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Research article

Effects of fungal secondary metabolites produced from Egyptian marine as hepatoprotective on female rats

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Abstract

The aim of this study was to evaluate the hepatoprotective effect of the secondary metabolites produced from Egyptian marine environment on female rats. Materials and Methods: Different marine specimens were collected from Egyptian seas then used for fungal isolation on biomalt agar medium. The isolated fungi were purified and identified. The four fungal isolates that secondary metabolites obtained through ethyl acetate extraction exhibited the highest antioxidative effects was subjected to full morphological identification and evaluation of *in vitro* and *in vivo* hepatoprotective activities. The hepatoprotective effects were evaluated *in vivo* on rats with determination of (liver function markers), AFP (tumor marker) and hematological profiles. Also, the intracellular MDA, GSH, CAT, and SOD levels were estimated in the rat liver tissue homogenate. The antioxidative activity was measured using DPPH radical scavenging assay. Results: Among the ninety fungi isolated from different marine sources, *Penicillium* represents more than 50% of the isolated fungal colonies followed by *Aspergillus* (30%) and *Fusarium* (14.4%). In this study, the protective effects of the tested fungal secondary metabolites were *in vitro* evaluated against CCl_4 induced HepG2 cytotoxicity. Incubating HepG2 cells with CCl_4 caused a significant loss in the cell viability. Treatment with the tested fungal metabolites resulted in a dose-dependent increase in cell viability. In the current study, fungal secondary metabolites showed protection in rats against hepatic lipid peroxidation and preserved GSH levels and activities of antioxidant enzymes namely, catalase (CAT), and superoxide dismutase (SOD). Conclusion: The results showed that the tested fungal metabolites had potent cytoprotective effect against oxidative damage induced by *CCl_4* in HepG2 cells and rats liver, thus suggesting their first time discovered potential use as liver protectant.

Introduction

In human body, liver is the main organ for various metabolic reactions including oxidation, sulfation, acetylation, hydrolysis and conjugation reactions. It has an important role in the maintenance, performance and regulating homeostasis mechanism of the body. Injury to liver and damage to the hepatic parenchyma cells were always proved to be associated with distortion of different metabolic functions of liver [1]. Etiologically various infectious agents including viruses and different hepatotoxins along with environmental pollutants are thought to be responsible for causing different types of liver damage and hepatic injuries [2-3].

Liver diseases are a major health problem worldwide, making it necessary to develop new molecules that help counteract or prevent such diseases. On account of this fact, investigations aiming to obtain natural and/or synthetic compounds possessing hepatoprotective activity have been undertaken. The development of new drugs consists of a variety of steps, ranging from the discovery of the pharmacological effects in cellular and animal models, to finally demonstrate their efficacy and safety in humans. Different models for assessment of the hepatoprotective activity *in vitro*, *ex vivo* and *in vivo* can be found in medical literature [4].

The pathophysiological role of free radicals and oxidative stress in liver damage, acute and chronic hepatic injury were clearly observed in recent studies on free radicals research [5-6]. The mechanism of actions of potent hepatotoxins such as CCl4, paracetamoletc, also indicated the role of oxidative stress and free radicals in the pathophysiology of hepatic injury. Excess production of reactive oxygen species (ROS) along with significant

decrease of antioxidant defense in these pathological conditions impairs and alters various cellular functions through the processes of lipid peroxidation. Accordingly, effects of antioxidants or free radical scavengers have been widely tested for the prevention and treatment of acute and chronic liver damages [7-9]. Free radicals mainly act by attacking the unsaturated fatty acids in the cell membranes causing membrane lipid peroxidation which is a hallmark sign of hepatotoxicity, and damaging membrane protein which finally triggers the cell inactivation and death [10-11]. Available data from earlier studies also indicated the beneficial effects of antioxidants specifically, for prevention and treatment of acute and chronic liver injury [9, 12]. Therefore, many natural products are tested for its antioxidant and hepatoprotective potential on liver damage by using various animal models [9, 13].

The marine environment may be explored as a rich source for novel drugs. A number of marine-derived compounds have been isolated and identified, and their therapeutic effects and pharmacological profiles are characterized [14].

This work was designed to investigate the pharmacological effects derived from fungal secondary metabolites produced from Egyptian marine environment, including *in vitro*, and *in vivo* hepatoprotective activities as well as antioxidant activities on liver damage induced by carbon tetrachloride (CCl4) hepatotoxicity.

Materials and methods

This research work was conducted in the Regional Center for Mycology & Biotechnology, Al-Azhar University, Nasr city, Cairo, Egypt during the period from October 2016 till August 2018.

Materials

Samples collection

The following samples were collected from different Egyptian marine sources (sea water, Sea shore Soil, Sea weeds, Wood decay, Sediments & Mollusks) from red sea (Ismailia, Suez & South Sinai), Mediterranean Sea (Alexandria, Damietta & Port Said governorate), different Lakes (El-Fayoum, Damietta & Port Said governorate). The sea water samples were collected in sterile tight bottles, soil samples were collected after 10–15 cm deep pits dug and solid samples in sterile zipper polythene bags and transferred to the laboratory in 24 h of duration and stored at 4°C until used [15].

Media used

Czapek's Dox Agar Medium, Czapek's Agar (Cz) Medium, Malt Extract Agar (MEA) Medium, Potato Dextrose Agar (PDA) Medium, Czapek Yeast (autolyzate) Extract Agar (CYA) and Nutrient Agar Medium[16].

Chemicals used

Glucose, L-glutamine, yeast extract, beef extract, malt extract, peptone, acetone, toluene, ethyl acetate, formic acid, petroleum ether, chloroform, methanol, crystal violet, dimethyl sulphoxide (DMSO), silica gel (100, 63-200 mesh), anisaldehyde, sulfuric acid, glacial acetic acid, EDTA, chloramphenicol, silymarin MTT (3-[4.5-dimethylthiazol-2yl]-2.5-diphenyltetrazolium bromide) and (2, 2-diphenyl-1-picrylhydrazyl) were obtained from Sigma-Aldrich, USA. Phosphate buffer saline (PBS), fetal bovine serum, DMEM, Trypsin/EDTA, HEPES buffer, gentamycin solution and Trypan blue stain were obtained from Life Technologies Inc., Grand Island, NY, USA. All other chemicals used in this study were analytical grade.

Animals used

One Hundred and four female albino Wister rats [17] weighing (120-190 g) were purchased from Helwan animal station, Ministry of Health, Egypt. Animals were allowed to adapt for two weeks and housed under standard experimental condition in cages in animal house of Zoology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.

All of the ethics documents or statements required for the manuscript type that was selected as the guidelines Committee of the National Research Center, Dokki, Cairo, Egypt, and the National Institutes and all animals received human care in agreement with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt, and the National Institutes of Health (NIH publication 86-23 revised 1985).

Cell line and culture condition

HepG2 cell line (hepatocellular carcinoma) (# HB-8065™) was procured from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in DMEM containing 10% FBS, 1 mm sodium pyruvate, and 2 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37°C.

Methods

Cultures isolation and purification

In case of non-aqueous marine sample, a suspension of 50 g (Sea shore Soil, Sea weeds, Wood decay, Sediments & Mollusks) in 90 mL of sterilized distilled water was prepared and 0.5 mL of this suspension were placed in Petri dishes in triplicate containing Malt extract Agar medium amended with an antibacterial agent (chloramphenicol, 0.5 g/L).

For isolation of fungi, 0.5 mL of sea water were placed on the surface of the same medium, in Petri dishes, which were prepared in triplicate and incubated for 5-7 days at 25°C. Fungi growing on the agar plates were purified and transferred to malt extract slants and then maintained as a

stock culture and submitted to macroscopic and microscopic observations.

Preparation of inocula (fungal material)

The pure isolates used in this investigation were inoculated on Petri-dishes containing malt extract agar medium (MEA) and incubated for 7-10 days at 28°C ±2 until sporulation. The spores were harvested by adding 10 ml of sterilized distilled water to the cultures on the surface of the agar slants and gently dislodging spores with a sterile inoculation loop. The spore suspension was filtered through four layers of sterile cheese cloth followed by filtration through Whatman No. 1 filter paper to remove mycelial debris. Spores were enumerated using an Improved Neubauer bright line hemocytometer. Appropriate dilutions were made from the stock spore suspension using sterile 0.1% (w/v) peptone water as diluent to obtain the desired inoculum's level of $4x10^2$ cells/ml [18].

Cultivation of isolates for production of secondary metabolites

For the production of secondary metabolites, biomalt liquid medium was used. Five Liter was used for each fungal strain. The medium was distributed into 250-ml Erlenmeyer flasks each containing 100 ml of the medium. The pH was adjusted to 6.5 ±0.2. The medium was then autoclaved at 121°C for 15 min. The medium (100 ml) was inoculated with 1ml spore suspension of the studied fungus and incubated under aerobic conditions at 25°C for 21 days [15].

Extraction of extracellular secondary metabolites

At the end of each incubation period, inoculated flasks were collected. The culture filtrates were separately subjected to solvent extraction as previously described by Younis *et al.* [19] with slight modifications. For each fungus the whole broth (5 L) was filtered through Whattman No.1 filter paper to separate culture filtrate and mycelia. The culture filtrates were mixed with petroleum ether in a separating funnel, shaken vigorously well and left to settle down for at least six hours until complete separation. The petroleum ether layer (top layer) was then separated. The residue was re-extracted twice for complete extraction; the solvent was evaporated and stored at 5°C. The other layer of filtrate was subjected to ethyl acetate solvent system three successive times then concentrated by using a rotary evaporator (Buchi RV 4) to dryness and stored at 5°C till tested [20].

Chromatographic investigation

Each fungal extracts were chromatographically investigated using three systems a, b and c, system (a) ethyl acetate: methanol: water 90: 5:4 v/v/v, system (b) chloroform: methanol 95: 5 v/v and system (c) benzene: ethyl acetate 86: 14 v/v. Visualization of the spots were carried out under UV. TLC is performed to analyze the fractions (compounds) present in the crude extract. Separation of the compound depends on the usage of solvents. Silica gel is prepared in slurry form and evenly spread on glass plate. Crude extract prepared with a concentration of 1 mg/ml was placed on the TLC plate and dried. After running with Hexane and Ethyl acetate solvents at different proportions, spots were identified with iodine crystal vapors [21]. All fractions were stored at 4°C. Bioactivity was used as a guide for purification hence all fractions were tested for their biological activities to determine the active ones. Some of the collected fractions were combined based on similarities in TLC properties. TLC was performed on aluminum sheet plates precoated with silica gel G-60 (GF254, layer thickness 0.2 mm, Merck, Darmstadt, Germany). Fractions having high activity were automatically spotted on TLC plates using CAMMAG® LINOMAT 5 application system and the developing processes were carried out with two solvent systems consisting of Chloroform: Methanol (9:1; v/v) or Toluene: Ethyl acetate: Formic acid (TEF, 7: 5: 1; v/v/v). The plates were dried at room temperature then the purity of fractions was scanned using CAMMAG ®TLC scanner unit and detected from their UV absorbance at 254 and 365 nm or appearance in visible light to detect the characteristics of all spots, i.e. color and Rf value were recorded.

Antioxidant activity assay

The antioxidant activity of the tested metabolites was determined by the DPPH free radical scavenging assay in triplicate and average values were considered. Briefly, freshly prepared (0.004%w/v) methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10°C in the dark. A methanol solution of the test compound was prepared. A 40 µL aliquot of the methanol solution was added to 3ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = [{(AC-AT)/AC}x 100]. Where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample + DPPH at t = 16 min.

The IC50 value, i.e., the concentration of the tested compound leading to 50% inhibition of the DPPH radical was estimated from graphical plots of DPPH radical scavenging vs compound concentrations [22].

Hepatoprotective study in HepG2 Cell line

HepG2 cell line is suitable for *in-vitro* model system for the study of polarized human hepatocytes. HepG2 cell line with proper culture conditions displays robust morphological and functional differentiation with a controlled formation of apical and basolateral cell surface domain in models. The HepG2 cell line was maintained in DMEM medium. The cells were incubated in 75 cm3 culture Corning® flask at 37°C in humidified water jacketed CO2 incubator (Shel Lab, Sheldon Manufacturing, Inc.®, USA) for 48 hrs. Cultures were viewed using an inverted microscope (CKX41; Olympus, Japan) to view the cell monolayer and confirm the absence of bacterial and fungal contaminants. To count the number of cells, the cell monolayer was washed with 5 ml phosphate buffer saline (PBS) without Ca^{2+}/Mg^{2+} then 2.5 ml of 0.53 mm trypsin/EDTA solution was added to the culture flask, and incubated for 7-15 min. When cells were displaced from the flask, 6 ml of maintenance media were added to stop the action of the trypsin. A hemocytometer was used to determine the number of viable cells using trypan blue staining [23].

The cells were seeded in 96-well plate, flat-bottomed microtiter plates (Falcon, NJ, USA), in 100µl of growth medium at a cell concentration of 1×10^4 cells per well. After 24 h of seeding, the confluent cell monolayers dispensed into 96-well plate were then washed with sterile phosphate buffered saline (0.01 M pH 7.2). The HepG2 cells were exposed to toxicant containing 1% CCl4 along with /without the tested fungal metabolites of different concentrations or the medium alone is considered as control. Serial two-fold dilutions of the tested metabolite (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 µg/ml) were added to confluent cell monolayers dispensed into 96-well microtiter plates using a multichannel pipette. Similar concentrations from silymarin were tested as reference drug for comparison. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 24 h. Three independent experiments were performed each containing three replicates for each concentration of the tested samples. At the end of the period, the viability of the HepG2 cells was assessed by the MTT reduction assay, CBB assay and LDH assay.

MTT assay

The metabolic activity of living cells was assessed by the activity of dehydrogenases [23-24]. The MTT (3-[4.5-dimethylthiazol-2yl]-2.5-diphenyltetrazolium bromide) was dissolved in a sterile phosphate buffer saline solution at the concentration of 5 mg/ml and sterilized by filtration through a 0.22 µm filter and protected from light by covering into aluminum foil. After incubation of the cells with the substances, 10 µl of the MTT solution was added to every well of 96-well plates and incubated for 3 h at 37°C in humidified atmosphere of 5% CO_2. Formazan crystals were solubilized overnight in an SDS buffer (10% SDS in 0.01N HCl) and the product was quantified by measuring absorbance at 570 nm wave length using Micro-plate Reader (SunRise, TECAN, Inc, USA).

The hepatoprotective percentage was calculated using the Microsoft Excel; according to the following equation: Hepatoprotective % = [(ODt/ODc)] × 100%, where ODt is the mean optical density of wells treated with the tested metabolite and ODc is the mean optical density of untreated cells. The test metabolites were also compared using the EC_{50} value, i.e., the effective median concentration of an individual metabolite leading to 50% hepatoprotection that was estimated from graphical plots of surviving cells vs metabolite concentrations.

Coomassie brilliant blue (CBB) assay

Total cellular protein was measured by the dye uptake being incorporated into the cells of the culture, which reflected the degree of cytotoxicity effects caused by the tested substances [25].

LDH assay

Lactate dehydrogenase (LDH) release assay was done to determine the effect of the secondary metabolites on membrane integrity and permeability in HepG2 cells. The amount of cytoplasmic LDH released into the medium was determined by using the commercially available Cytotoxicity Detection (LDH) Kit (Roche Diagnostics, Poland) [26].

In vivo experimental procedure

Rats were weighted and randomized into 7 groups of 8 rats each. Blood samples were collected before and after treatment and analyzed for various biochemical and hematological parameters. Group (I) animals received CMC (0.3%) and served as negative control. Group (II) animals served as CCl_4 treated positive control. Group (III), (IV), (V), and (VI) received metabolites S3, S64, S67 and S73 at a dose of 200 mg/kg, respectively. Group (VII) animals received standard Sylimarin (200 mg/kg). The animals were treated for 7 days as per the study design mentioned above and on the 7th day after one hour of dosing, the toxicant 30% of CCl_4 (1ml/kg i.p.) was administered to all the groups except Group (I). After 24 h, the animals were anesthetized and blood was collected from retro orbital plexus [16] using Halothane and heparinized capillary tubes. Blood drops were collected, gently, serum was separated by centrifugation (2500 rpm for 15 min), and EDTA was used as an anticoagulant for haematological parameters. Total Bilirubin, Alkaline phosphatase (ALP), Alanine amino transeferase (ALT), Aspartate amino transferase (AST), GGT, albumin, urea, creatinine, and total protein were determined in the serum using diagnostic kits [27]. Haemoglobin concentration (Hb),), red blood cells count (RBCs), total leucocytic

count (TLC) and platelets were measured in EDTA samples.

The animals were sacrificed later and the liver was perfused and excised. The liver portion was rinsed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation. A part of homogenate after precipitating proteins with Trichloroacetic acid was used for estimation of glutathione. The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of SOD and CAT activity.

Determination of Reduced Glutathione (GSH)

To measure the reduced glutathione (GSH) 0.2 ml of liver tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml precipitating reagent (1.67 g of met phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2.0 ml of the supernatant, 4.0 ml of 0.3-M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5,5-dithio bis 2-nitro benzoic acid) reagent were added and was read at 412 nm [28].

Determination of Super Oxide Dismutase (SOD)

The assay mixture contained 1.2 ml sodiumpyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1ml phenazine methosulphate (186 mM), 0.3 ml NBT (300 mM), 0.2 ml NADH (780 mM), diluted enzyme preparation and water in a total volumeof 3 ml. After incubation at 30ºC for 90 sec, the reaction was terminated by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol and concentration of SOD was expressed as units/mg protein [29].

Determination of Catalase (CAT)

The liver tissue was homogenized in phosphate buffer (pH 7.0) at 4ºC and centrifuged at 5000 rpm. Thereaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2mM H2O2and the enzyme extract. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The specific activity of catalase was expressed in terms of U/mg protein [30].

Animal toxicity

A toxicological study of the tested secondary metabolites of the fungi which possesses hepatoprotective activity was conducted by acute oral toxicity assay performed according to the OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001). Wister Rats were weighted and randomized into 5 groups of 8 rats each then fasted overnight prior to the experiment and maintained under standard laboratory conditions. The control group was given only distilled water while the tested groups were received the active metabolites administrated orally in increasing dose up to 1000mg/kg. The behavior and the illness signs were recorded through 2 weeks of treatment.

Identification of most active fungal isolates

The isolated fungi were identified to the genus and the species level on the basis of their morphological characters and microscopic analysis by using suitable media, slide cultures and the most taxonomic guides and standard procedures [31-34].

The selected four fungal isolates were subjected for certain morphological studies by an Image Analysis System using Soft-Imaging GmbH software (analysis Pro ver. 3.0) at the Regional Center for Mycology and Biotechnology, Al-Azhar University. The plates (in triplicates) were incubated at 25°C for 7 to 15 days (15 days for the production of sclerotia and ascospores). After seven days of incubation, the gross morphology on different growth media viz. the rate of growth, colony diameter, colony texture, colony color, and reverse pigmentation. The measurements of the diagnostic structures that characterized the species were taken at 40X lens (10-20 units of each structure).

Statistical analysis

The data were expressed as mean ±S.D. The statistical significance of the difference between mean values was determined by Student's unpaired t-test. Data were considered statistically significant at a significance level of $P < 0.05$. STATA statistical analysis package was used for the dose response curve drawing in order to EC_{50} and IC_{50} calculations.

Results

Isolation of fungal cultures from different Egyptian marine sources

In the present investigation, ninety fungal isolates were isolated from different marine sources. The sources of samples from different areas of Egypt were obtained from sea water (at 1 miter depth), Woody decay (in the sea bottom), mollusks, sea weeds and sea shore sands when inoculated on the Biomalt solid medium amended with antibacterial agents and the fungal species were isolated and purified for identification. Mollusks represented the highest source of fungal isolates followed by marine water (Figure 1). Ninety marine fungi were isolated from thirty nine samples collected from different areas of Mediterranean sea of Alexanderia governorate (16.7%), Port said governorate (14.4%), Matrouh governorate, Damietta governorate (3.3%) and Red sea of Ismaalia

governorate (21.1%), (Ain Sokhna) Suez Governorate (1.1%), Sharm El-Sheikh of South Sinai Governorate (4.44%), Hurghada of South Sinai Governorate (4.44%) and Qaroun lake in El Fayoum Governorate (13.3%) as represented in Figure 2. Among the isolated fungi, *Penicillium* represent more than 50% of the isolated fungal colonies (Figure 3) followed by *Aspergillus* (30%) and *Fusarium* (14.4%).

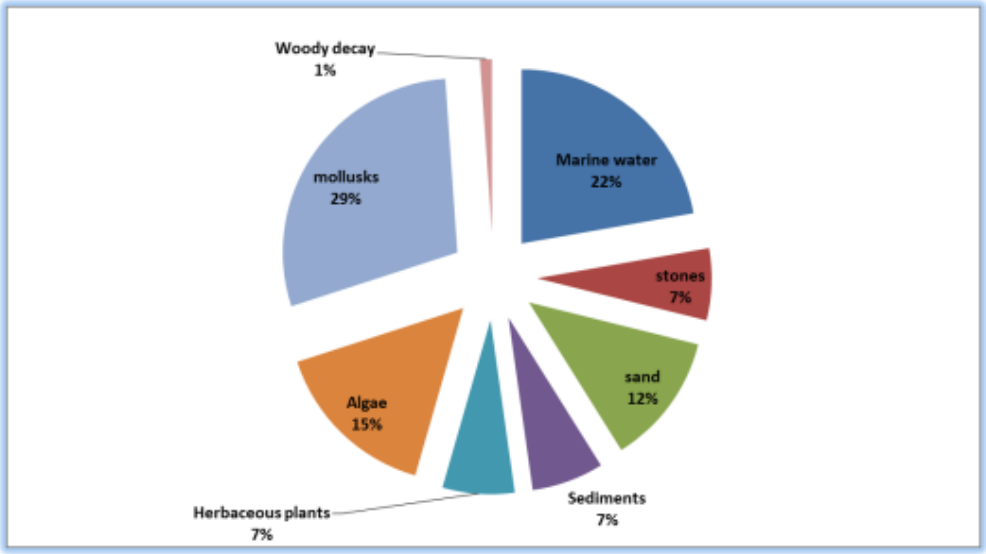


Figure 1. Distribution of the isolated fungi among different marine sample sources.

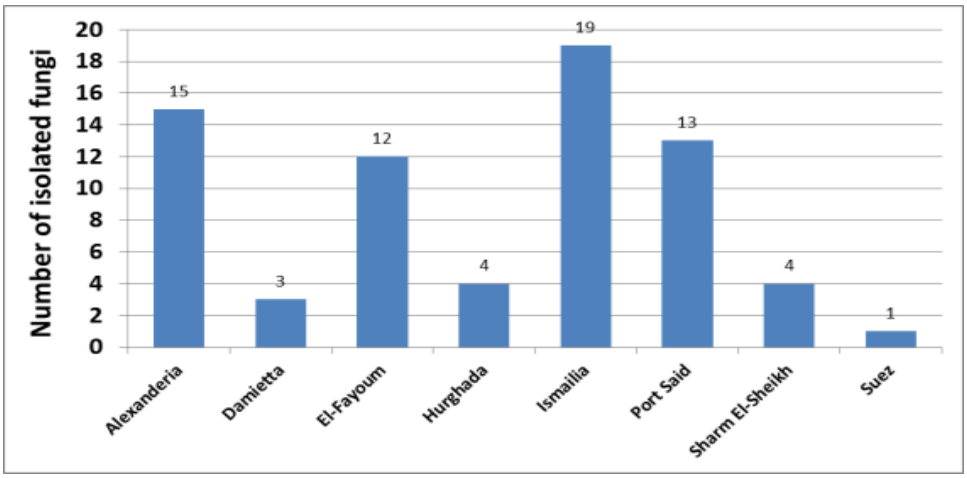


Figure 2. Frequency of the isolated fungi among different marine Egyptian sites.

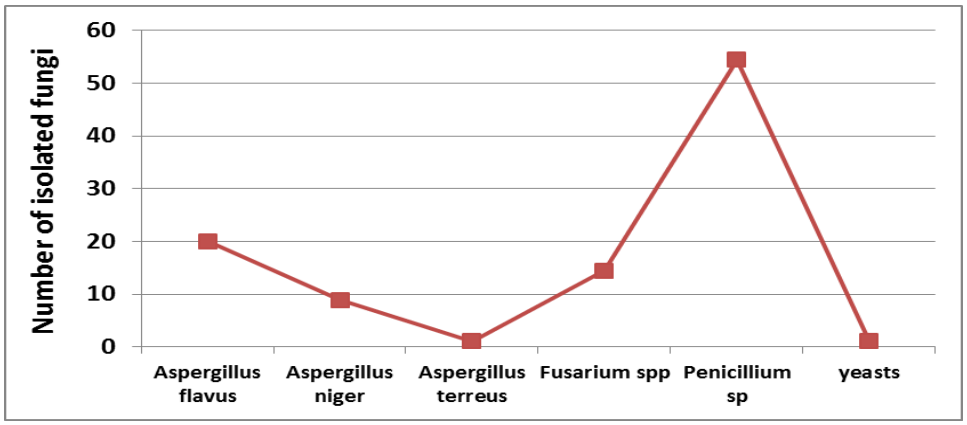


Figure 3. Number of isolated fungal genera among different marine Egyptian isolates encountered in this study from different sources.

In the present investigation, the extracellular secondary metabolites obtained from Ninety isolates were defatted with hexane then extracted three times by ethyl acetate. The crude ethyl acetate extracts were concentrated to dryness then tested for antioxidant activities. The antioxidant activity of the ethyl acetate extracts obtained from investigated marine fungi was performed by DPPH free radical scavenging assay at 1 mg/ml and the results are presented in Figure 4. The highest radical scavenging percentage was detected for 12 isolates that showed maximum antioxidant activities. However, nine isolates showed very good antioxidant activities. On the other hand, 24 isolates showed good antioxidant activities. Moreover, twenty isolates showed weak antioxidant activities. Additionally, 25 isolates showed non-significant antioxidant activities.

The four fungal isolates that those secondary metabolites exhibited the highest antioxidative effects was subjected to full morphological identification and evaluation of *in vitro* and *in vivo* hepatoprotective activities. Two active isolated fungal samples encoded S64 and S67 were identified as *Penicillium* sp. and *Fusarium* sp. However, 2 Aspergilli were also exhibited highest potency encoded S73 and S3 that identified as *Aspergillus flavus* and *A. terreus*, respectively (Figure 5).

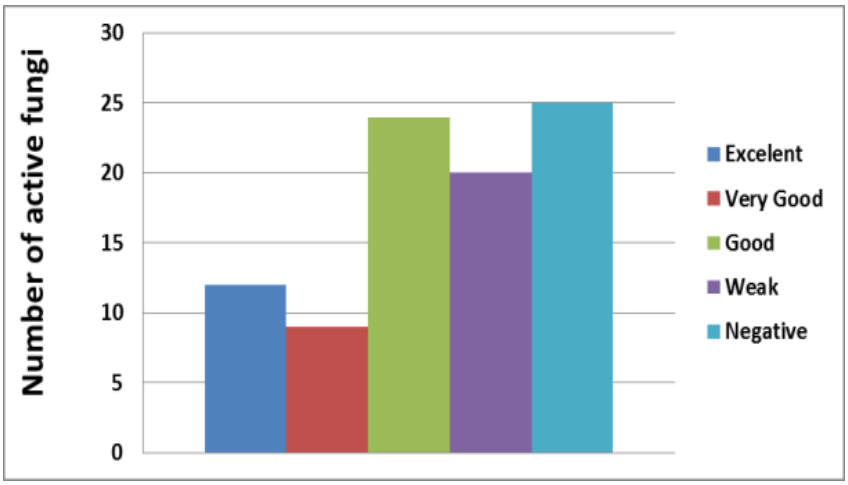
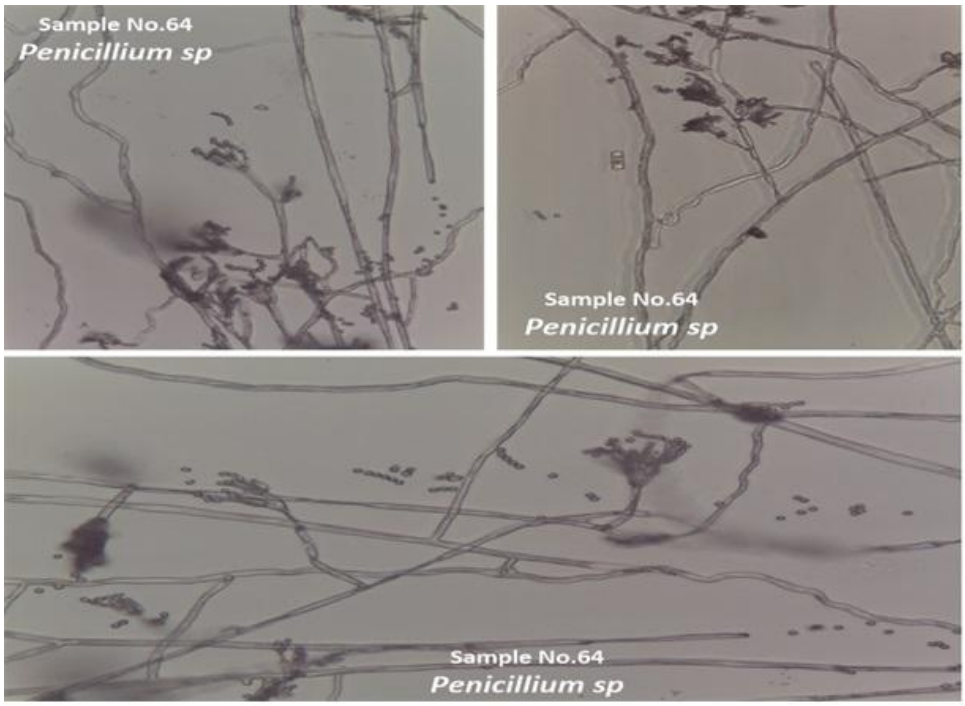
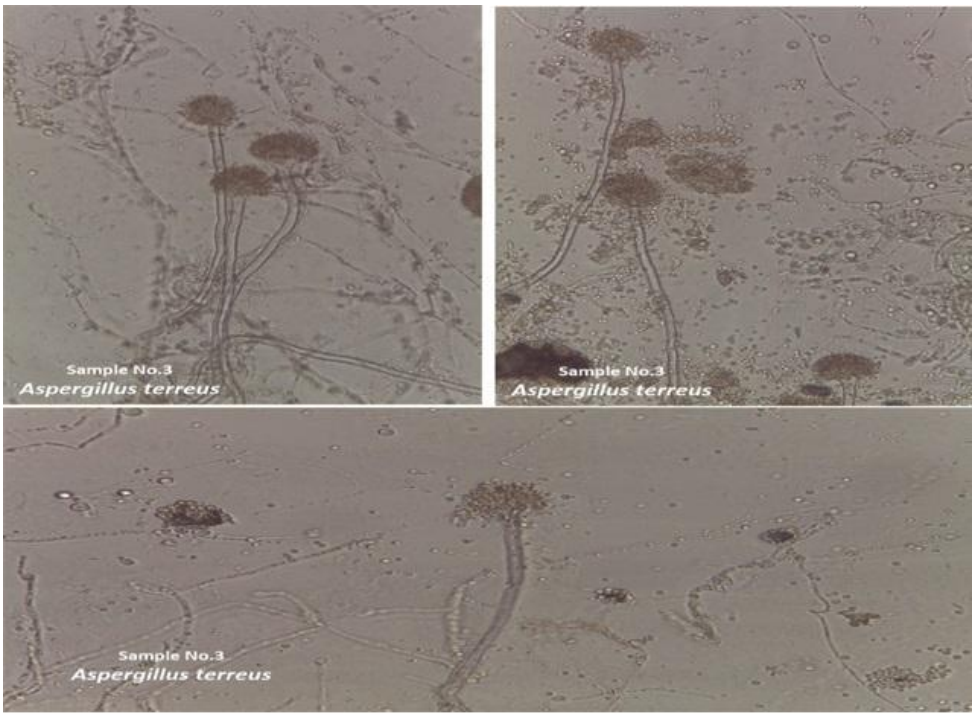


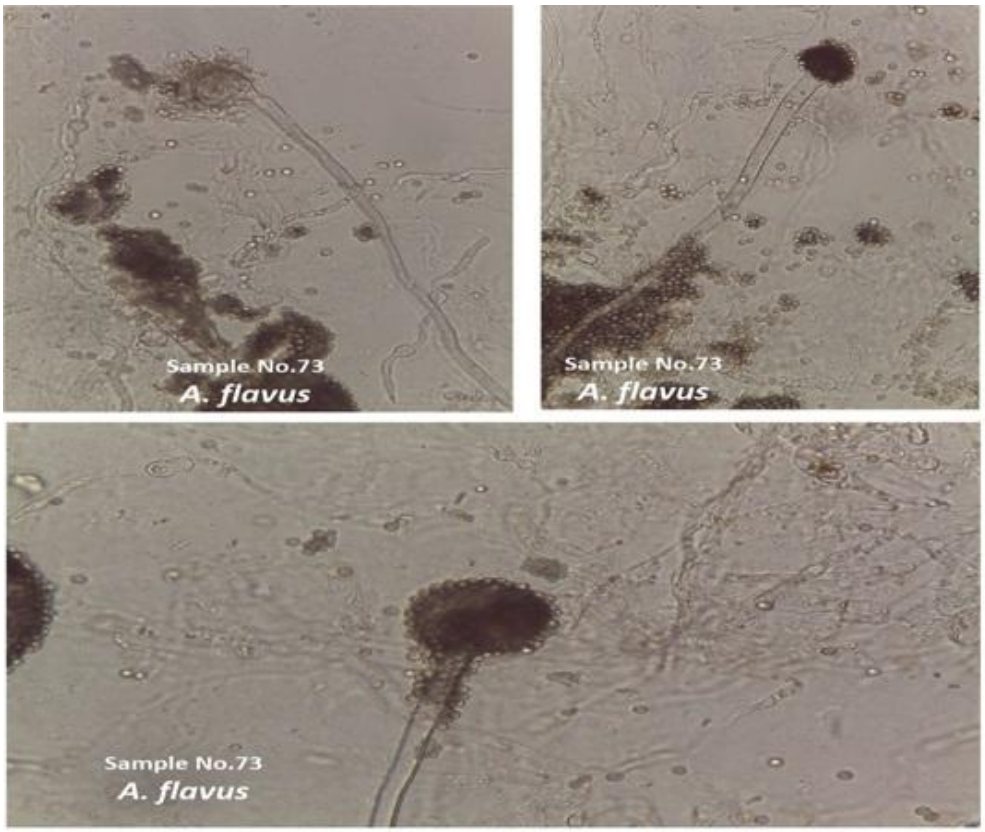
Figure 4. The radical scavenging percentage showing the variation in antioxidant activities among the secondary metabolites obtained from isolated fungi. Where Excellent: ++++; Very good: +++; good: ++; weak: +; Negative: No radical scavenging activity.



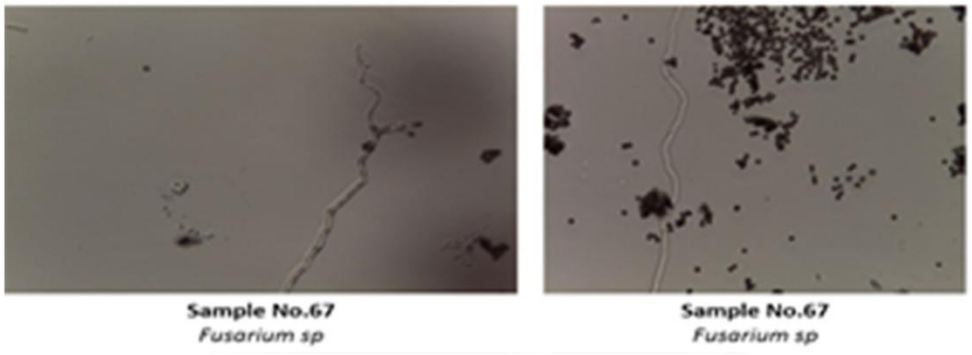
Sample No. 64 isolated from water of Mediterranean Sea at Port Said Governorate was identified as *Penicillium sp.*



Sample No. 3 isolated from stones of red sea at Ismailia Governorate was identified as *Aspergillus terreus.*



Sample No. 73 isolated from water of Qarun lake at El-Fayoum Governorate was identified as *Aspergillus flavus.*



Sample No. 67 isolated from sediment of Mediterranean Sea at Port said Governorate was identifies as *Fusarium sp.*

Figure 5. The culture characteristics of the four bioactive isolated fungi when grown on malt extract agar medium for 7 days at 28°C.

Hepatoprotective effect of secondary metabolites on HepG2 Cell line

In the *in vitro* study, HepG2 cells exposed with CCl_4 showed depletion of viability by 83% whereas fungal secondary metabolites received HepG2 cell after exposure to CCl_4 showed a dose-dependent increased percentage viability ($P<0.001$) at concentrations ranging from 4 to 800 µg/ml as shown in Figure 6. The cytotoxic effects of the tested fungal metabolites on HepG2 cells exposed for 24 h were also performed in the same assay experimental conditions and showed that all these metabolites exerted non-significant toxic effects on HepG2 cells (ranged from 6 to 28% decrease in cell viability compared with untreated cells).

The cell metabolism (MTT assay, Figure 6), cellular protein content, and the integrity of cell membrane (LDH release, Figure 7) were significantly affected in a concentration-dependent manner after 24 h exposure of intoxicated HepG2 cells in tested concentrations when compared to the control ($P<0.05$). The results of MTT, cellular protein content and LDH assays with EC_{50} in intoxicated HepG2 cells are shown in Table 1. The mean values of median effective concentrations in MTT assay were lower than for other performed tests and statistically different ($P<0.05$). Then, best value of 50% protection was attained at a concentration of 162.6 µg/ml by metabolite S73. In comparison with silymarin standard, the order of hepatoprotective activities of the tested secondary metabolites was S73, S67, S3 and S64, respectively.

In vivo hepatoprotective activity

The obtained results (Figures 8 & 9) further indicate their hepatoprotective effects. However, acute administration of CCl_4 produced a marked elevation of the serum levels of AST, ALT, ALP, and serum bilirubin in animals (Group II) when compared with normal control (Group I). Treatment with the tested fungal metabolites at a dose of 200 mg/kg significantly reduced the elevated levels of ALT, AST, ALP and bilirubin ($p<0.01$) towards the respective normal value (Figure 9).

Also, fungal metabolite received groups showed increased levels of total protein and albumin which indicates its hepatoprotective activity comparable with standard drug Sylimarin (Figure 9).

The serum levels of the kidney function tests (urea and creatinine) as well as Hemoglobin and platelets levels were still in the normal range even after inducing the animals with CCl_4 (Figures 8 & 9). However, non-significant effect was observed in tumor marker AFP after treatment (Data not shown) that can be attributed to the tumor marker needs prolonged exposure periods to decrease its levels.

Table 1. The mean values of the effective concentrations from the tested secondary metabolites that exhibited hepatoprotective activities of on HepG2 cell line exposed to CCl_4 when tested using different assays.

Compound Tested	MTT assay	Total Protein assay	LDH release assay
		EC_{50} values (µg/ml)	
S3	356.7 ± 22.5	411.2 ± 30.4	389.1 ± 19.7
S64	737.5 ± 33.8	> 800	> 800
S67	283.4 ± 15.4	342.6 ± 21.5	378.3 ± 17.1
S73	162.6 ± 5.9	189.3 ± 7.6	213.4 ± 10.8
Silymarin	192.8 ± 4.7	218.9 ± 13.1	205.6 ± 9.4

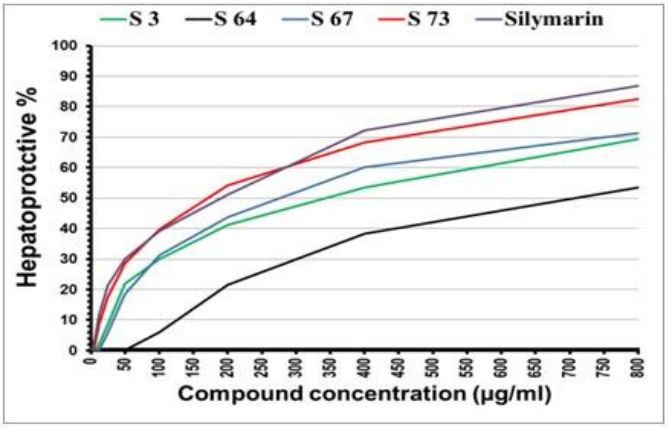


Figure 6. The in vitro hepatoprotective activities of the secondary metabolites obtained from isolated fungi on HepG2 cell line exposed to CCl_4 compared with silymarin standard when tested using MTT viability assay.

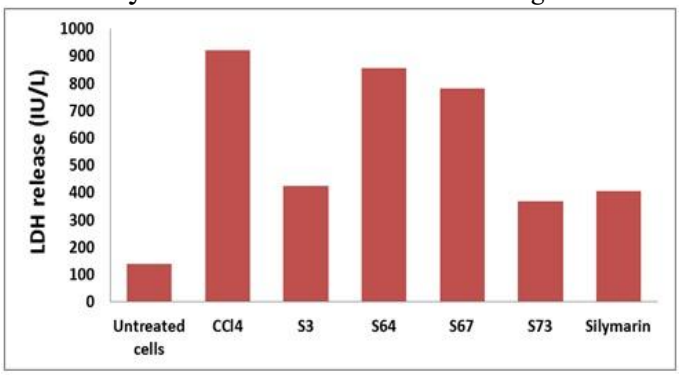


Figure 7. The concentrations of lactate dehydrogenase enzyme released after 24 h treatment of the HepG2 cell line intoxicated with CCl_4 by 200 µg/ml from the tested secondary metabolites compared with silymarin standard.

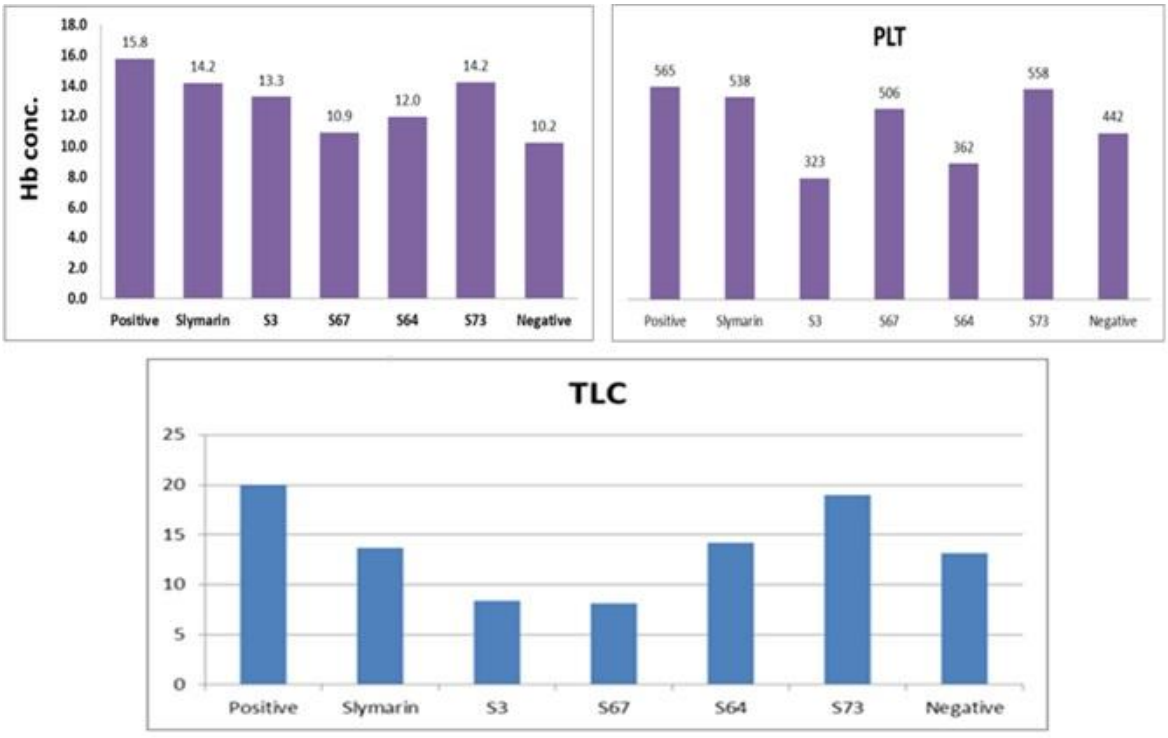


Figure 8. The effect of the secondary metabolites obtained from isolated fungi on haematological parameters of rat blood showing hepatoprotective activities on rats exposed to CCl_4 compared with silymarin standard. The data are expressed as the mean values of six replicates of each parameter.

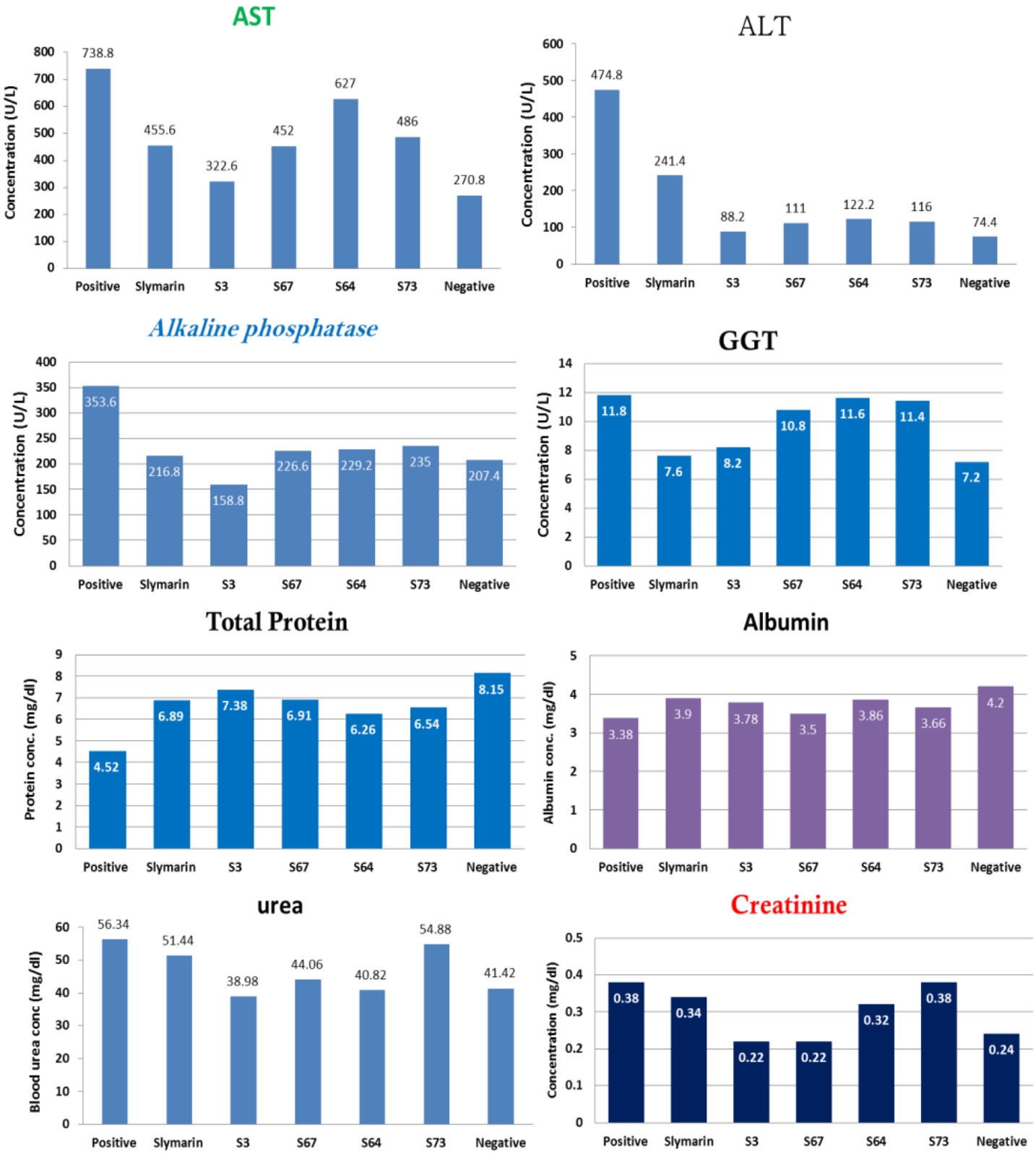


Figure 9. The mean values of the biochemical parameters measured in rat serum samples showing the hepatoprotective effect of the tested fungal secondary metabolites on CCl_4 intoxicated rats compared with silymarin standard.

Effect of metabolites on antioxidant status

An increase in lipid peroxidation MDA level (LPO from 0.4 to 2.1, $p < 0.001$) in liver homogenate was detected in CCl_4 intoxicated rats suggests enhanced lipid peroxidation which leads to tissue damage and failure of antioxidant defense mechanisms resulting in excessive free radicals (Figure 10B). However, treatment with the tested metabolites at a dose of 200 mg/kg, produced significant ($P<0.01$) reductions in lipid peroxidation when compared to CCl_4 administered rats. On the other hand, it was observed that the fungal metabolites caused a significantly increased in the hepatic catalase and SOD ($p< 0.05$) activity of the CCl_4 intoxicated rats (Figure 10C & D). Also, a significant elevation of GSH level ($p < 0.05$) was observed in the metabolite treated CCl_4 rats which indicate that the tested fungal metabolites (S 73 and S3 (Figure 10A). Treatment of cells with silymarin, and fungal metabolites (especially S73 and S3) for 24 h was able to ameliorate the increase in lipid peroxidation content (Figure 10B) and decrease in GSH content in comparison to Group II (Figure 10A). Furthermore, the cells treated with silymarin showed about 60% increase in GSH content, and 74, 56, 45, 34% increase when treated with S73, S3, S67 and S64 in comparison to Group II (CCl_4-induced rats' positive control). On contrary, the cells treated with silymarin, S73, S3 and S67 showed significant decrease whereas treatment with S64 showed 39% decrease in lipid peroxidation (MDA) levels.

Toxicity test

Also, in the current study, another experiment was carried out on normal healthy rats. The behavior of the treated rats appeared normal when tested up to 1000 mg/kg. No toxic effect was reported at doses up to 3-5 times of effective dose of the metabolites and there was no death in any of these groups.

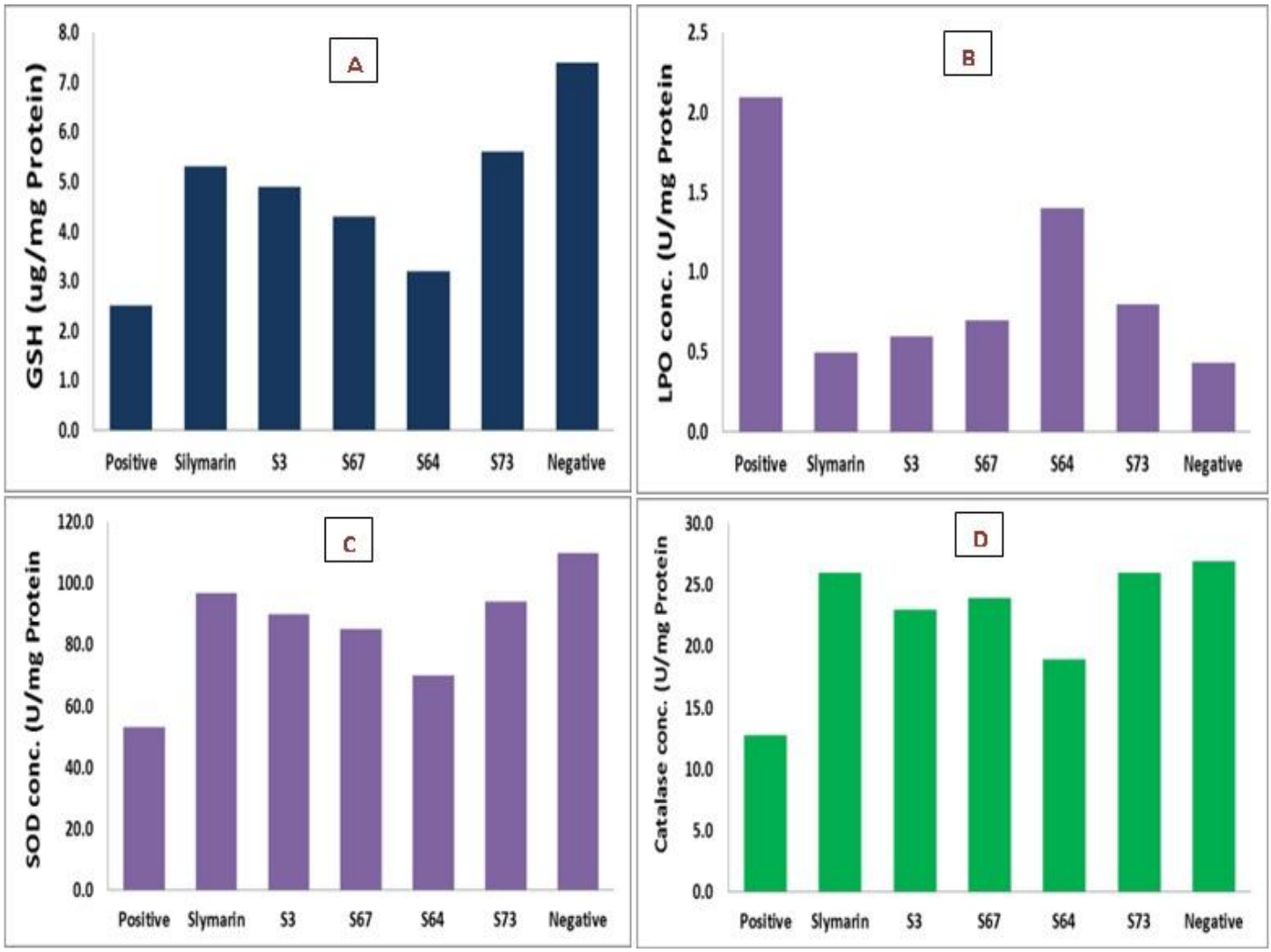


Figure 10. The effect of the secondary metabolites obtained from isolated fungi on glutathione levels (A) and oxidative enzymes [lipid peroxidation (B); superoxide dismutase (C) and catalase (D)] of CCl_4 intoxicated rat showing hepatoprotective activities compared with silymarin standard.

In vitro Antioxidant activity

The fungal secondary metabolites were evaluated by their reactivity towards a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results from *in-vitro* antioxidant studies revealed that the tested metabolites were found to possess good DPPH radical scavenging activity were found to possess good activity and scavenged free radicals in a concentration dependent manner in the models studied. Maximum percentage inhibition of DPPH radicals by S3 and S73 was more than 80% from 1000 till 300 µg/mL and demonstrated dose dependent antioxidant activity. The IC_{50} values as depicted in Figure 11 showed the following order of radical scavenging activity: *A. terreus*> *A. flavus* > *Fusarium* sp.> *Penicillium* sp. Further, on comparing the 50% scavenging activity with the reference standard ascorbic acid, we observed that *A. terreus* S3 metabolites (IC_{50} = 14.3±0.09 µg/ml) was less potent than ascorbic acid (IC_{50} = 8.36 ± 0.06 µg/ml).

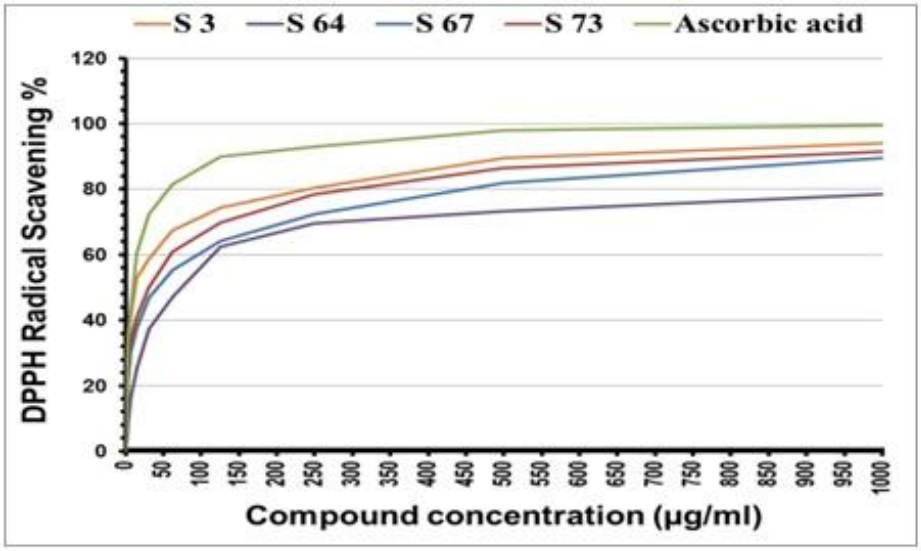


Figure 11. The dose response curves of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the tested fungal secondary metabolites showing good antioxidant activities when compared with ascorbic acid reference standard drug.

Discussion

Liver damage mainly occurs due to excessive alcohol consumption, viral infections; and as a consequence of drug adverse effects. Nowadays, liver diseases constitute a major medical problem of worldwide proportions [35-36].

The liver mainly detoxifies toxic chemicals, drugs and becomes the main target organ for all possible toxic xenobiotics. Carban tetrachloride is one of the most commonly used hepatotoxin in experimental study of liver diseases [37]. CCl_4 is biotransformed by cytochrome p-450 in liver to produce highly reactive trichloromethyl free radical leading to loss of integrity of cell membranes and damage of hepatic tissue [38].

Cytoprotective agents are regarded as suitable, biologically active substances preventing pathogenesis of chemically-induced injury of human or animal's cells by restoring physiological and biochemical cell function [39, 40]. Naturally occurring compounds have received increased attention in the past few years because of their chemopreventive properties.

Studies on animals and humans indicated that silymarin has got a broad spectrum of hepatoprotective effects in cases of the liver injured by acetaminophen, carbon tetrachloride, ethanol, iron overload, amanita mushroom poison and radiation. Chemopreventive efficacy has been demonstrated for different cell cultures [41, 42].

In this study, four active fungal secondary metabolites obtained through ethyl acetate extraction from marine fungi and exhibited the highest antioxidative effects was then subjected to evaluation of in vitro and in vivo hepatoprotective activities.

In this study, the protective effects of the tested fungal secondary metabolites were *in vitro* evaluated against CCl_4 induced cytotoxicity. Incubating HepG2 cells with CCl_4 caused a significant loss in the cell viability. Treatment with the tested fungal metabolites resulted in a dose-dependent increase in cell viability. Further, on comparing with silymarin, S73 was found more potent in protecting the HepG2 cells against CCl_4-induced oxidative damage.

HepG2 cells retain several specialized functions which are characteristic of normal hepatocytes and hence are used as a model for extensive toxicity studies of the liver [36, 43].

The effect of CCl_4 that caused damage of hepatic tissue was evidenced by increased levels of serum marker

enzymes, namely AST, ALT and ALP and total bilirubin which are related to the normal function of hepatic cell [44, 45]. In this study, the same trend of elevated levels of serum marker enzymes in CCl_4 treated rats groups were detected confirming cellular breakage and loss of functional integrity of cell membranes in liver. Treatment with the tested fungal secondary metabolites showed a significant restoration of the altered biochemical marker enzymes when compared with normal groups ($p<0.01$) which is an indication of plasma membrane stabilization as well as repair of hepatic tissue damage caused by carbon tetrachloride. Interestingly, no toxic effect was reported at doses up to 4-5 times of hepatoprotective effective dose of the tested metabolites.

Similarly, in a previous study, treatment with methanolic extract of *Polyalthiya longifolia* (MEPL) at a dose of 200 and 400 mg/kg significantly reduced the elevated levels of ALT, AST, and ALP towards the normal value [9].

Serum bilirubin is a breakdown product of haem in red blood cells and regarded as one of the most important clinical and pathophysiological indicator of necrosis of liver tissues. Treatment with the fungal metabolites resulted in a significant decrease in total bilirubin levels as compared to CCl_4 treated group (Group II) and this effect was possibly due to stabilization of hepatic cellular membranes by the tested active metabolites. The effective control of alkaline phosphatase (ALP) and bilirubin levels towards normal is good indication suggesting improvement in the secretary mechanism of the hepatic cell.

Also, fungal metabolite received groups showed increased levels of total protein and albumin. These alterations can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. Available data from earlier studies states that, stimulation of protein synthesis accelerates regeneration process and production of liver cells [46].

In order to elucidate the possible mechanism of hepatoprotection, we further evaluated the antioxidant potential of the fungal metabolites. The results depicted that in the chemical-based and the cell-based assays, the fungal secondary metabolites showed significant antioxidant and radical quenching potential.

The results observed from the DPPH radical scavenging method shows, the definite scavenging activity of the fungal metabolites towards DPPH radicals in comparison with ascorbic acid and the scavenging effect increases with increasing concentration used.

In the current study, fungal secondary metabolites showed protection in rats against hepatic lipid peroxidation and preserved GSH levels and activities of antioxidant enzymes namely, catalase (CAT), and superoxide dismutase (SOD). Glutathione is known to protect the cellular system against toxic effects of lipid peroxidation [47]. GSH plays an important role of intracellular antioxidant in cells and prevents damage by ROS to important cellular components by reducing them. During induced toxicity excess binding to NAPQI leads to depletion of GSH. This depletion in turn leads to excessive generation of ROS which ultimately leads to LPO and increase in the levels of MDA [43]. Decreased level of GSH in the liver tissue on CCl_4 exposure represents its increased utilization due to oxidative stress [48]. In the present study, a significant elevation of GSH level was observed in the treated CCl_4 rats which indicate that fungal metabolites can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH.

This protection against CCl_4-induced oxidative stress is in agreement with a similar study [36], where treatment of HepG2 cells with plant extracts of *Lavandula coronopifolia* (10–50 µg/ml) for 24 h was able to alleviate the increase in LPO and decrease in GSH induced by ethanol.

Superoxide dismutase is also an important indicator of hepatocellular damage in both acute and chronic conditions. SOD has potential role in scavenging the superoxide anion to form hydrogen peroxide and thus diminishes the toxic effect caused by this radical [9]. CAT is a hemeprotein, which catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals [49]. This decrease in CAT activity could result from inactivation by glycation of the enzyme [50]. Further, an increase in the SOD activity may protect CAT against enzyme inactivation by superoxide radical as these radicals have been shown to inactivate CAT [51]. Thus, the increase in SOD activity in our study may indirectly play an important protective role in preserving the activity of CAT. Previous reports also confirm that the antioxidant - rich secondary metabolites were able to show significant per cent cytoprotection, reduction in leakage of SGOT and SGPT, decrease in MDA (lipid peroxidation) content and increase in GSH content in comparison to CCl_4-treated cells. The secondary metabolites showed effective protection against radical damage accompanied with membrane stabilization. As shown in the present study, a significant hepatoprotection for the tested metabolites was detected in comparison to silymarin treatment. Peng *et al.* [52] have shown that post-treatment with the fractions isolated from *Ganoderma resinaceum* was able to protect HepG2 cells against oxidative damage induced by hydrogen peroxide (H_2O_2). In their study, three fungal metabolites – ganoderesin B, ganoderol B and lucidone A – showed inhibitory effects against the increase of SGPT and SGOT levels in HepG2 cell culture medium induced by H_2O_2 compared to a control group treated only with H_2O_2.

Previous work has established the presence of flavonoids, phenols, tannins or lignans in extracts of *M. koenigii* and

Phyllanthus niruri, which have been known for their rich antioxidant and hepatoprotective properties [53-55].

Similarly, the hepatoprotective and antioxidant activity of *Bauhinia hookeri* ethanol extract (BHE) against CCl_4-induced liver injury was reported in mice by enhancing the antioxidant defense status, reducing lipid peroxidation, and protecting against the pathological changes of the liver. BHE treatment significantly inhibited the CCl_4-induced increase in ALT (44 and 64%), AST (36 and 46%), ALP (28 and 42%), and MDA (39 and 51%) levels at the tested doses, respectively. Moreover, BHE treatment markedly increased the activity of antioxidant parameters GSH, GPx, GR, GST, and SOD [56]. Earlier studies have also shown that five phenolic compounds, namely luteolin, quercetin, rosmarinic acid, luteolin-7-glucoside and caffeic acid were able to protect HepG2 cells from oxidative stress against tert-butyl hydroperoxide (t-BHP) by restoring the decreased levels of GSH [55-57].

Conclusion

There is a beneficial influence of the effect of the extract of marine-derived fungus produced from Egyptian marine environment against liver diseases. In this study, the EC_{50} values for silymarin, as well fungal metabolites S73, S67, S3 and S64 were found to be 192.8, 162.6, 283.4, 356.7 and 737.5 µg/ml, respectively when tested *in vitro* against intoxicated HepG2 cells.

We could confirm that these metabolites possess hepatoprotective property due to its proven antioxidant and free radical scavenging properties and the order of activity: *A. terreus* >*A. flavus* > *Fusarium* sp. > *Penicillium* sp. Administration of these metabolites inhibited the degree of hepatic necrosis and concomitantly decreased the leakage of intracellular enzymes by stabilizing hepatic cellular membranes. Inhibition of lipid peroxidation to a significant degree is also a predominant mechanism of hepatoprotection. Increase in the SOD level was also suggestive of repairment of antioxidant defense system, which plays an important role in hepatoprotection. These hepatoprotective activities were discovered for the first time from these marine metabolites obtained from *Aspergillus flavus*, *A. terreus*, *Penicillium* sp. and *Fusarium* sp. with no toxic effects on tested animals.

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