Journal of Pharmaceutical Research International

33(24A): 24-48, 2021; Article no.JPRI.67485
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

The Preventive Effects and Mode of Actions of Ulva Fasciata Synthesized Silver Nanoparticles in Doxorubicin-Induced Hepatotoxicity in Wistar Rats

Asmaa M. Mahmoud[1], Osama M. Ahmed[2], Ibraheem B. Mohamed[3], Hanan A. Soliman[1] and Basant M. Mohamed[1*]

[1]Biochemistry Department, Faculty of Science, Beni-Suef University, P.O.Box 62521, Beni-Suef, Egypt.
[2]Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, P.O.Box 62521, Beni-Suef, Egypt.
[3]Botany and Microbiology Department, Faculty of Science, Beni-Suef University, P.O.Box 62521, Beni-Suef, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. Author OMA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors IBM and AMM managed the analyses of the study. Authors BMM and HAS managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i24A31429
Editor(s):
(1) Dr. Prabakaran Nagarajan, The Ohio State University, USA.
Reviewers:
(1) Sahar Hassan Orabi, University of Sadat City, Egypt.
(2) Gîtea Daniela, University of Oradea, Romania.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67485

Original Research Article

Received 05 February 2021
Accepted 11 April 2021
Published 16 April 2021

ABSTRACT

Background: Hepatotoxicity was one of the major side effects associated with doxorubicin treatment in cancer chemotherapy. The synthesized silver nanoparticles (AgNPs) from natural products such as algae especially green algae is one of the favorable means to minimize the deleterious effects of the chemotherapy. Thus, this study aimed to evaluate the preventive role of AgNPs synthesized by *Ulva fasciata* (*U. fasciata*) against doxorubicin-induced hepatotoxicity and oxidative stress in the liver of male Wistar rats.
Materials and Methods: In the present study, the green macroalga *U. fasciata* ethanolic extract was used as reducing agents to reduce Ag ions to Ag^0. Doxorubicin-injected male Wistar rats were

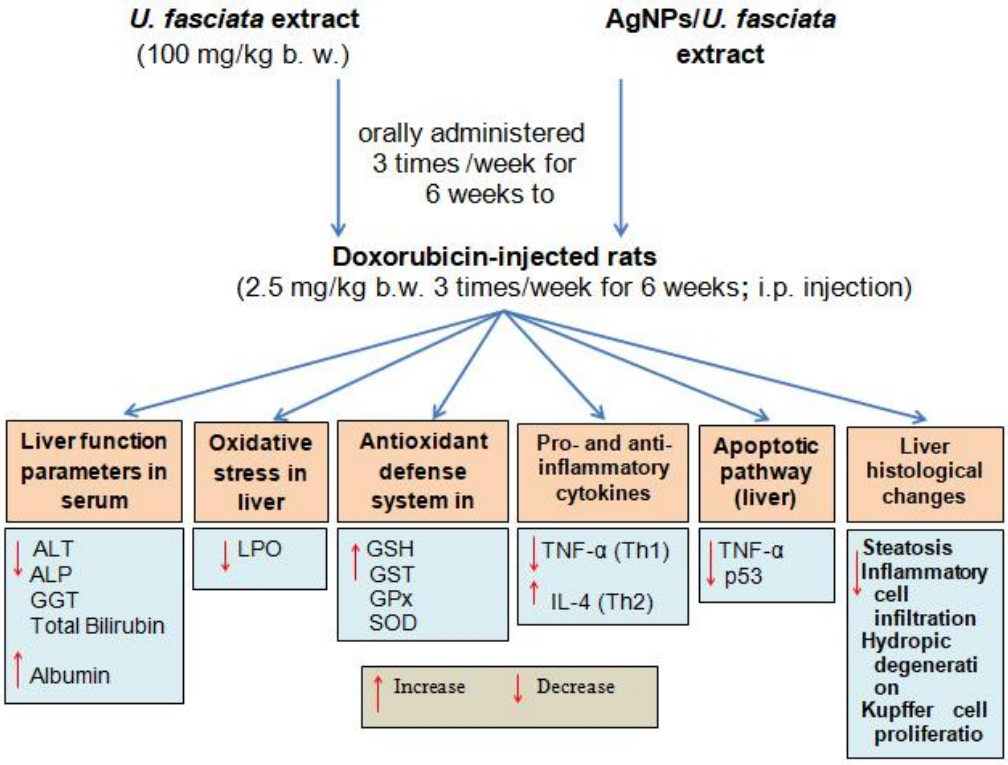
**Corresponding author: E-mail: soma198855@yahoo.com;*

concomitantly treated with *U. fasciata* ethanolic extract and AgNPs synthesized by *U. fasciata* extract (AgNPs/*U. fasciata*) 3 times/week by oral gavage for 6 weeks.

Results: The results showed that male Wistar rats injected with doxorubicin showed a significant increase in ALT, ALP and GGT activities and total bilirubin level as well as a reduction in the serum albumin level. The concurrent treatments of doxorubicin-injected rats with *U. fasciata* ethanolic extract and AgNPs/*U. fasciata* significantly abrogate these alterations. The altered levels of tumor biomarkers CA19.9 and AFP as well as pro-inflammatory cytokine, TNF-α, and anti-inflammatory cytokine, IL-4, in doxorubicin-injected animals were significantly ameliorated by concurrent treatment with *U. fasciata* and AgNPs/*U. fasciata*. Moreover, the elevated mRNA expression of p53 significantly decreased by treatment. In association, the doxorubicin-induced deleterious histological changes represented by severe hydropic degenerative changes, steatosis, inflammatory cell infiltration, Kupffer cell proliferation and apoptosis were remarkably improved by concurrent treatment with *U. fasciata* extract and AgNPs/*U. fasciata* which was more potent.

Conclusion: Based on results of this study, it can be concluded that *U. fasciata* extract and AgNPs/*U. fasciata* counteracts doxorubicin-induced toxicity by suppression of inflammation, oxidative stress and apoptosis. AgNPs/*U. fasciata* was the most potent in improving hepatocyte integrity and liver histological architecture.

Graphical Abstract



Keywords: Hepatotoxicity; doxorubicin; U. fasciata; AgNPs; oxidative stress.

ABBEREVIATIONS

AgNPs : Silver nanoparticles
U. fasciata : Ulva fasciata
U. lactuca : Ulva lactuca
$AgNO_3$: Silver nitrate
NaCl : saline
CMC : carboxymethylcellulose
ALT : Alanine Aminotransferase
ALP : alkaline phosphatase
GGT : Gamma glutamyl transferase
CA19.9 : carbohydrate antigen 19.9

AFP : α-fetoprotein
IL-4 : Interlukin-4
GSH : glutathione
GST : Glutathione-S-transferase
GPx : Glutathione peroxidase
LPO : lipid peroxidation;
TBARS :Thiobarbituric acid reactive substances
SOD : superoxide dismutase
NCI : National Cancer Institute;
H&E : hematoxylin and eosin
FC : Fatty changes
DC : Degenerative changes
IC : Inflammatory cells
KC : Kupffer cells
DNA : deoxyribonucleic acid
HCC : hepatocellular carcinoma

1. INTRODUCTION

Some drugs are widely used in cancer therapy such as doxorubicin which is one of the commonly used drugs because it's fighting various types of tumor including hematological cancers, sarcomas and carcinomas [1,2,3]. Regardless of its efficacy in fighting a variety of tumors, doxorubicin had side effects on several organs including liver causing high toxicity [4,5].

It had been revealed that most patients having a liver tumor and treated with doxorubicin were suffering from liver damage owing to induced oxidative stress and blocking of the cell cycle [6,7]. Even though, the biological mechanism of these negative activities of doxorubicin on hepatocytes was not understood [1]. Therefore, minimizing the toxicity of doxorubicin by using antioxidant natural products, chelating compounds and cytokines are one of the most important roles of drug findings of Biologists [8,9,10]. Moreover, one of the most challenges for pharmacologists is finding new products that reduce doxorubicin's harmful effects and improve its therapeutic actions [1].

The mechanism of doxorubicin anticancer activity includes alterations of deoxyribonucleic acid (DNA) as well as excess production of free radicals [11]. Nicotinamide adenine dinucleotide phosphate dependent cellular reductase enzyme had been shown to transform doxorubicin to semiquinone free radicals that can generate ROS involving superoxide, hydroxyl radicals and hydrogen peroxide [12]. Excess ROS production was critically implicated in doxorubicin cytotoxicity, especially hepatotoxicity [5,13,14].

The controlling of liver disorders is still challenging in the modern medicine [15]. Algae have been reported to have antioxidant [16], anti-inflammatory [17,18] and hepatoprotective effects in diseased animal models [19].

Marine algae have valuable contents such as polysaccharides (alginates, laminarians and fucans) and other pharmaceutical and nutraceutical compounds [20]. The green macroalgal genus *Ulva* commonly called sea lettuce was widely distributed in marine and fresh water throughout the world [21]. *Ulva fasciata* (*U. fasciata*) Delile that belongs to phylum Chlorophycota, class *Ulvophyceae*, order *Ulvales* and family *Ulvaceae* [22] has shown antioxidant [23], antileishmanial [24], antihyperglycemic [25], antibacterial [26,27], antifungal [28], antihyperlipidemic [29,30], nematicidal and insecticidal activities [31].

The green synthesis of AgNPs using algae is considered as ecofriendly procedures without using expensive cost and toxic chemicals [32]. *Ulva species* were applied to mediate reduction of silver into AgNPs using *Ulva lactuca* (*U. lactuca*) [33] and *U. fasciata* [34,35,36]. AgNPs prepared by green synthesis is highly effective against bacteria, fungi and cancer cells [36,37]. Also, they were used as antiviral, anti-inflammatory, anti-angioneogenesis, anti-platelet and anti-permeability activities [33,38,39,40].

Consequently, the present study was designed to assess the possible counteraction effects and mode of actions of AgNPs/*U. fasciata* against doxorubicin-induced hepatotoxicity.

2. METHODS

2.1 Alga Collection and Isolation

U. fasciata was collected on 4 March 2014 from the shallow water beside the shore of the Mediterranean Sea at Abo Qir coast, Alexandria, Egypt.

2.2 Preparation of Algal Extract

The collected *U. fasciata* was washed with tap water followed by distilled water, blotted, spread out and dried at room temperature for two weeks in a well aerated area. Shade dried *U. fasciata* was ground to fine powder by an electric grinder. The powdered sample was stored in sterilized containers for further usage. Powdered sample

of *U. fasciata* (100 g) was extracted with 400 ml of ethanol (99% purity) for 6–8 hours (hrs) in a conventional Soxhlet apparatus (500 mL boiler). At the end of the extraction, the liquid extract was filtered and evaporated to complete dryness in a vacuum at 35 °C using a Rota-vapor apparatus. The dried extract was stored at 4 °C for further studies.

2.3 Synthesis of AgNPs

Formation of AgNPs was done by adding 10 ml of algal extract to 90 ml 0.001M of $AgNO_3$ at room temperature. Once after addition, the color changed to greenish yellow color. Then, the color changes bit by bit till becomes dark brown color which indicated the formation of AgNPs [41].

2.4 Chemicals

Doxorubicin (Adriblastina® produced by Carlo Erba, Turkey) was purchased from a local pharmacy in the form of 10 mg/ampoule. Silver nitrate ($AgNO_3$) and Ethanol (99.9%) were obtained from Sigma Chemicals Company, St. Louis, MO, USA. Sterile saline (NaCl 0.9%) and carboxymethylcellulose (CMC) and other chemicals used in this investigation were of analytical grade and were commercially obtained.

2.5 Experimental Animals

Male rats of Wistar strain, weighing between 100-120 g and 9-11 weeks old, were used in the present investigation. They were obtained from the Animal House of Abou Rawash, El-Giza, Egypt. They were maintained under observation for 2 weeks before starting the experiment to eliminate any intercurrent infection. The chosen animals were housed in polypropylene cages with good aerated stainless steel covers at normal atmospheric room temperature (25±5ºC) under good ventilation. The rats were supplied with the access of water and a standard balanced diet *ad libitum*. All attempts were done to minimize the number of used animals.

2.6 Doses and Treatment

The animals were treated with doxorubicin by an intraperitoneal route at a dose level of 2.5 mg/kg b. wt. three days/week for 6 weeks. The doxorubicin was dissolved in 0.9 % NaCl [42, 43]. The dose of *U. fasciata* and AgNPs/*U. fasciata* used in this study was 100 mg/kg b. wt. [44] which was given three times/week by oral gavage for 6 weeks. The treatments were dissolved in 1% CMC (a vehicle) at a concentration 100 mg *U. fasciata* and AgNPs of *U. fasciata*/5 ml 1% CMC.

2.7 Experimental Design

The number of rats used in the present study was 40. They were allocated into 4 groups (n = 10) designed as follow:

Group 1: The rats of this group were given the equivalent volume of vehicles 0.9% NaCl by intraperitoneal injection and 1% CMC three times per week by oral gavage for 6 weeks.
Group 2: (Doxorubicin–administered control): The rats of this group were intraperitoneally injected with doxorubicin at a dose of 2.5 mg/kg b. wt. (dissolved in 0.9% NaCl) three times per week for 6 weeks.
Group 3: (Doxorubicin-administered group treated with *U. fasciata* extract): This group was intraperitoneally injected with doxorubicin at a dose of 2.5 mg/kg b.w. three times per week for 6 weeks and was orally treated with algal extract at a dose level of 100 mg/kg b.w. three times per week by oral gavage for 6 weeks.
Group 4: (Doxorubicin-administered group treated with *U. fasciata* AgNPs): This group was intraperitoneally injected with doxorubicin at a dose of 2.5 mg/kg b.w. three times per week for 6 weeks and was orally treated with *U. fasciata* AgNPs at a dose level of 100 mg/kg b.w. three times per week by oral gavage for 6 weeks.

2.8 Sampling and Tissue Preparation

By the end of the experiment, 6 animals from each group were sacrificed under mild diethyl ether inhalation anesthesia. Blood from each rat was withdrawn from the jugular vein in a gel and clot activator tube. Sera were separated by centrifugation at 3000 r.p.m. at 30ºC for 15 minutes and kept frozen at -30ºC pending biochemical analyses. The liver from each animal was rapidly excised after dissection. One part was fixed in neutral buffered formalin for 24 hrs, and then transferred into 70% alcohol for histopathological examination. 0.5 g was homogenized in 5 ml 0.9% sterilized isotonic saline (10% w/v) using Teflon Homogenizer (Glas-Col, Terre Haute, USA).

2.9 Biochemical Analyses

1. Alanine Aminotransferase (ALT) and alkaline phosphatase (ALP) activities in

serum were measured by kit obtained from BioSystem Company (Spain) according to the procedures of [45] and [46]. Gamma glutamyl transferase (GGT) activity in serum was measured by kits obtained from Spectrum Diagnostics (Egypt) according to [47]. Total bilirubin level in serum was determined by kits obtained from HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany according to [48]. Serum albumin level was measured by colorimetric method using reagent kits purchased from Diamond Diagnostics Company (Egypt) according to [49]. Serum carbohydrate antigen 19.9 (CA19.9) level was assayed by Sandwich-ELISA using kits purchased from R&D Systems (USA). Serum α-fetoprotein (AFP) and serum Tumor necrosis factor-α (TNF-α) were measured by colorimetric method using kits developed by Quantikine 614 McKinley Place NE, USA according to manufacturer's instructions. Serum IL-4 level was assayed by RayBio® Rat IL-4 ELISA kit. Liver glutathione (GSH) content was determined according to the method of [50]. Glutathione-S-transferase (GST) activity in liver was determined according to Mannervik and Gutenberg [51]. Glutathione peroxidase (GPx) activity in liver was determined according to Matkovics et al [52]. Liver lipid peroxidation (LPO) product was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Matkovics et al [53]. Liver superoxide dismutase (SOD) activity was assayed according to the method of Preuss et al [54]. Total RNA was isolated from the liver tissue according to the method of Giannopolitis and Ries [55], and mRNA expression of p53 and β-actin were determined by Thermo Scientific Verso 1-Step RT-PCR Reddy Mix Kit (Applied Biosystems, Foster City, CA, USA) according manufacturer's instruction in the presence of specific primers. The forward primer sequence for p53 is 5′-CAGCGTGATGATGGTAAGGA-3′ and the reverse sequence is 5′-GCGTTGCTCTGATGGTGA-3′ [56]. The forward primer sequence for β-actin is 5′-TCACCCTGAAGTACCCCATGGAG-3′ and the reverse sequence is 5′-TTGGCCTTGGGGTTCAGGGGG-3′ [57].

2.10 Histological Investigation

Fixed liver tissue samples were transferred to the pathology department, National Cancer Institute (NCI), Cairo University, Egypt for embedding after dehydration in paraffin wax sectioned of thickness of 5 µm and stained with hematoxylin and eosin (H&E) for general histopathological examination using the light microscope.

2.11 Statistical Analysis

Data are expressed as mean ± SE. Analysis of variance (one-way ANOVA) was used to identify statistically significant differences between groups. One way-ANOVA was followed by Tukey's test to compare various groups at $P<0.05$. All statistical analyses were performed using SPSS 15.0 software.

3. RESULTS

3.1 Effects on Serum Parameters Related to Liver Function

Changes in different serum biochemical biomarkers related to liver function are represented in Tables 1 and 2. Serum ALT (Fig. 1), ALP (Fig. 2) and GGT (Fig. 3) activities and total bilirubin level exhibited a significant ($p<0.05$) increase as a result of administration of doxorubicin. These elevated levels decreased in the rats administered doxorubicin and treated with *U. fasciata* extract and AgNPs/*U. fasciata* extract. The treatment with AgNPs/*U. fasciata* extract was more effective in reducing the elevated ALT activity (-58.64) and total bilirubin level (-82.14%) (Fig. 4) of doxorubicin-administered rats while the treatment with *U. fasciata* extract was more effective in decreasing the elevated ALP activity (-65.20%). On the other hand, serum albumin level (Table 2) remarkably decreased in doxorubicin-administered rats. The treatment of doxorubicin-administered rats with *U. fasciata* extract and AgNPs/*U. fasciata* extract potentially increased the lowered serum albumin level (Fig. 5).

Table 1. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* extract on serum ALT, ALP and GGT activities in doxorubicin-administered rats

Parameters / Groups	ALT (U/L)	% Change	ALP (U/L)	% Change	GGT (U/L)	% Change
Control	30.90 ± 0.83[b]	-	400.50 ± 5.10[a]	-	0.50 ± 0.08[a]	-
Doxorubicin	48.60 ± 1.96[d]	57.28	1166.80 ± 84.40[c]	191.34	1.70 ± 0.06[b]	240.00
Doxorubicin + *U. fasciata* extract	39.90 ± 0.84[c]	-17.90	406.00 ± 6.00[a]	-65.20	0.50 ± 0.04[a]	-70.59
Doxorubicin + AgNPs/*U. fasciata* extract	20.10 ± 0.86[a]	-58.64	656.00 ± 22.60[b]	-43.78	0.60 ± 0.08[a]	-64.71
LSD at the 5% level	1.56		55.99		0.09	
F- probability	P<0.001		P<0.001		P<0.001	

administered rats

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

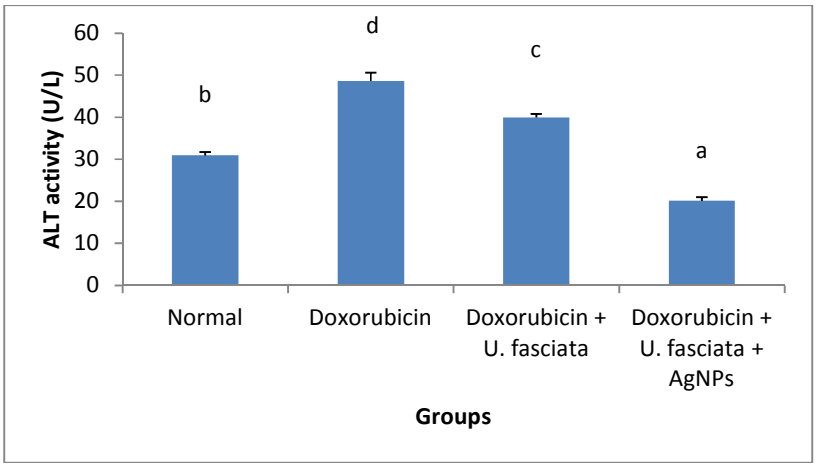


Fig. 1. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum ALT activity (U/L) in doxorubicin-administered rats

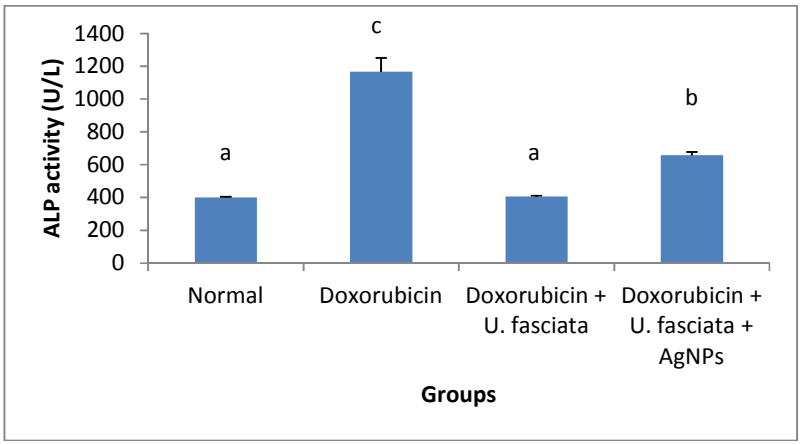


Fig. 2. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum ALP activity (U/L) in doxorubicin-administered rats

Table 2. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* on serum albumin and total Bilirubin levels in doxorubicin-administered rats

Parameters / Groups	Total bilirubin (g/dl)	% Change	Albumin (mg/dl)	% Change
Control	1.40 ± 0.02^{b}	-	3.60 ± 0.09^{c}	-
Doxorubicin	2.80 ± 0.11^{c}	100.00	2.40 ± 0.13^{a}	-33.33
Doxorubicin + *U. fasciata*	1.30 ± 0.16^{b}	-53.57	4.20 ± 0.02^{d}	75.00
Doxorubicin + AgNPs/*U. fasciata*	0.50 ± 0.07^{a}	-82.14	3.50 ± 0.04^{c}	45.83
LSD at the 5% level	0.15		0.11	
F- probability	P<0.001		P<0.001	

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

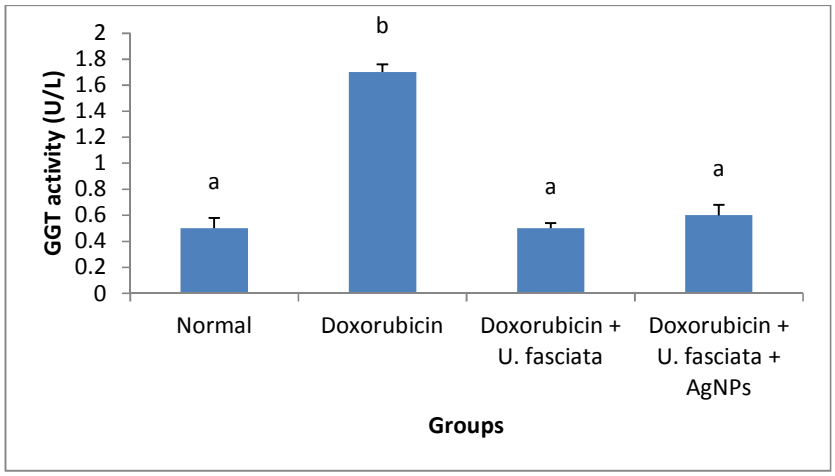


Fig. 3. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum GGT activity (U/L) in doxorubicin-administered rats

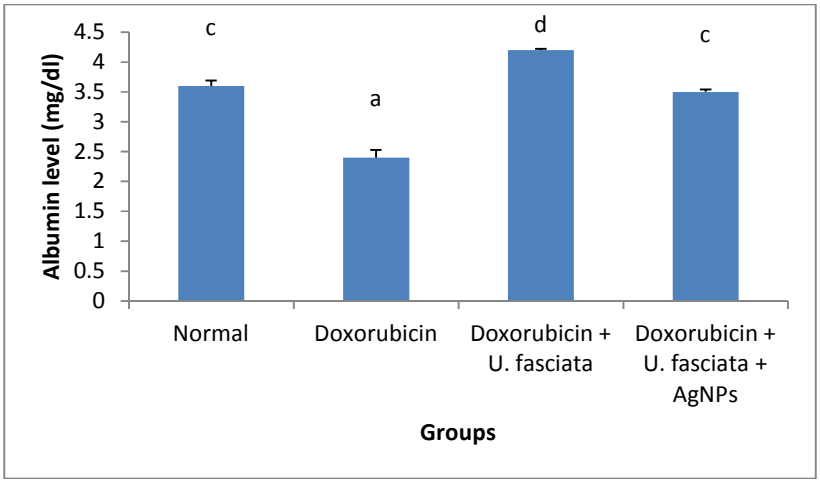


Fig. 4. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum albumin concentration (mg/dl) in doxorubicin-administered rats

Table 3. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* extract on serum CA19.9 and AFP levels in doxorubicin-administered rats

Parameters / Groups	CA19.9 (U/ml)	% Change	AFP (ng/ml)	% Change
Control	0.60 ± 0.08[a]	-	0.20 ± 0.04[a]	-
Doxorubicin	3.60 ± 0.15[c]	500.00	1.40 ± 0.07[d]	600.00
Doxorubicin + *U. fasciata*	1.70 ± 0.05[b]	-52.78	0.80 ± 0.05[b]	-42.86
Doxorubicin + AgNPs/*U. fasciata*	1.80 ± 0.05[b]	-50.00	0.90 ± 0.06[c]	-35.71
LSD at the 5% level	0.12		0.08	
F- probability	P<0.001		P<0.001	

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

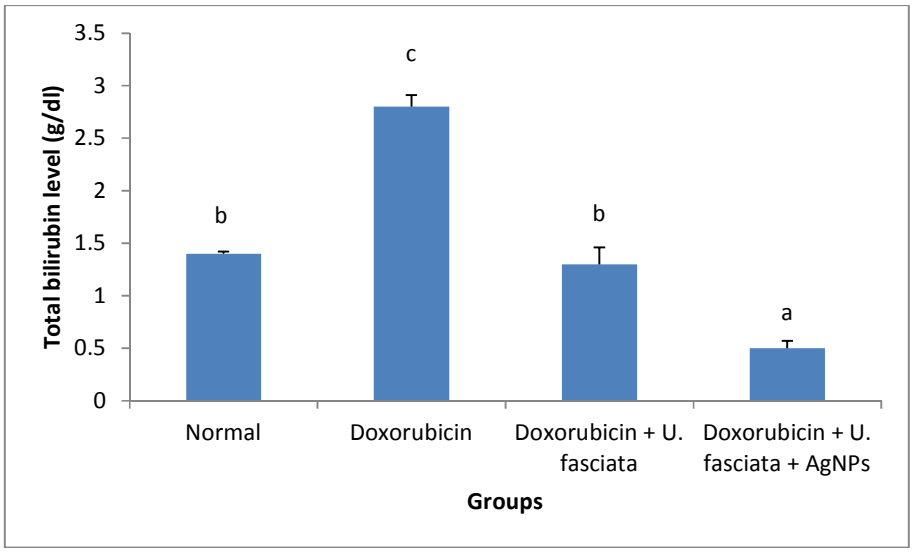


Fig. 5. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum total bilirubin concentration (g/dl) in doxorubicin-administered rats

3.2 Effects on Serum Tumor Markers

Changes in serum CA19.9 and AFP levels are represented in Table 3. Serum CA19.9 (Fig. 6) and AFP (Fig. 7) levels significantly ($p<0.05$) elevated in doxorubicin-administered rats; the recorded percentage increases were 500.00 and 600.00 respectively as compared with normal control. On the other hand, treatments of doxorubicin-administered rats with *U. fasciata* and AgNPs/*U. fasciata* lowered the elevated CA19.9 and AFP levels. The effect of treatment with AgNPs of *U. fasciata* (-42.86%) was more potent than *U. fasciata* extract (-35.71%) in decreasing the elevated AFP level.

3.3 Effects on Serum Markers Related to Inflammation

Changes in serum TNF-α and IL-4 are represented in Table 4. Serum TNF-α level (Fig. 8) significantly ($p<0.05$) elevated in doxorubicin-administered rats while IL-4 level (Fig. 9) significantly ($p<0.05$) decreased as a result of doxorubicin injection. On the other hand, treatment of doxorubicin-administered rats with *U. fasciata* and AgNPs/*U. fasciata* lowered the elevated TNF-α level and ameliorated the lowered serum IL-4 level. The treatment with AgNPs/*U. fasciata* was more potent than *U. fasciata* extract in decreasing the elevated TNF-α level.

3.4 Effects on Liver Oxidative Stress and Antioxidant Defense Biomarkers

Tables 5 and 6 show the effect of *U. fasciata* extract and AgNPs/*U. fasciata* on the liver oxidative stress and antioxidant defense system biomarkers in doxorubicin-administered rats.

The doxorubicin administration produced a significant decrease in GSH (Fig. 10), GST (Fig.11) and GPx (Fig.12) levels; the recorded percentage changes were -70.37, -28.12 and -24.59 respectively. The treatment of these animals with *U. fasciata* produced a significant ($p<0.05$) increase of the GSH content and GST activity as compared to doxorubicin-administered rats. The treatment of doxorubicin-administered rats with AgNPs/*U. fasciata* only induced a significant increase of liver GST activity (Table 5).

In Table 6, liver LPO product (Fig.13) significantly ($p<0.05$) increased (138.43%) while SOD (Fig. 14) significantly ($p<0.05$) decreased (-40.00%) as a result of doxorubicin-administration. On the other hand, the treatment of doxorubicin-administered rats with *U. fasciata* and AgNPs/*U. fasciata* extract induced a significant ($p<0.05$) decrease of the elevated LPO and a significant increase of the lowered SOD activity. The treatment with *U. fasciata extract* was more effective in improving the lowered liver GSH content and GST activity as well as the elevated LPO.

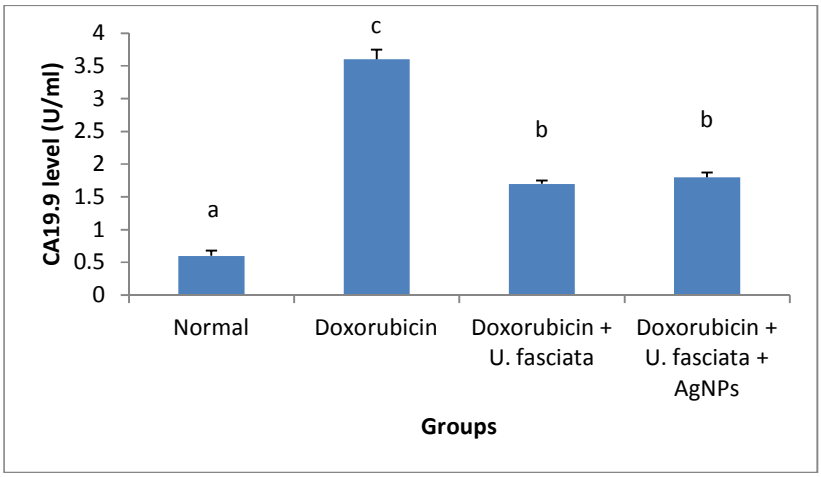


Fig. 6. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum CA19.9 concentration (u/ml) in doxorubicin-administered rats

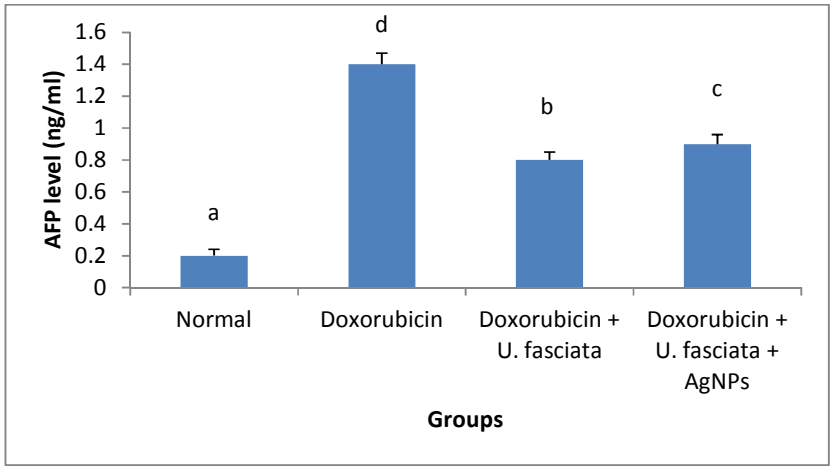


Fig. 7. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum AFP concentration (ng/ml) in doxorubicin-administered rats

Table 4. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* extract on serum TNF-α and IL-4 levels in doxorubicin-administered rats

Parameters / Groups	TNF-α (pg/ml)	% Change	IL-4 (pg/ml)	% Change
Control	16.30 ± 1.00^{a}	-	121.50 ± 2.50^{c}	-
Doxorubicin	140.70 ± 19.40^{c}	763.19	66.40 ± 1.40^{a}	-45.35
Doxorubicin + *U. fasciata*	79.10 ± 5.70^{b}	-43.78	97.20 ± 3.50^{b}	46.39
Doxorubicin + AgNPs/*U. fasciata*	55.10 ± 2.90^{b}	-60.84	95.60 ± 3.50^{b}	43.98
LSD at the 5% level	13.15		4.56	
F- probability	P<0.001		P<0.001	

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

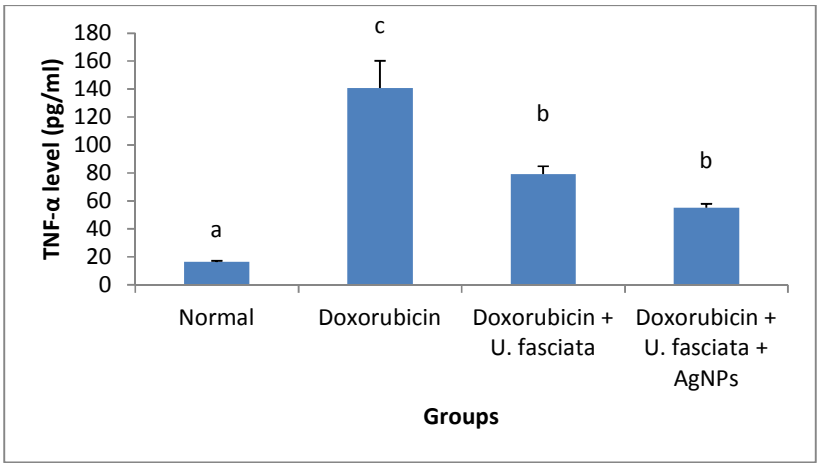


Fig. 8. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum TNF-α concentration (pg/ml) in doxorubicin-administered rats

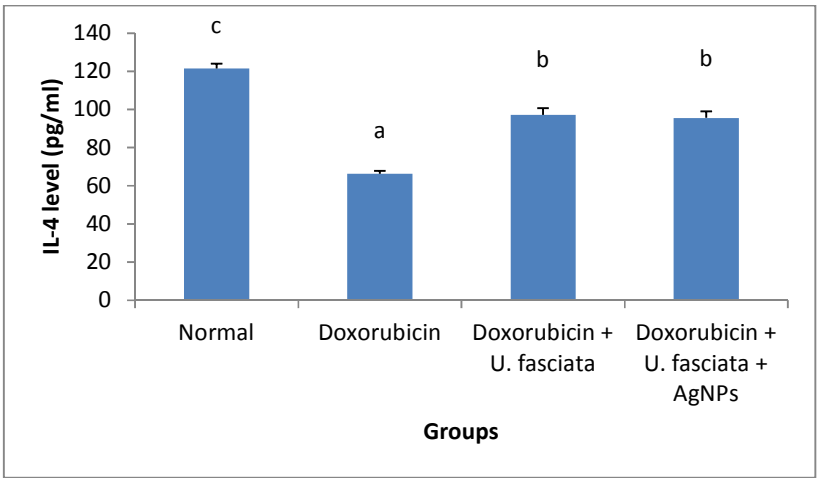


Fig. 9. Effect of *U. fasciata* and AgNPs / *U. fasciata* on serum IL-4 concentration (pg/ml) in doxorubicin-administered rats

3.5 Effects on Liver Tissue mRNA Expression of p53

Fig. 15 shows the effect of *U. fasciata* and AgNPs/*U. fasciata* on the liver tissue mRNA gene expression of p53 in doxorubicin-administered rats. Hepatic p53 level significantly ($p<0.05$) increased in doxorubicin-administered rats. On the other hand, doxorubicin-administered groups treated with *U. fasciata* and AgNPs/*U. fasciata* exhibited a significant decrease of the elevated hepatic p53.

3.6 Histological Changes

Liver histological examinations of control, doxorubicin-injected group and doxorubicin-injected groups treated with *U. fasciata* and AgNPs/*U. fasciata* were depicted in Fig. 16 (Photomicrographs A – D).

Photomicrograph of liver section of normal rats showed normal histological structure of hepatic lobule (Photomicrograph A). The liver of animals injected with doxorubicin showed severe hydropic degenerative changes, fatty change of hepatocytes, apoptotic cells, Kupffer cell proliferation and inflammatory cells infiltration (Photomicrograph B). The liver of animals injected with doxorubicin and treated with *U. fasciata* showed moderate hydropic degenerative changes (Photomicrograph C). On the other hand, the liver of animals injected with doxorubicin and treated with AgNPs/*U. fasciata* showed nearly normal histological changes and Kupffer cell proliferation (Photomicrograph D).

Table 5. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* on liver GSH content and GPx and GST activities in doxorubicin-administered rats

Parameters / Groups	GSH (nmole/100 mg tissue)	% Change	GST (U/100 mg tissue)	% Change	GPx (mU/100 mg tissue)	% Change
Control	24.30 ± 0.40^{c}	-	267.80 ± 6.40^{d}	-	337.00 ± 9.50^{c}	-
Doxorubicin	7.20 ± 0.10^{a}	-70.37	192.50 ± 2.10^{a}	-28.12	254.10 ± 3.80^{a}	-24.59
Doxorubicin + *U. fasciata*	13.70 ± 0.20^{b}	90.28	253.00 ± 11.20^{cd}	31.43	260.70 ± 1.40^{a}	2.59
Doxorubicin + AgNPs/*U. fasciata*	8.90 ± 1.50^{a}	23.61	218.10 ± 5.50^{b}	13.29	264.80 ± 1.00^{a}	4.21
LSD at the 5% level	1.03		10.9		7.83	
F- probability	P<0.001		P<0.001		P<0.001	

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

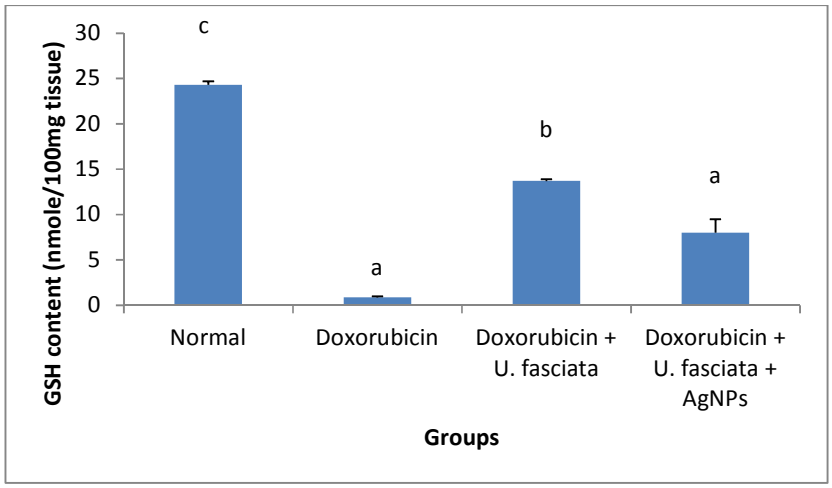


Fig. 10. Effect of *U. fasciata* and AgNPs / *U. fasciata* on liver GSH content (nmol/100mg tissue) in doxorubicin-administered rats

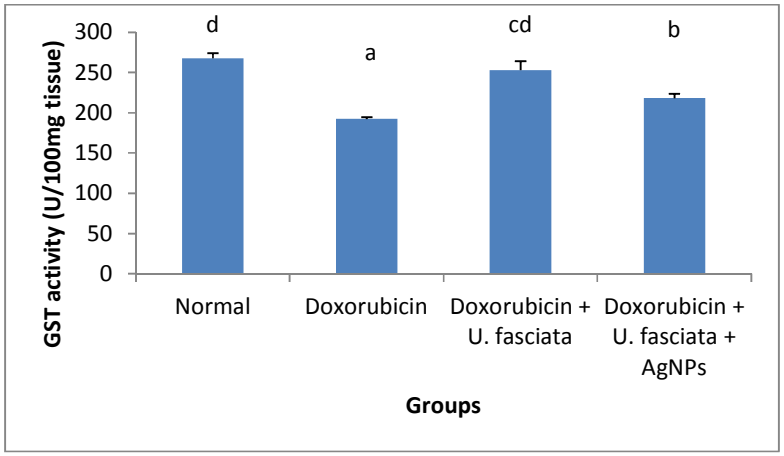


Fig. 11. Effect of *U. fasciata* and AgNPs / *U. fasciata* on liver GST activity (U/100mg tissue) in doxorubicin-administered rats

Table 6. Effect of *U. fasciata* extract and AgNPs/*U. fasciata* extract on liver LPO product and SOD activity in doxorubicin-administered rats

Parameters / Groups	LPO (nmole MDA /100 mg tissue/hour)	% Change	SOD (mU/100 mg tissue)	% Change
Control	43.20 ± 1.00^{ab}	-	10.00 ± 0.07^{c}	-
Doxorubicin	103.00 ± 0.70^{c}	138.43	6.00 ± 0.07^{a}	-40.00
Doxorubicin + *U. fasciata*	40.00 ± 1.40^{a}	-61.17	7.50 ± 0.20^{b}	25.00
Doxorubicin + AgNPs/*U. fasciata*	46.70 ± 3.80^{b}	-54.66	7.10 ± 0.20^{b}	18.33
LSD at the 5% level	2.87		0.23	
F- probability	P<0.001		P<0.001	

- *Data are expressed as Mean ± SE. The numbers of detected samples in each group is six.*
- *Means, which have the same superscript symbol(s), are not significantly different.*
- *Percentage changes were calculated by comparing doxorubicin-administered group with normal control and doxorubicin- administered groups treated with U. fasciata extract and AgNPs/U. fasciata with doxorubicin-administrated control groups*

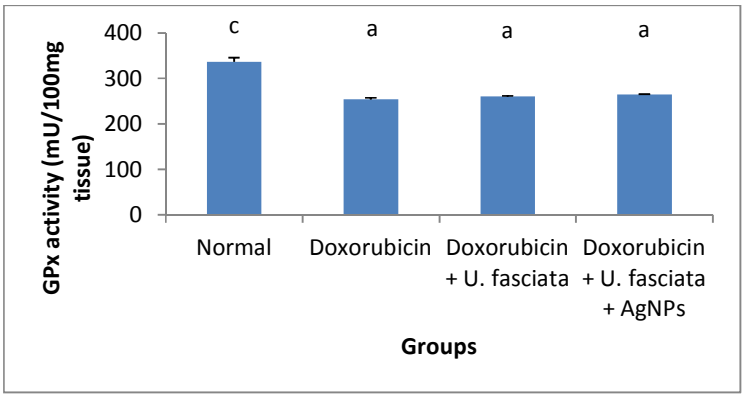


Fig. 12. Effect of *U. fasciata* and AgNPs / *U. fasciata* on liver GPx activity (mU/100mg tissue) in doxorubicin-administered rats

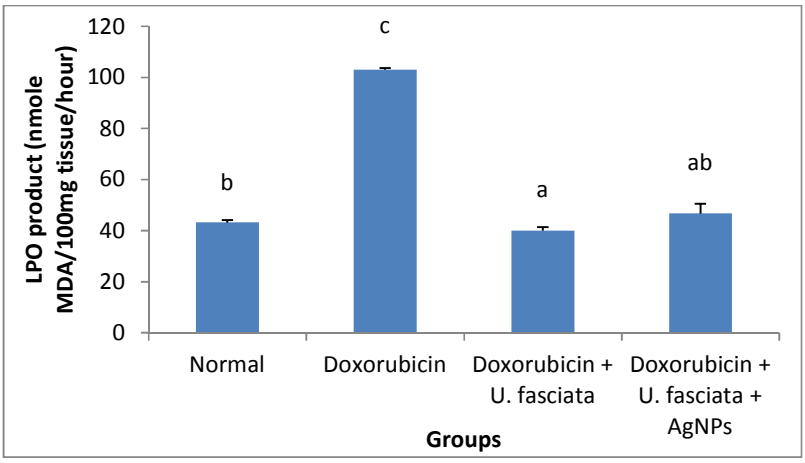


Fig. 13. Effect of *U. fasciata* and AgNPs / *U. fasciata* on liver LPO product (nmole MDA/100mg tissue/hour) in doxorubicin-administered rats

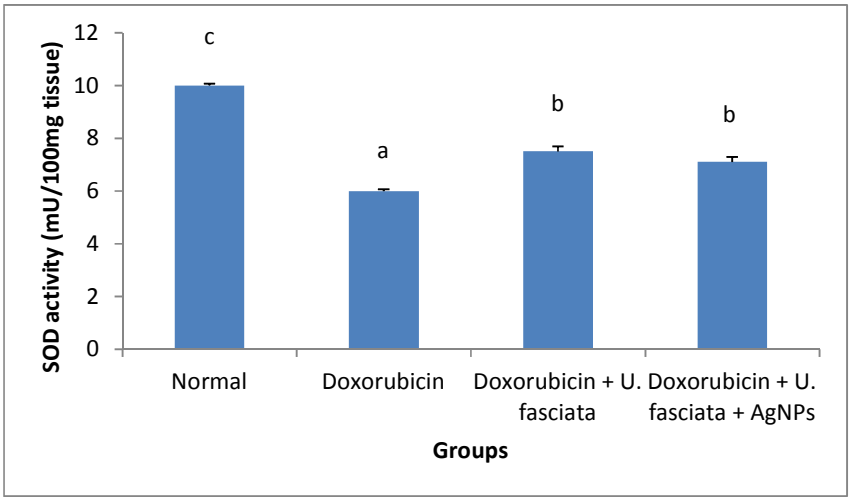


Fig. 14. Effect of *U. fasciata* and AgNPs / *U. fasciata* on liver SOD activity (mU/100 mg tissuer) in doxorubicin-administered rats

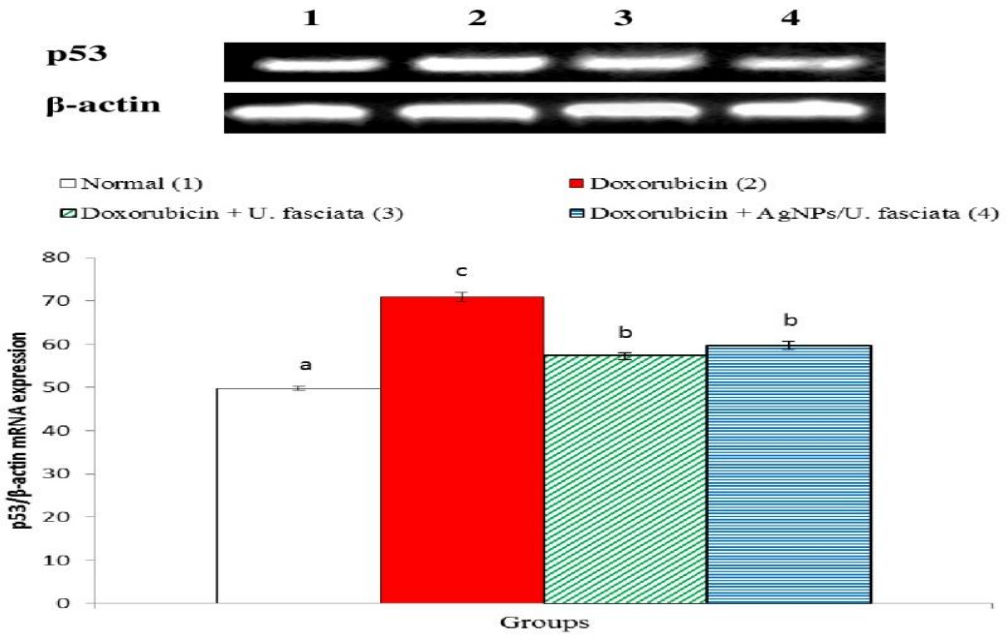


Fig. 15. The effect of *U. fasciata* and AgNPs/*U. fasciata* on the liver tissue mRNA gene expression of p53 in doxorubicin-administered rats

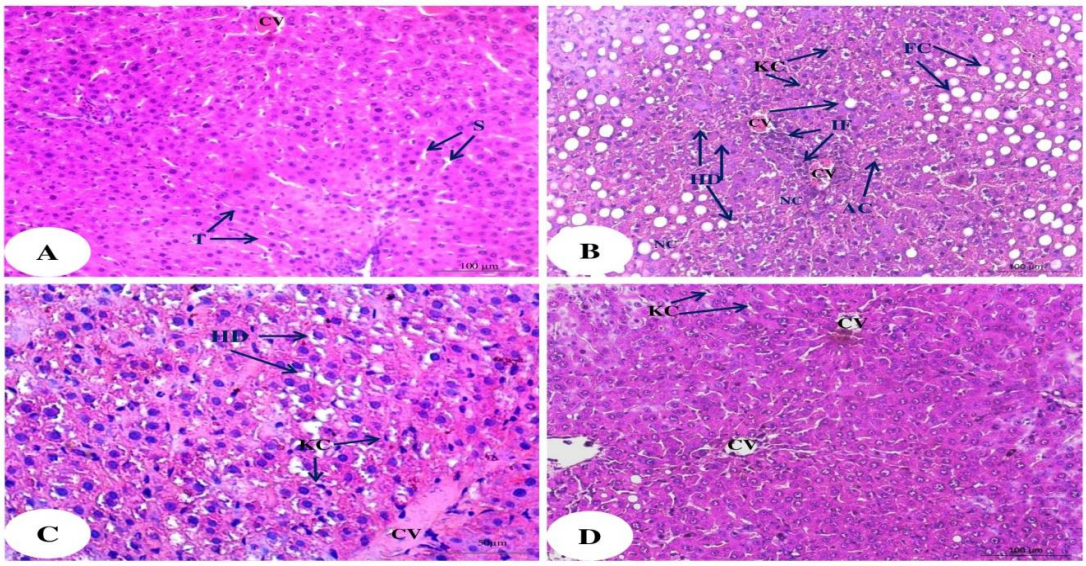


Fig. 16. photomicrographs of H&E stained liver sections of control (A), doxorubicin-administered group (B) and doxorubicin-administered groups treated with *U. fasciata* (C) and AgNPs / *U.fasciata* (D). FC: Fatty changes; DC: Degenerative changes; IC: Inflammatory cells; and KC: Kupffer cells

4. DISCUSSION

Doxorubicin is used frequently for the therapy of different varieties of malignancies. Liver toxicity is one of the serious side effects of doxorubicin [43]. In the present investigation, administration of doxorubicin resulted in liver function impairment manifested by significant elevations in serum ALT, ALP, GGT and total bilirubin levels and decrease in serum albumin

level as well as deleterious histopathological changes represented by hydropic degenerative changes of hepatocytes, inflammatory cell proliferation, Kupffer cell proliferation, steatosis, apoptosis and necrosis. These manifestations were in agreement with [58,59] who revealed hepatoxic effects of doxorubicin and attributed this toxicity to doxorubicin capability to induce certain genetic and biochemical variations in the liver of mice. The results of the present study are in concordance with those of [60] who stated that doxorubicin caused direct cytotoxicity to the hepatocytes with significant impairment of the liver function tests and liver histological changes. Oxidative stress had been incriminated to be the major mechanism responsible for doxorubicin-induced liver toxicity [61]. In this regard, [59] reported that doxorubicin induced an oxidative stress status characterized by enhanced production of ROS and/or suppression of the antioxidant defense leading to an imbalance in the oxygen normal metabolism. This elucidation was concomitant with the results of the present study which found an increase in liver LPO and a decrease in liver GSH content and GST, GPx and SOD activities. Adding of an electron to the quinone moiety of doxorubicin leads to the production of a semiquinone form which induces inhibition of conversion of the molecular oxygen to ROS which attack and oxidize DNA and induce apoptosis in the liver cells [62]. Redox cycling between the quinone and semiquinone forms of doxorubicin leads to oxygen radicals' formation [63]. These free radicals, in turn, attack and oxidize DNA and induce apoptosis in liver cells. Moreover, doxorubicin had found to interfere with the non-enzymatic metabolic reactions that iron was involved in leading to change of iron metabolism which subsequently leads to enhance formation of ROS [63].

ALT is a cytosolic enzyme of hepatocytes. Thus, its elevation in serum may be due to its increased leakage from the cytosol to plasma as a result of membrane damage and necrosis. On the other hand, GGT and ALP are enzymes embedded in the plasma membrane mainly in the canalicular domain and their release into serum indicates injury to the canicular cells resulting in biliary damage [64].

On the other hand, doxorubicin-administered rats were treated with *U. fasciata* and AgNPs/*U. fasciata* exhibited a significant decrease in activities of serum ALT, ALP and GGT and level of serum total bilirubin while they showed an increase in the lowered serum albumin level. These results are in agreement with [65] who stated that crude extract of *Ulva reticulata* (as type of green algae) preserve the structural integrity of the hepatocyte plasma membrane to protect the membrane from injury by the toxic reactive metabolites caused by drugs. Additionally, numerous studies in animals have proved that AgNPs can be translocated in the blood circulation and distributed to some organs, including the liver, kidney and lung, after exposure through subcutaneous injection [66], inhalation [67] and oral administration [68,69]. In most cases, the liver is suggested to be the major target organ for AgNPs [67,68,69].

In the current study, the histological deteriorations of the liver were surprisingly alleviated secondary to treatment with *U. fasciata* extract and AgNPs/*U. fasciata*. Our results are in concurrence with [70] who detected that *U. fasciata* treated groups showed a positive effect on the liver. No histological injuries were proved in the liver of treated rats at both the beginning and the end of the experiment as compared to normal rats.

In the present study, the treatment of doxorubicin-injected rats with AgNPs*/U. fasciata* appeared to be more potent than *U. fasciata* extract in improving liver histological changes. While the liver of doxorubicin-injected rats treated with AgNPs*/U. fasciata* showed nearly normal histological integrity, the liver of doxorubicin-injected rats treated with *U. fasciata* extract still exhibited hydropic degenerative changes and Kupffer cells proliferation. In association, the effect of AgNPs*/U. fasciata* on serum ALT activity and total bilirubin level was more potent than the effect of *U. fasciata* extract. In light of these results, it can be suggested that AgNPs*/U. fasciata* may be more potent in improving liver function and histological integrity.

In the current study, the serum levels of tumor markers CA19.9 and AFP exhibited a marked increase in serum of doxorubicin-administered rats as compared to the normal group. On the other hand, the treatment of doxorubicin-injected rats with *U. fasciata* and AgNPs*/U. fasciata* induced a significant decrease in the elevated levels of CA 19.9 and AFP. It is related here to mention that CA 19.9 was a tumor marker raised in hepatocellular carcinoma (HCC) [71]. AFP is a fetal specific glycoprotein that falls rapidly after birth and high concentration of AFP is suspicious of HCC but may be raised in chronic viral hepatitis [72]. Their decrease in doxorubicin-administered rats treated with *U. fasciata* and AgNPs/*U.*

fasciata may provide evidence for decreased probability for HCC as compared with doxorubicin-administered control rats which showed profound elevation of serum CA 19.9 and AFP.

In the current study, the serum level of pro-inflammatory cytokine, TNF-α, exhibited a marked increase in serum of doxorubicin-administered rats as compared to the normal group while serum level of the anti-inflammatory cytokine, IL-4, significantly decreased. In association with the elevation of TNF-α and diminution of IL-4, the liver histological section of doxorubicin-injected rats exhibited noticeable inflammatory cells infiltration and Kupffer cell proliferation. In accordance, many previous publications stated that doxorubicin causes an increase of peripheral TNF-α level [73,74,75]. Since TNF-α is a Th1 cytokine and IL-4 is produced by Th2 CD^{4+} T cells, the increase in TNF-α and decrease in IL-4 reflected the induced inflammation and dominance of Th1 on Th2 as a result of doxorubicin administration. TNF-α together with other cytokines had roles in chronic and acute inflammation [76]. The release of pro-inflammmatory cytokines, including the critical early mediators of organ damage such as IL-1β and TNF-α from activated Kupffer, stellate and sinusoidal endothelial cells, induced different pathophysiologic responses in the liver [77,78]. These cytokines contributed to the intra-hepatic recruitment and activation of granulocytes that were characteristically found in hepatic inflammation [79]. It is also worth mentioning here that stimulation of inflammation might give rise to trigger apoptosis [80]. In this regard, TNF-α may stimulate apoptosis *via* TNF receptor or death receptor, thereby activate the extrinsic pathway of apoptosis [81]. This elucidation is supported by the results of the present study which revealed that doxorubicin injection stimulates the expression of pro-apoptotic mediator in the intrinsic pathway of apoptosis (Fig. 17).

The changes in IL-4 and TNF-α may be interrelated since it was reported by previous publication that IL-4 suppressed macrophage IL-1β and TNF-α production [82]. Moreover, IL-4 stimulated macrophage lipoxygenase activity, which may reduce synthesis of the pro-inflammmatory leukotriene B4 [83]. IL-4 also inhibits many functions of activated macrophages, such as the secretion of reactive oxygen intermediates [84] and nitric oxide [85].

The treatments of doxorubicin-injected rats with *U. fasciata* and AgNPs/*U. fasciata*, in the present study, caused a marked amelioration of the elevated serum TNF-α and the lowered serum IL-4 levels. Thus, the concurrent treatments with *U. fasciata* and AgNPs/*U. fasciata* caused bias towards the preponderance of Th2 on Th1 pattern. These results agree well with [44,86,87] who reported the anti-inflammatory effects of green algae and their constituents. It was also noticed in the results of the present study that while the effects of both treatments were more or less similar on serum IL-4 level, the effect of AgNPs/*U. fasciata* was more potent on serum TNF- α level. In association with the decrease in serum level of TNF-α, p53 (pro-apoptotic mediator in the intrinsic pathway) expression decreased by treatments of doxorubicin-injected rats with *U. fasciata* and AgNPs/*U. fasciata*. Based on these evidence, it can be suggested that the reduction in apoptosis may be mediated, at least in part, by the suppression in the levels of TNF-α which activates the extrinsic pathway of apoptosis through cell death/TNF receptors (Fig. 17).

Oxidative stress was caused by an imbalance between the cellular production of ROS and the antioxidant defense mechanisms that eliminate ROS [88]. ROS are various forms of activated oxygen inevitably produced in living organisms as by-products during oxidative process and catabolism or as exogenous sources. These oxygen forms can easily react with most biological molecules including lipids, proteins, lipoproteins and nucleic acids causing progressive decline in cell function and leading to a variety of pathophysiological disorders [89].

Antioxidants protected membrane from ROS toxicity by prevention of ROS formation by interruption of ROS attack, facilitating the repair caused by ROS and providing cofactors for the effective functioning of other antioxidants [90].

LPO had been reported as the main contributor to the loss of cell function under oxidative stress conditions [91]. Oxidative stress produces deleterious effects by initiating LPO directly or by acting as second messengers for the primary free radicals that initiate LPO [92]. In the present study, thiobarbituric acid reactive species (TBARS) or MDA level was measured as indicator or index for lipid peroxidation and oxidative stress [93].

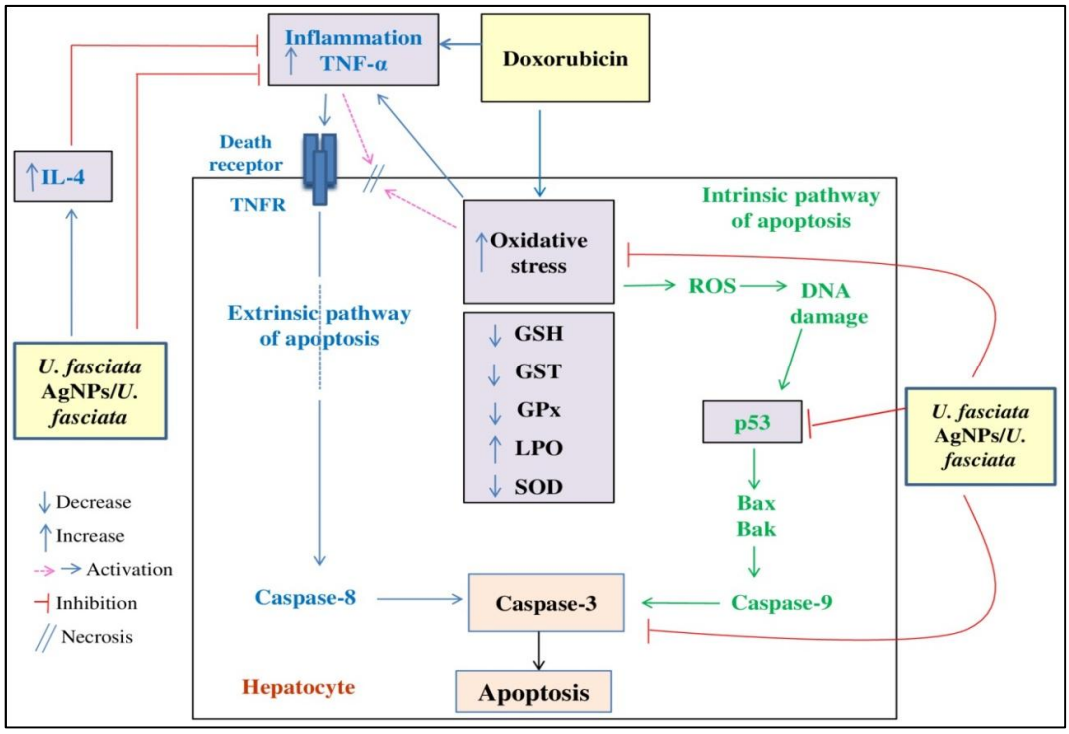


Fig. 17. Elucidation is supported by the results of the present study which revealed that doxorubicin injection stimulates the expression of pro- apoptotic mediator in the intrinsic pathway of apoptosis

MDA level, in the present study, markedly elevated in liver homogenate of rats consumed doxorubicin as compared to normal ones. These data are in accordance with those of many publications [60,94,95].

Doxorubicin cytotoxicity and genotoxicity could be mediated by elevations in free radicals and its ability to activate apoptosis through a wide variety of mechanisms including production of ROS, alkylation of cellular macromolecules, DNA intercalation and crosslinking, LPO, cell membrane damage, ceramide production and p53 induction in various organs including liver [96,97,98]. In association with the elevated LPO as a subsequent effect of doxorubicin administration in the present study, there was an increased apoptosis which was marked by histological investigation and by an increased expression of pro-apoptotic protein, p53.

On the other hand, treatment of doxorubicin-injected rats with *U. fasciata* extract and AgNPs/*U. fasciata* extract exhibited a decrease in MDA level as compared with normal ones. This agrees with many investigators [65,99,100,101]. Many studies had shown that the inhibition of LPO and oxidative damage is a mechanism of liver protection [95,102,103].

The current study also showed a decrease in the activity of hepatic SOD, GPx, and GST activities and GSH content in doxorubicin-administered group; this decline may be an indicator for the increasing the formation of $O_2^{\bullet}$ radicals in liver tissues. The decline of GPx activity may lead to the accumulation of H_2O_2 which in turn feeds back and inhibits SOD [104]. These results are in agreement with other previous publications [60,95].

The decrease in liver GSH content in doxorubicin-administered rats may be due to the increased oxidative stress. This caused insufficient synthesis of GSH to maintain its adequate intracellular levels and in turn led to further GSH depletion [105,106]. The depletion in GSH content as well as antioxidant enzyme activities including SOD, GPx and GST activities may influence the ability of the liver to provide protection against oxidative injury [107]. Also, the decreased in GSH content may be due to its

increased consumption by GST and GPx [105,106]. These data are in accordance with those reported by previous investigators [108,109,110]. The observed GSH deficiency and the rise of the level of GSSG caused by doxorubicin might be owing to GSH consumption in the interactions of doxorubicin-induced free radicals with bio-membranes and the subsequent LPO.

The involvement of doxorubicin in the production of ROS has been widely accepted as the mechanism of action of doxorubicin-induced cardiotoxicity and hepatotoxicity [111,112,113,114]. The present study strongly support this elucidation as the present results showed a significant increase of LPO product and a significant decrease in GSH content and SOD, GST and GPx activities in liver, kidney and heart as a result of doxorubicin administration.

In the present study, the treatment of doxorubicin-administered rats with *U. fasciata* extract and AgNPs/*U. fasciata* extract increased hepatic content of GSH content and activities of SOD, GPx and GST as compared to doxorubicin-administered group. These results are in agreement with past publications [99,115] which reported the antioxidant effects of *U. fasciata* and *U. lactuca*. In our opinion, the suppression of oxidative stress as represented by decreased LPO and enhancement of the antioxidant defense system represented by increasing in liver GSH content and antioxidant enzymes may have an important role in the hepatopreventive effects of *U. fasciata* extract and AgNPs/*U. fasciata* extract against doxorubicin-induced toxicity.

In association with the improvement effects of *U. fasciata* and AgNPs/*U. fasciata* on the liver oxidative stress and antioxidant defense system in doxorubicin-administered rats in the present study, liver p53 significantly decreased. Concomitantly, serum TNF-α significantly decreased in doxorubicin-administered rats treated with *U. fasciata* and AgNPs/*U. fasciata*. As p53 is an intermediate in the intrinsic pathway of apoptosis that was triggered by oxidative stress [5,81,116] and TNF-α triggers extrinsic apoptosis *via* cell death/TNF receptors [5], this led us to suggest that *U. fasciata* and AgNPs/*U. fasciata* may decrease apoptosis in doxorubicin-administered rats through affecting both intrinsic and extrinsic apoptotic pathways (Fig. 17).

5. CONCLUSION

In conclusion, the results of this study demonstrated that *U. fasciata* and its biogenic synthesized AgNPs have effective preventive action against doxorubicin-induced liver toxicity *via* their antioxidant, anti-inflammatory and anti-apoptatic effects. The AgNPs generated by *U. fasciata* extract seemed to be more potent than *U. fasciata* extract improving liver function and histological integrity. However, further studies are required to assess the efficacy and safety of AgNPs/*U. fasciata* extract in human beings.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experiment was approved by the Experimental Animal Ethics Committee, Faculty of Science, Beni-Suef University, Egypt (Ethics approval number: BSU/FS/2017/18.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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