 $\pm$ 0.27	0
II	14.11 $\pm$ 0.9	71.27 $\pm$ 0.11	20.31 $\pm$ 0.01	5.27 $\pm$ 0.02	3.1 $\pm$ 0.10	0
III	15.03 $\pm$ 0.33	72.44 $\pm$ 0.42	17.85 $\pm$ 0.21	5.41 $\pm$ 0.12	6.2 $\pm$ 0.05	0
IV	6.95 $\pm$ 0.48	71.09 $\pm$ 0.39	25.46 $\pm$ 0.17	2.05 $\pm$ 0.05	2.18 $\pm$ 0.1	0
V	7.30 $\pm$ 0.42	60.72 $\pm$ 0.85	37.24 $\pm$ 0.83	1.11 $\pm$ 0.00	2.31 $\pm$ 0.04	0
VI	11.54 $\pm$ 0.32	75.67 $\pm$ 0.63	19.77 $\pm$ 0.82	4.75 $\pm$ 0.07	3.30 $\pm$ 0.82	0
VII	7.21 $\pm$ 0.06	73.22 $\pm$ 0.53	25.21 $\pm$ 0.51	2.66 $\pm$ 0.01	1.52 $\pm$ 0.02	0
VIII	7.72 $\pm$ 0.34	66.58 $\pm$ 0.37	31.82 $\pm$ 0.22	3.12 $\pm$ 0.62	5.5 $\pm$ 0.52	0

WBCs: White blood corpuscles, NEUT: Neutrophils, LYMPH: Lymphocytes, MONO: Monocytes, EO: Eosinophils. Values are means  $\pm$  S.D. \*ANOVA test is significant at  $p \leq 0.05$ .

**Table 7.** Effect of the bacterial extract (plain and chitosan-loaded nanoparticles) on PLT, PDW and MPV among the eight rat groups.

Group	PLT ( $\times 10^3 \mu\text{l}$ )	PDW (fl)	MPV (fl)
I	632.22 $\pm$ 71.72	27.31 $\pm$ 0.21	11.31 $\pm$ 0.05
II	155.13 $\pm$ 5.28	18.34 $\pm$ 0.62	6.420 $\pm$ 0.66
III	170.48 $\pm$ 0.55	29.54 $\pm$ 0.73	6.32 $\pm$ 0.91
IV	553.56 $\pm$ 74.56	27.5 $\pm$ 0.91	11.26 $\pm$ 0.61
V	467.51 $\pm$ 57.01	13.34 $\pm$ 0.89	10.64 $\pm$ 0.49
VI	335.2 $\pm$ 0.37	13.53 $\pm$ 0.23	6.45 $\pm$ 0.63
VII	548.75 $\pm$ 57.18	26.74 $\pm$ 0.52	10.86 $\pm$ 0.44
VIII	580.02 $\pm$ 0.61	13.41 $\pm$ 0.11	10.91 $\pm$ 0.70

PLT: Platelets, PDW: Platelet distribution width, MPV: Mean platelet volume. Values are means  $\pm$  S.D. \*ANOVA test is significant at  $p \leq 0.05$ .

(Vitamin B<sub>6</sub>), may influence the serum AST level since the concentration of AST in erythrocyte is roughly tenfold than normal serum level [56]. The concurrent elevations of serum AST together with ALT indicates that the above factors may likely not be the cause of the elevated serum enzyme activities, but rather it is more likely to be due to the toxicity induced by CCl<sub>4</sub>. Treatment with the ethyl acetate bacterial extract decreased significantly the serum levels of ALT and AST. At the same time, the group treated with the extract-chitosan nanoparticles, decreased significantly the serum levels of ALT and AST comparing to +ve control group and approaching their values of -ve control group. This result could be due to the stabilization of plasma membrane as well as the repair of hepatic tissue damage caused by CCl<sub>4</sub> (Table 2 and Figure 7). The biochemical findings were further substantiated by the liver histological examinations for the liver. CCl<sub>4</sub> produces an experimental damage that histologically resembles viral hepatitis [57]. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures

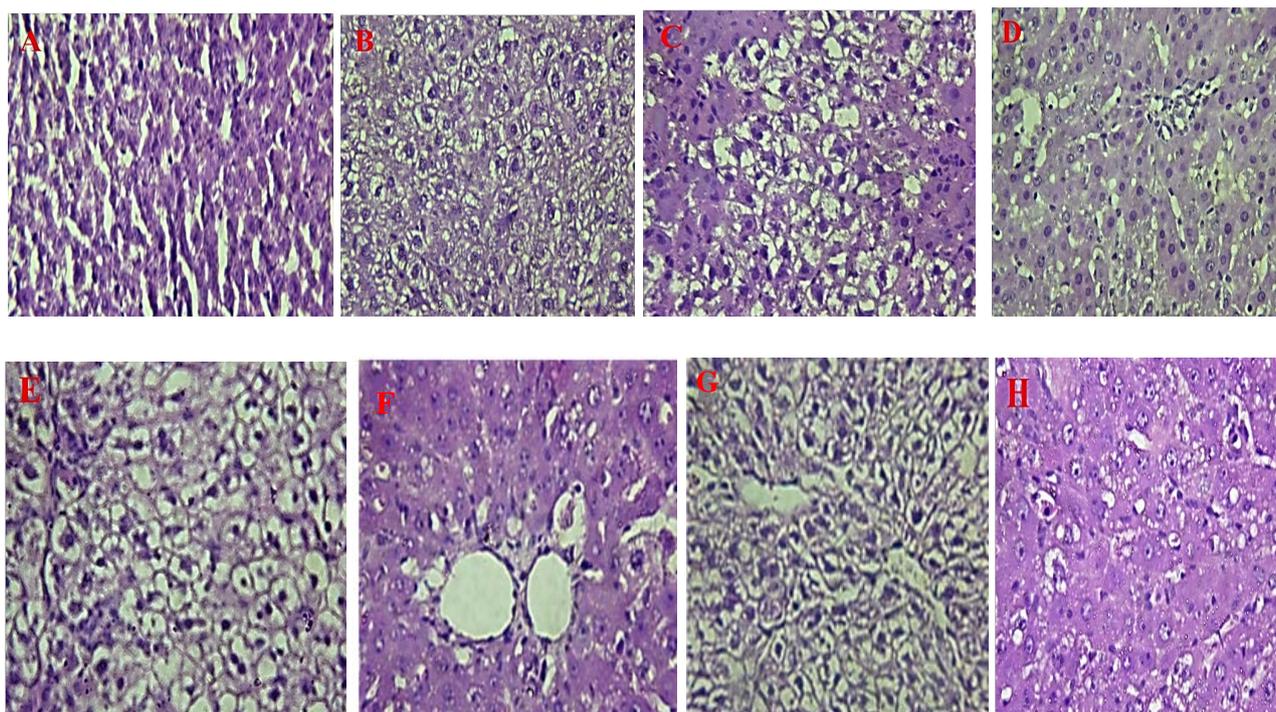
[51]. The toxic metabolite  $\text{CCl}_3$  radical is produced which is further converted to trichloromethylperoxy radical by cytochrome P450 2E1 enzyme. This radical binds covalently to the macromolecules and causes peroxidative degradation of cellular membrane leading to the necrosis of hepatocytes [58].

The concentration of serum direct bilirubin level in Group II (+ve control group, **Table 4** and **Figure 8**) increased significantly comparing to its level in the -ve control group (Group I). The value of serum direct bilirubin indicates the presence of hyperbilirubinaemia, which could be due to liver dysfunction. However, hyperbilirubinaemia is not always present in well compensated cirrhosis [59] and the presence of increased direct bilirubin is probably an indication of acute hepatitis induced by  $\text{CCl}_4$ . The serum levels of albumin in +ve control group (Group II) was found to be significantly decreased comparing to its corresponding value of the -ve control one (Group I). The decrease in serum albumin level in the induced toxicity group *i.e.*, +ve control group (Group II) is associated with hepatic damage which may lead to impaired synthesis of albumin due to impairment of hepatic function and the effect may be severe in chronic hepatitis. The administration of the bacterial extract to Groups III and IV for 7 days using  $\text{CCl}_4$  as well as to Groups V and VII induced hepatotoxicity for 14 days, reduced significantly ( $p < 0.05$ ) the levels of serum AST, ALT as well as direct bilirubin comparing to their levels in Group II (+ve control group). The significant decrease in liver function indices in the bacterial extract administered animals might be due to the decreased leakage of liver hepatocytes enzymes. Accordingly, this indicates that the bacterial extract may reduce and/or reversed the toxic effect of  $\text{CCl}_4$  and prevent leakage of hepatocyte enzymes into blood circulation. The reduction in serum albumin in Group II (administered with 1 ml/Kg  $\text{CCl}_4$ ; +ve control group) was significantly elevated when the rats treated with bacterial extract as shown in Groups III and IV, as well as Groups V and VI treated with nano-extract. This elevation could be related to the curative effect of bacterial extract against liver cell damage induced by  $\text{CCl}_4$ . Clawson (1989) [60] reported that, the decline in protein content may be due to defect in protein biosynthesis as well as disruption and disassociation of polyribosomes from endoplasmic reticulum following administration of  $\text{CCl}_4$ . According to Rajesh and Latha (2004) [61], the improvements in the level of serum albumin of induced hepatotoxicity rats after their treatment with the natural products may be due to the promotion of ribosome assembly on endoplasmic reticulum which facilitates uninterrupted protein biosynthesis [55].

The histopathological changes were observed in the present study including ballooning and fatty degeneration, congestion, reactive changes and loss of normal structure of hepatocytes of rats induced toxicity group (+ve control group), in comparison with Group I (-ve control group). These changes were nearly normalized, when bacterial extract, in dose of 10 mg/Kg b.w. was administered either loaded in chitosan nanoparticles (Groups VI and VII) or not loaded in chitosan nanoparticles (Groups III and V). Many phytoconstituents present in the bacterial extract, are reported to possess hepatoprotective property, like

phenolic compounds [61]. Flavonoids and polyphenols [62], terpenoids [63] and glycosides [64] also were reported to possess antioxidant properties which in turn could exert hepatoprotective effects.

Hepatocytes of -ve control rats group have a regular morphological structure (Figure 9(A)). In case of Group II (+ve control group), when bacterial extract, in dose of 10 mg/Kg b.w. was co-administered with CCl<sub>4</sub> for one week, some of the liver cells were damaged while the rest were nearly normalized as shown in Figure 9(C). In Group III (extract treated control): treated by the 10 mg/Kg b.w. bacterial extract; showed normal hepatic architecture (Figure 9(D)). In case of Group IV (control extract treated group) where rats were treated by 10 mg/Kg b.w. extract with CCl<sub>4</sub> for one week revealed some hepatic cells in normal case but the others were damaged (Figure 9(E)). When the rats were treated by the same extract dose for week more without CCl<sub>4</sub> intoxication, the rats' hepatocytes were normal. In case of Group V (two weeks bacterial extract treated group) the liver cells showed the central vein and necrosis while some cells appeared in normal shape as shown in Figure 9(F). The liver of Group VI (one week nano



**Figure 9.** (A) Histopathological examination of -ve control group (Group I). The liver is showing normal hepatic architecture with discrete hepatic cells, well preserved cytoplasm and sinusoidal spaces; (B) Histopathological examination of +ve control group (Group II). The liver is showing disarrangement of normal hepatic cells with intense centrilobular necrosis and ballooning degeneration of hepatocytes; (C) Histopathological examination of one week extract-treated group (Group III). The liver is showing mild necrosis; (D) Histopathological examination of the control extract-treated group (Group IV). The liver is showing mild necrosis; (E) Histopathological examination of two weeks extract-treated group (Group V). The liver is showing moderate accumulation of fatty lobules and cellular necrosis; (F) Histopathological examination of two weeks extract-treated group (Group VI). The liver is showing moderate accumulation of fatty lobules and cellular necrosis with decreased necrotic zones and hepatocellular degeneration; (G) Histopathological examination of two weeks extract-treated group (Group VII). The liver is showing decreased necrotic zones and hepatocellular degeneration; (H) Histopathological examination of two weeks extract-treated group (Group VIII). The liver is showing moderate accumulation of fatty lobules and cellular necrosis.

extract-chitosan treated group), necrotic and lipid cells were observed as shown in **Figure 9(G)**, while Group VIII (control nano-chitosan treated group): treated by the 10 mg/Kg b.w. nano-chitosan (no extract) with CCl<sub>4</sub> for one week; their liver showed necrotic cells (**Figure 9(H)**). Accordingly, we could say that the bacterial extract, is the more responsible factor for the hepatic protection against CCl<sub>4</sub> toxicity rather than chitosan.

### c. Effect of the bacterial extract on Blood parameters

Although no available literature could be obtained concerning the effect of the tested bacterial ethyl acetate extracts on blood cellular elements, however the increased RBCs count and Hb as well as HCT of rats' blood in extract-treated rats could be attributed to its active constituents. The increased monocyte counts in blood of rats treated with ethyl acetate bacterial extract maybe related to the anti-inflammatory effect of their active constituents [65] [66] and to their immune protectant effect [67] [68]. The present data clearly demonstrate that the administration of ethyl acetate bacterial extract either as plain emulsion or loaded in chitosan nanoparticles has restored the platelets counts which were decreased after CCl<sub>4</sub>-induced intoxication.

## 4. Conclusion

In conclusion, the results of the present study suggest that the bacterial extract has an ability to protect the liver against CCl<sub>4</sub>-induced liver damage, and it could be associated with decreased oxidative stress as well as free radical-mediated tissue damage. Delivery of the bacterial extract through chitosan nanoparticles is a better way to improve the efficacy of result in expression of bioactivity of the bacterial extract as a hepatoprotective agent.

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