



Ameliorating effect of *Azolla pinnata* extract and its nanosized bilosomes against 5-Fluorouracil-induced hepato-renal toxicity in male rats

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ABSTRACT

Key words:

Azolla pinnata nanosized bilosomes; Sodium deoxycholate; Fluorouracil; Antioxidants; Histopathological studies.

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The present study aimed to show the ameliorating effect of *Azolla pinnata* against hepatorenal toxicity caused by fluorouracil (5-FU). Forty-nine male Wistar rats, weighing 100-115g, were randomly distributed into seven groups (n=7). Group I received 1ml distilled water (DW) orally daily, Group II received 1ml Sodium deoxycholate (SDCh) orally daily, Group III received fluorouracil (5-FU) IP on the 11th experimental day in dose 150mg/kg body weight, Group IV received an *Azolla pinnata* (AP) orally daily in dose 10mg/kg body weight, Group V received an APN (*Azolla pinnata* nanosized bilosomes) orally daily 10% of 10mg/kg body weight, Group VI received an AP orally daily in dose 10mg/kg body weight and 5-FU IP on the 11th experimental day in dose 150mg/kg body weight, Group VII received an APN orally daily 10% of 10mg/kg body weight and 5-FU IP on the 11th experimental day in dose 150mg/kg body weight. The experiment lasted 21 days. On the 22nd day, blood and tissues were gathered for determination of liver and kidney functions, antioxidants and histopathological studies. Our results showed that elevated ALT, AST, urea, creatinine, uric acid, MDA and NO and reduced SOD, GSH, CAT and TAC in 5-FU group when compared to other groups. Abnormal changes in liver and kidney tissues than other groups according to histopathological studies. AP has anti-inflammatory and antioxidant effects that play role in decreasing hepatorenal toxicity caused by 5-FU.

1. INTRODUCTION

The liver is one of the most remarkable solid organs in the body. It is considered a gland that is characterized by its size, which is large. It is considered a metabolism center for nourishing substances and the removal of waste metabolites. Complete forfeiture of liver fundamental roles leads to death immediately [1]. Hepatic tissues play a necessary role in adaptative and innate immunity together [2]. It contains the highest number of phagocytes that act as a conclusive barrier against

the external environment. It is the groundwork of immunity as it has anti-inflammatory effects and is responsible for tolerance of immunity [3]. Insinuation to toxins, pesticides, drugs and heavy metals is considered the main purpose of hepatic toxicity in all living organisms [4-7]. Kidneys are one of endocrine organs that secrete hormones such as renin, erythropoietin and kallikreins [8]. They are responsible for removing waste and maintenance of homeostasis [9]. Insinuation to toxins, heavy metals, and drugs is the main purpose of renal toxicity in all living organisms [10,11].

Fluorouracil is one of the most widespread anticancer drugs that is known for its ability in the treatment of neck, head, and breast cancers even after 1957 [12]. It is one of the most widely exercised anticancer and antimetabolite drugs recently. Regrettably, it has many abuses, such as shortage of half-life, increased cytotoxic effect, and bad absorption [13]. It is symmetrical to uracil but has fluorine instead of a hydrogen atom at carbon number 5. DPD (Dihydropyrimidine dehydrogenase) is the enzyme that is responsible for catabolism of fluorouracil that occurs in the liver [14]. It has the ability to damage the liver [15,16]. It has the ability to damage the kidney [17].

Herbal medicine turns off more widespread as it is dynamic, safe and affordable. One of hepatorenal prophylactic factors are plants and algae which are considerable well in research and applications [18].

Azolla pinnata is a genus that has belonged to the salvinaceae family that is widespread. It can be grown in Asia and Africa as tropical and temperate climate [7]. It contains several compounds such as phenols, flavonoids, alkaloids, hormones, amino acids, proteins, minerals, vitamins, saponins, fatty acids, triterpenoids and tannins that make *Azolla pinnata* with anticarcinogenic, antidiabetic, antihypertensive, antiviral, antimicrobial, insecticidal, antiparasitic, antioxidant, anti-inflammatory, cardioprotective, neuroprotective, gastroprotective, hepatoprotective and alternative source of protein [19]. It is an inexpensive drug with less side effects [20].

Nanotechnology is widely used in medicine, engineering, electronics, physics, biology and chemistry. It is used in diagnosis of diseases and delivery of drugs to target sites [21]. Nanoencapsulation is important therapeutically in improvement of efficiency, ability in delivering of drugs to target sites, taking of drugs by cells and bioavailability of drugs. It has ability to protect drugs from degradations [22]. Bilosomes (bile salt stabilized nanovesicles) have been widely utilized as a drug delivery system. Different types of bile salts were utilized for bilosomes fabrication, such as sodium deoxycholate (SDCh), sodium glycolate (SGCh), and sodium taurocholate (STCh). Among the bile salts, SDCh is the most widely used bile salt as its considered non-toxic and higher capacity for drug permeation enhancing [23].

The goal of the work is to investigate the effect of *Azolla pinnata* against hepatorenal toxicity in rats intoxicated with fluorouracil through evaluation of liver functions (ALT and AST), kidney functions (urea, creatinine, and uric acid), histopathological inspection of liver and kidney, and evaluation of

antioxidants (MDA, NO, SOD, CAT, GSH and TAC).

2. MATERIALS AND METHODS

2.1. *Azolla pinnata*

Green fresh *Azolla pinnata* leaves were gained from Agriculture Research Center at Giza, Egypt. Ethanolic *Azolla pinnata* extract was prepared as [24-26]. It were dried under shadow at 22°C room temperature and relative humidity 65% for 7 days after that dried *Azolla pinnata* were grinded into powder which steeped into 70% ethyl alcohol in shaker incubator for 48 hours for shaking gently then filtration by filter paper Whatman number 1 then evaporation of filtrate till became dried and finally the obtained extract was preserved in airtight dark bottle at 4°C in the refrigerator till using it.

2.2. Fluorouracil

It was obtained as 5 FlucelTM500® as one vial packing as 500mg fluorouracil/10ml; manufactured by CELON LABS PVT, LTD, Plot NO:2, ALEAP Industrial Estate, Gajularamaram, Medchal District – 500090, Telangana State, India. Mfg. Lic. No.: 14/RR/AP/2008/F/CC.

2.3. Other used substances

Sorbitan monostearate (Span 60), potassium dihydrogen orthophosphate anhydrous, and dipotassium hydrogen orthophosphate anhydrous were obtained from Elnasr Pharmaceutical Chemical Co., Cairo, Egypt. Soy phosphatidylcholine (SPC) was purchased from Carlo Erba Reagents, Spain. Cholesterol and sodium deoxycholate were obtained from Thermo Fisher Scientific, India. Diethyl ether, chloroform, and methanol were procured from the Sigma Aldrich, St Louis, MO63103, USA. Trypsin enzyme was obtained from ACG, ALFA Chemicals, Egypt. Other chemicals and reagents were of analytical quality and obtained from reliable sources.

2.4. Experimental animals

Forty-nine distinctly healthy male Wistar rats, weighing from 100-115g, were obtained from animal facility. Rats were settled in cages made from polypropylene in laboratory animal house in Faculty of Veterinary Medicine, Menoufia university, Egypt. Rats were kept under sterilized main circumstances. Rats received diet in the form of pellets that were equiponderant and free access of water. Husbandry conditions of rats such as 12 hrs. light/12 hrs. dark, temperature of 18-25°C and normal good ventilation. The adaptation period of

rats was 14 days before the beginning of the experiment. MNVETPh253802.

2.5. Screening studies

Span 60, Span 60 to SPC ratio (1:1), CHOL (25mg), and SDCh concentrations (0, 20, 40, and 60mg) were chosen for the present investigation according to the trial experiments that give minimum vesicle size, minimum polydispersity index, maximum zeta potential, and maximum entrapment efficiency.

2.6. *Azolla pinnata* Assay Method: UV Spectroscopy

Accurately weighed amount of AP (20mg) was transferred into a 100ml volumetric flask and dissolved in 40ml methanol with sonication for 10 minutes to obtain a clear solution. The final volume was adjusted with phosphate buffer, pH 6.8 (200µg/ml) to obtain the UV maximum absorbance of AP (λ_{max}) (UV-1601, Shimadzu Co.; Kyoto, Japan) and the AP calibration curve [27,28].

2.7. Assay Method Validation

The utilized UV spectrophotometric analysis method of AP was validated by various parameters such as linearity range, accuracy, and precision [29].

2.8. Construction of *Azolla pinnata* Nanosized Bilosomes

AP-loaded nanosized bilosomes were fabricated utilizing the thin-film hydration technique [23,27,30]. Composition of the various AP-loaded bilosomal formulations is listed in Table (1).

Table (1): Composition of the AP various bilosomes batches.

Run code	AP (mg)	Span 60 (mg)	SDCh (mg)	SPC (mg)	CHOL (mg)	DW to
BILO-1	20	150	10	150	25	20ml
BILO-2	20	150	20	150	25	20ml
BILO-3	20	150	40	150	25	20ml
BILO-4	20	150	60	150	25	20ml

AP; *Azolla pinnata*, SDCh; Sodium deoxycholate, SPC; Soy phosphatidylcholine, CHOL; Cholesterol, DW; Distilled water

2.9. Characterization of the Formulated AP-Loaded Bilosomes

2.9.1. Vesicle Size, Polydispersity index, and Zeta Potential

Vesicle size, polydispersity index (size distribution) (PDI), and zeta potential of the fabricated AP-loaded nanosized bilosomal vesicles were investigated via dynamic light scattering (DLS) on Malvern Zetasizer, ZEN 1690, Nano-S90 (Malvern Instruments Limited, UK) after appropriate dilution with distilled water [27].

2.9.2. Vesicle Size, Polydispersity index, and Zeta Potential

For investigation the entrapped AP into bilosomes utilizing indirect method [23,30]. Entrapment efficiency (%) =

$$\frac{\text{Total amount of AP} - \text{Free amount of AP in the supernatant}}{\text{Total amount of AP}} \times 100$$

2.9.3. In-vitro AP Release

In-vitro release studies of AP-loaded bilosomes according to [27,29,30].

2.9.4. Choosing the Best Bilosomal Formula

Rank order method was used for choosing the best bilosomal formula based on maximizing EE %, zeta potential, and in-vitro amount released, in addition to minimizing particle size and PDI. The best formula was taken for further investigation.

2.9.5. Animals

For conducting the ex-vivo studies, one male healthy New Zealand white rabbit weighing about 2.25kg was utilized as an animal model. MNVETPh253802. The rabbit was housed under standard laboratory conditions, accommodating temperatures of 25±3°C, and 12 hrs. light/12 hrs. dark cycle and was fed with free access to a standard diet and water [30]. All animal studies complied with the guidelines for the care and use of laboratory animals.

2.9.6. Ex-Vivo Permeation Study

The small intestine of New Zealand rabbits was utilized to assess the enhancement in the intestinal permeability of the selected AP-loaded bilosomes (BILO-3) compared to free AP suspension [23,29,30]. The amount of AP permeated through the rabbit intestine per unit area (µg/cm²) against each time interval (hrs.) was plotted. The apparent permeability coefficient (PC, cmhr⁻¹) of AP-loaded bilosomes and free AP suspension were determined utilizing the following equation [30].

$$PC = \frac{F}{A} \times \text{Initial } Azolla \text{ pinnata concentration}$$

Where, F is permeation flux ($\mu\text{g}/\text{cm}^2\text{hr}$), and A is the total surface area of the rabbit intestinal sac. The enhancement ratio, ER of *Azolla pinnata* was calculated using the following equation [23].

$$\text{Enhancement ratio (ER)} = \frac{\text{PC of bilosomes}}{\text{PC of free AP suspension}}$$

2.9.7. Transmission electron microscopy (TEM) Analysis

The morphological characterization of the selected AP-loaded bilosomes, BILO-3 was conducted utilizing TEM analysis. This was conducted using TEM (Jeol; JEM-2100 plus) at 200KV [29].

2.9.8. Lyophilization of the selected Bilosomal Formula, BILO-3

The selected AP-loaded bilosomal formula, BILO-3 was frozen at (-20°C) followed by lyophilization at (-45°C) under lowered pressure for 72 hrs. (FD55-10S freeze-dryer, Acculab, NEW YORK, USA). The lyophilized formula was utilized for conduction of differential scanning calorimetry (DSC) analysis [27].

2.9.9. Differential Scanning Calorimetry (DSC) Analysis

DSC thermograms were recorded by DSC60, Shimadzu, Kyoto, Japan according to [27].

2.9.10. Biological Stability Study

The biological stability study of BILO-3 formulation in the presence of simulated intestinal fluid (SIF) was conducted. SIF was prepared according to [27]. The tested bilosomal dispersion, BILO-3 was mixed with SIF in a ratio of 1:1 v/v. The stability (digestibility) of the tested bilosomal formulation was investigated by observing the changing in vesicle size and zeta potential at various time intervals (0, 0.5, 1, and 2 hrs.) (n=3) [23].

2.10. Experimental design

Forty-nine male Wistar rats, weighing 100-115g, were randomly distributed into seven groups as 7 rats of each.

Group I (control negative group) received 1ml distilled water (DW) orally daily for 21 days.

Group II received (solvent) orally 1ml sodium deoxycholate (SDCh) daily for 21 days.

Group III (control positive group) received fluorouracil (5-FU) IP single dose on the 11th experimental day in dose 150mg/kg body weight [31-35].

Group IV received an *Azolla pinnata* (AP) orally daily in dose 10mg/kg body weight for 21 days [7,36].

Group V received an *Azolla pinnata* nanosized bilosomes (APN) orally daily for 21 days 10% of 10mg/kg body weight.

Group VI received AP orally daily in dose 10mg/kg body weight for 21 days and 5-FU IP single dose on the 11th experimental day in dose 150mg/kg body weight.

Group VII received APN orally daily for 21 days 10% of 10mg/kg body weight and 5-FU IP single dose on the 11th experimental day in dose 150mg/kg body weight.

2.11. Mortality

During this study, occurred death to number of rats. Data of dead rats were excluded from statical analysis.

Table (2): Illustrating the date, number of deaths

Mortality	Experimental groups						
	Control (G1)	Solvent (G2)	5-FU (G3)	AP (G4)	APN (G5)	5-FU+ AP (G6)	5-FU+ APN (G7)
Number	-	-	2	-	-	1	1
Date (day of experiment)	-	-	at 16 th at 18 th	-	-	at 22 nd	at 22 nd
Rate (%)	0	0	28.57	0	0	14.29	14.29

and rate in each group.

5-FU: fluorouracil, AP: *Azolla pinnata*, APN: *Azolla pinnata* nanosized bilosomes, 5-FU + AP: fluorouracil + *Azolla pinnata*, 5-FU + APN: fluorouracil + *Azolla pinnata* nanosized bilosomes.

2.12. Serum biochemical analysis

Liver and kidney functions such as (ALT, AST, urea, creatinine and uric acid). The most common indicators of liver diseases are alanine transaminase and aspartate aminotransferase [37]. Urea and creatinine tests which are more frequent diagnostic tests of kidney diseases [38]. Evaluation of AST and ALT according to [39], evaluation of urea and creatinine according to [40] and evaluation of uric acid according to [41].

2.13. Histopathological inspection

Fixation of tissues (liver and kidney) in buffer formalin 10%. Tissues were processed and treated according to [42,43].

2.14. Antioxidants inspection

Liver and kidney tissues were collected and preserved at -80°C till using them. Evaluation of lipid peroxidation (MDA) according to [44,45], evaluation of nitric oxide (NO) according to [46], evaluation of reduced glutathione (GSH) according to [47], evaluation of super oxide dismutase (SOD) according to [48], evaluation of catalase (CAT) according to [49,50] and evaluation of total antioxidant capacity (TAC) according to [51].

2.15. Statistical analysis

Data was statistically analyzed by mean $\pm$ SE through using of ANOVA (one way variance analysis test) and Duncan's post hoc to assure differences of groups statistically. Variations among groups means were determined as $p \leq 0.05$ that was significant statistically. Statistical analysis was performed through SPSS program (software version 16) [52].

3. RESULTS

3.1. *Azolla pinnata* nanosized bilosomes

3.1.1. UV-Spectroscopy of *Azolla pinnata*

AP exhibited peak absorbance at 413 nm when measured in methanol: PBS, pH of 6.8 (4:6 v/v). The constructed calibration curve was linear in the 20–200 $\mu\text{g}/\text{mL}$ concentration range with a correlation coefficient (R^2) of 0.9986. The analysis spectrophotometric method was validated, and the intraday recorded % recovery ranged from 97.1 to 102.2% of the nominal values.

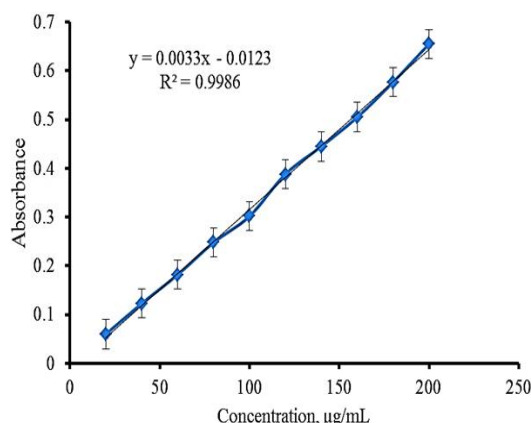


Figure (1): Calibration curve of *Azolla pinnata* in PBS, pH 6.8 at λ_{max} 413nm.

3.1.2. Linearity Range

The value of the correlation coefficient (0.9986) indicates excellent linearity between absorbance and AP concentration over the range of 20–200 $\mu\text{g}/\text{mL}$.

3.1.3. Accuracy

The utilized method accuracy was performed as the percentage of recovery of known amounts of AP. It is performed at AP concentrations of 50, 100, and 150 $\mu\text{g}/\text{mL}$. Each sample was taken three times, and the result range was 98.57–101.22% Table (3), high recovery indicated that the utilized assay method has a high degree of accuracy.

3.1.4. Precision

The precision of the utilized assay method was determined by repeatability (intra-day) precision and intermediate (inter-day) precision. Precision was expressed as the relative standard deviation (RSD). Three different AP concentrations (in the linear range) were investigated for intra-day and two inter-day precision ($n=3$). Both intra- and inter-day RSD values were less than 2 confirming good precision of the utilized assay method Table (3).

3.1.5. Vesicle size, PDI, and Zeta Potential

The obtained results of vesicle size (nm), PDI, and zeta potential (mV) of the formulated AP-loaded bilosomal formulations are presented in Table (4), with the particle size distribution graph shown in Figure (2). The vesicle size of the constructed bilosomal formulations was found in the range of 220.9 – 292.6nm. The PDI value reflexes the homogeneity (distribution) of the vesicles size in the fabricated bilosomal dispersion. The obtained results revealed the homogenous distribution of the vesicles in all formulations except BILO-4 formulation (PDI = 0.605) Table (3). With regard to the zeta potential, all the fabricated AP-loaded bilosomal vesicles were negatively charged with zeta potential values ranging from -32.8 ± 2.21 to $-41.4 \pm 3.44 \text{ mV}$ Table (3). The stability of the vesicle dispersion is investigated by zeta potential value in terms of electric repulsion. The stable vesicular system has a zeta potential of $\pm 30 \text{ mV}$.

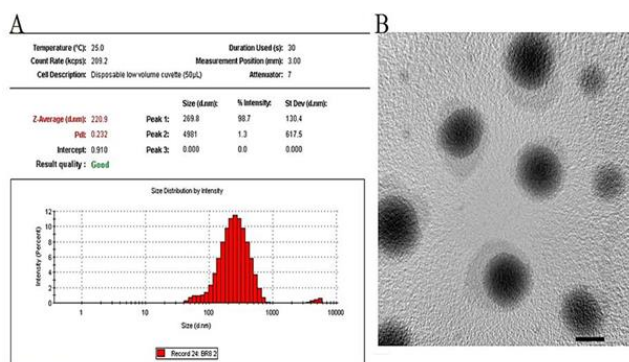


Figure (2): A) Vesicle size distribution graph, B) TEM image of the selected AP-loaded bilosomes, BILO-3.

Table (4): The obtained characteristics of the constructed AP-loaded bilosomal various formulations.

Run code	Vesicle size (nm)	PDI [#]	Zeta potential (mV)	EE [#] (%)
BILO-1	252.2±44.78	0.418±0.03	-32.8±2.21	46.36±4.12
BILO-2	239.1±31.38	0.261±0.05	-34.2±2.58	60.17±2.68
BILO-3	220.9±29.67	0.232±0.14	-37.6±1.12	78.22±4.08
BILO-4	292.6±45.11	0.605±0.09	-41.4±3.44	43.82±3.42

[#] PDI: Polydispersity index, EE: Entrapment efficiency, the results are the mean ± SD, n=3.

3.1.6. EE %

The EE % of the constructed AP-loaded bilosomes was determined by centrifugation/ indirect method. The obtained result showed that the minimum and maximum EE % was found to be 43.82±3.42% (BILO-4) and 78.22±4.08% (BILO-3), respectively, as listed in Table (4).

3.1.7. In-vitro AP Release Study

The amount of AP released from the various formulations were quantified spectrophotometrically due to the maintained sink conditions. The obtained results of BILO-3 formulation showed that a cumulative percent AP amount of 93.55±5.38% released within 12 hrs. A biphasic *in-vitro* release profile with initial burst release in the first 4 hrs (64.11±4.33%) followed with a sustained release for 12 hrs (93.55±5.38%) was noted Figure (3A). Furthermore, *in-vitro* release of AP from free AP suspension (performed in the same experimental

conditions) showed 37.26±3.75% over a period of 12 hrs. The obtained results revealed that the nanosized bilosomes showed significantly higher AP amount released ($p<0.05$) than free AP suspension. The comparative *in-vitro* release study of AP from the constructed nanosized bilosomes and free AP suspension was illustrated graphically illustrated in Figure (3A).

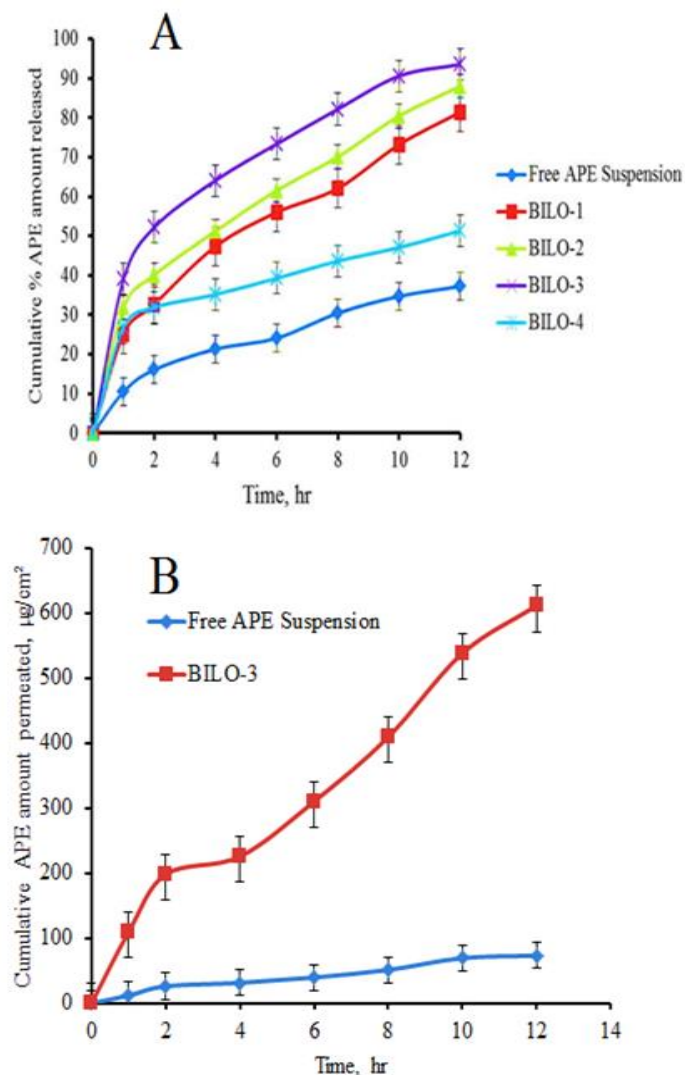


Figure (3): A) *In-vitro* release profile of *Azolla pinnata* from various AP-loaded bilosomes and free AP-suspension, B) The ex-vivo permeability profiles of BILO-3 compared to that from free AP suspension through rabbit intestine in PBS, pH 6.8 at 37±1°C (the data expressed as the mean ±SD, n=3).

3.1.8. Ex-vivo Permeation Studies

The intestinal permeation study was conducted on a rabbit intestine to estimate the intestinal permeation efficiency of AP from BILO-3 compared to the free AP suspension. The AP-loaded bilosomes, BILO-3

was selected as the best formula based on the best rank order of the formula (The ex-vivo permeation profiles of AP from BILO-3 formula and free AP suspension through rabbit intestine were graphically illustrated in Figure (3B). The permeated amount of AP from BILO-3 after 12 hrs from the beginning of the experiment was found to be $611.25 \pm 15.47 \mu\text{g}/\text{cm}^2$ which is significantly higher ($p < 0.05$) than the amount permeated from free AP suspension at the same time point ($73.19 \pm 3.87 \mu\text{g}/\text{cm}^2$). The obtained ex-vivo AP permeation profiles distinctly revealed the potential of the constructed AP-loaded bilosomal vesicles to enhance AP permeation through GIT membrane. The permeation flux (F) value was found to be $9.914 \mu\text{g}/\text{cm}^2\text{hr}^{-1}$ and $1.186 \mu\text{g}/\text{cm}^2\text{hr}^{-1}$ for BILO-3 and free AP suspension, respectively. Consequently, the PC of BILO-3 was found to be 0.00276 cm/hr which is markedly higher compared to free AP suspension that showed PC of 0.00033 cm/hr . The enhancement ratio (ER) of the selected AP-loaded bilosomal formulations is 8.36.

3.1.9. TEM

Representative TEM micrograph of the selected AP-loaded bilosome formulation (BILO-3) is shown in Figure (2). The obtained micrograph exposed the spherical nanostructured bilosomal vesicles with no agglomeration and low degree of polydispersity.

3.1.10. DSC

DSC thermogram of AP; Span 60; SDCh; CHOL; SPC; and BILO-3 formula were analyzed and depicted in Figure (4). The thermogram of AP Figure (4A), Span 60 Figure (4B), SDCh Figure (4C), CHOL Figure (4D), and SPC Figure (4E), exhibited its characteristic peak at 417.11°C , 53.18°C , 214°C , 148.8°C , and 165.2°C , respectively corresponding to their melting points. The thermogram of BILO-3 showed only one characteristic endothermic peak at 146.2°C Figure (4F), which could belong to the endothermic peak of CHOL. The thermal behavior of BILO-3 showed the disappearance of the AP characteristic peak, indicates that AP was completely entrapped into the bilayer structure (The reason for high EE % of AP). The obtained findings also demonstrate that AP exists in its amorphous state.

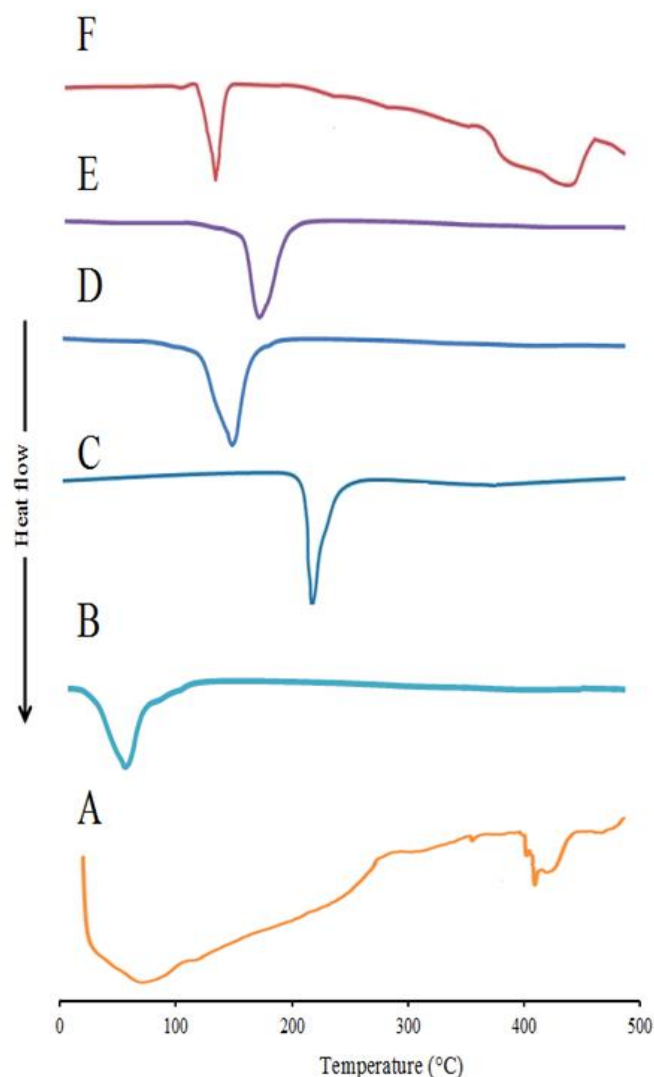


Figure (4): DSC thermograms of: AP (A); Span 60 (B); SDCh (C); CHOL (D); SPC (E); and BILO-3 formula (F).

3.1.11. BILO-3 Biological Stability Studies

To obtain an effective oral drug delivery system, AP-loaded bilosomes should be resistant to the harsh conditions of the GIT. To investigate that, the stability of BILO-3 was evaluated in SIF, pH 6.8. The obtained results Figure (5) revealed that the SIF media did not affect the BILO-3 integrity after 2 hrs of incubation. The obtained results revealed that the vesicle size of BILO-3 formulation remained constant (insignificantly changed) at the study conditions. The zeta potential was decreased immediately after incubation but remained constant during the sampling time points, assuring a good stability (repulsion) between the bilosomal vesicles.

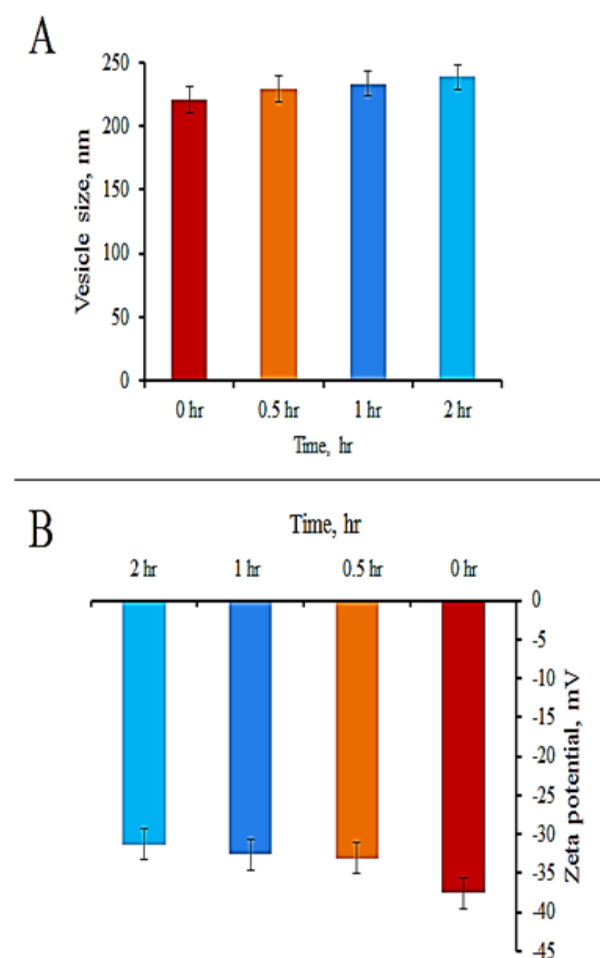


Figure (5): Biological stability studies of BILO-3 formulation in the presence of simulated intestinal fluid (SIF): A) Vesicle size, B) Zeta potential. The data represents the mean $\pm$ SD, $n=3$.

3.2. Effect of *Azolla pinnata* and its nanosized bilosomes on liver and kidney functions

Ameliorating effect of AP and APN against hepatorenal toxicity induced by 5-FU in rats was shown in Table (5). Results showed that there were no significant decrease in ALT, AST, urea, creatinine and uric acid among control negative, solvent, AP and APN compared to 5-FU group (G3). Administration of 5-FU (G3) increased significantly ALT, AST, urea, creatinine and uric acid when compared to control group. Treated groups with 5-FU and AP or APN simultaneously showed significant reduction in ALT, AST, urea, creatinine and uric acid when compared to 5-FU group, treated group with 5-FU and APN simultaneously (G7) showed significant reduction in elevated ALT, AST, urea, creatinine and uric acid than treated group with 5-FU and AP simultaneously (G6), ($p<0.05$).

3.3. Effect of *Azolla pinnata* and its nanosized bilosomes on antioxidants

Ameliorating effect of AP and APN against oxidative stress induced by 5-FU in rats was shown in Table (6). Results showed that there were no significant variations in MDA, NO, CAT, SOD, GSH and TAC among control negative and solvent, significant elevation in CAT, SOD, GSH and TAC with significant reduction in MDA and NO in AP and APN but APN was more significant than AP. Administration of 5-FU (G3) increased significantly MDA and NO with decreasing significantly in CAT, SOD, GSH and TAC when compared to control group. Treated groups with 5-FU and AP or APN simultaneously showed significant reduction MDA and NO with significant increasing in CAT, SOD, GSH and TAC when compared to 5-FU group, treated group with 5-FU and APN (G7) simultaneously showed significant reduction in elevated MDA and NO with significant increasing in CAT, SOD, GSH and TAC than treated group with 5-FU and AP (G6) simultaneously, ($p<0.05$).

3.4. Effect of *Azolla pinnata* and its nanosized bilosomes on histopathological examination

3.4.1. Liver histopathological examination

Microscopical examination of hepatic tissue samples of male Wistar rats from 5-FU group showed severe diffuse vacuolar degeneration and coagulative necrosis of hepatocytes, also there was dilatation of hepatic sinusoids Figure (6B). Examination of 5-FU group treated with AP showed diffuse vacuolar degeneration of hepatocytes and dilatation of hepatic sinusoids Figure (6C). Examination of 5-FU group treated with APN showed nearly normal hepatocytes with slight dilatation of hepatic sinusoids Figure (6D). Examination AP group, APN group and solvent group showed normal hepatic tissue structure Figure (6E, F and G) respectively.

3.4.2. Kidney histopathological examination

Microscopical examination of renal tissue samples of male Wistar rats from 5-FU group showed glomerular vacuolation, severe hydropic degeneration of renal tubular epithelium, renal tubular necrosis and desquamation of epithelium lining forming cellular cast in the tubular lumen Figure (7B). Examination of 5-FU group treated with AP showed severe diffuse hydropic degeneration of renal tubular epithelium with moderate renal tubular necrosis and desquamation of epithelium Figure (7C). Examination of 5-FU group treated with APN showed nearly normal renal tissue with slight vacuolation of renal tubular epithelium Figure (7D).

Examination AP group, APN group and solvent group showed normal renal tissue structure Figure (7E, F and G) respectively.

Figure (6): Hepatic tissue samples of male Wistar from different experimental groups. H&E staining, scale bar 100 μ m, (x40).

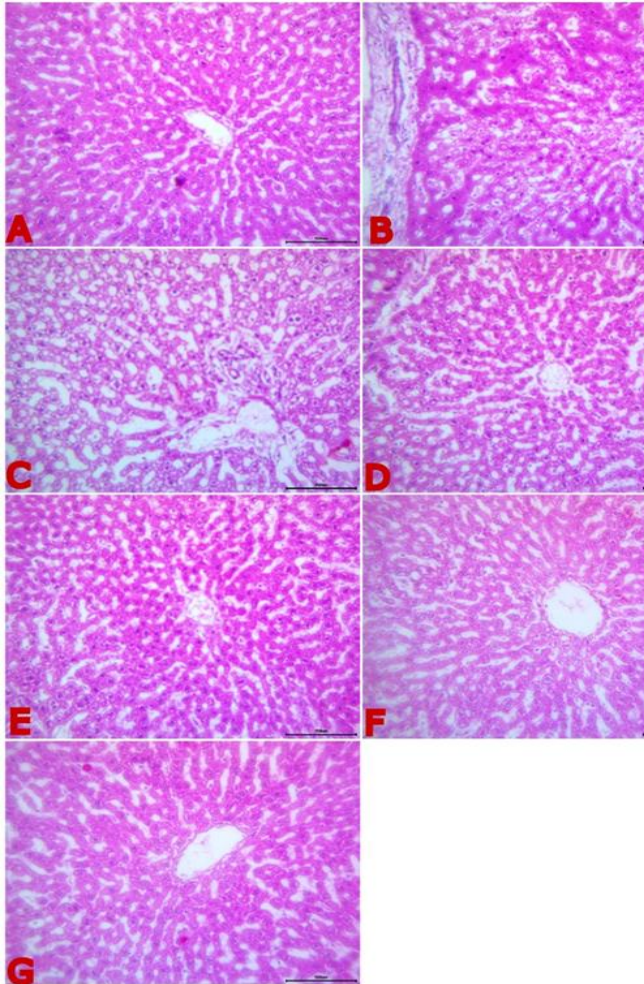


Figure (7): Renal tissue samples of male Wistar from different experimental groups. H&E staining, scale bar 100 μ m, (x40)

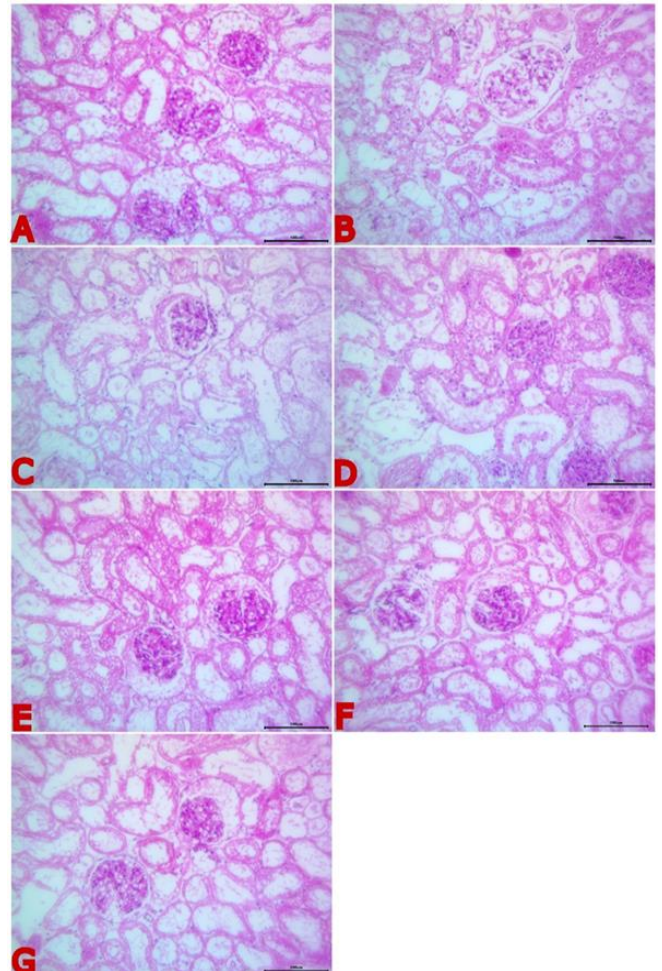


Table (3): Assessment of the accuracy, intra-day precision, and inter-day precision of the assay method at three *Azolla pinnata* concentration values within the linear range.

Taken Conc. (µg/ml)	Found Conc. (µg/ml)	Recovery ^a (±SD)	% Error	Intra-day precision		Inter-day precision	
				Recovery ^a ± SD	RSD	Recovery ^a ± SD	RSD
50	49.525	99.05± 0.79	-0.95	99.23±1.57	1.58	98.38±1.14	1.17
100	98.57	98.57±3.58	-1.43	99.18±1.82	1.83	99.07±1.33	1.34
150	151.83	101.22±1.39	+1.83	98.64±1.44	1.46	99.51±1.84	1.85

^a is the mean of three measurements, SD; the standard deviation, RSD; the relative standard deviation, and Conc. is the concentration

Table (5): The effect of *Azolla pinnata* (AP), *Azolla pinnata* nanosized bilosomes (APN) and fluorouracil (5FU) administration on ALT, AST, urea, creatinine and uric acid activity.

Parameters	Experimental groups						
	Control (G1)	Solvent (G2)	5-FU (G3)	AP (G4)	APN (G5)	5-FU+AP (G6)	5-FU+APN (G7)
ALT (U/L)	38.5±1.13 ^d	42.41±1.12 ^d	94.89±2.04 ^a	45.21±1.64 ^d	43.30±1.61 ^d	71.31±1.36 ^b	58.25±2.44 ^c
AST (U/L)	179.30±3.72 ^d	186.12±3.8 ^d	327.59±7.78 ^a	186.40±3.56 ^d	180.20±3.59 ^d	267.69±3.66 ^b	266.31±5.72 ^c
Urea (mg/dL)	30.94±1.16 ^d	32.56±1.02 ^d	85.35±1.92 ^a	37.44±1.44 ^d	34.90±1.11 ^d	64.16±1.76 ^b	47.89±1.26 ^c
Creatinine (mg/dL)	0.40±0.01 ^d	0.40±0.01 ^d	0.85±0.02 ^a	0.46±0.01 ^c	0.42±0.01 ^d	0.59±0.01 ^b	0.48±0.01 ^c
Uric acid (mg/dL)	2.01±0.03 ^d	2.09±0.03 ^d	5.48±0.11 ^a	2.16±0.03 ^d	2.05±0.03 ^d	4.42±0.1 ^b	3.52±0.1 ^c

Values conveyed as mean ± SE, different alphabets expressed as statistical differences as p<0.05. 5-FU: fluorouracil, AP: *Azolla pinnata*, APN: *Azolla pinnata* nanosized bilosomes, 5-FU + AP: fluorouracil + *Azolla pinnata*, 5-FU + APN: fluorouracil + *Azolla pinnata* nanosized bilosomes, AST: aspartate amino transferase, ALT: alanine transaminase.

Table (6): The effect of *Azolla pinnata* (AP), *Azolla pinnata* nanosized bilosomes (APN) and fluorouracil

Parameters	Experimental groups						
	Control (G1)	Solvent (G2)	5-FU (G3)	AP (G4)	APN (G5)	5-FU+AP (G6)	5-FU+APN (G7)
MDA(Liver) (nmol/gm)	8.03±0.42 ^c	9±0.06 ^c	22.23±0.18 ^a	6.93±0.23 ^c	4.97±0.18 ^c	11±0.40 ^b	8.63±0.23 ^c
NO(Liver) (µmol/L)	3.5±0.21 ^b	3.43±0.03 ^b	14.63±1.75 ^a	2.73±0.09 ^c	1.9±0.06 ^c	4.87±0.28 ^b	3.67±0.03 ^b
CAT(Liver) (U/gm)	3.27±0.27 ^c	3.63±0.07 ^c	2±0.06 ^d	4.6±0.25 ^b	6.03±0.12 ^a	3±0.06 ^c	3.9±0.12 ^c
CAT (Kidney) (U/gm)	7.93±0.80 ^c	8.9±0.21 ^c	1.43±0.12 ^d	11.63±0.35 ^b	14.37±0.84 ^a	8.37±0.45 ^c	9.47±0.18 ^c
SOD(Liver) (U/gm)	30.73±1.86 ^b	30.17±0.48 ^b	10.13±0.55 ^d	31.77±1.11 ^b	39.33±0.41 ^a	24.8±0.98 ^c	31.07±0.24 ^b
GSH(Liver) (mmol/gm)	23.6±2.14 ^c	24.37±1.44 ^c	10.8±0.71 ^d	34.8±2.83 ^b	46±2.19 ^a	21.63±0.50 ^c	27.17±1.10 ^c
TAC(Liver) (mmol/gm)	311.67±13.0 ^{2d}	326.67±4.41 ^d	213±2 ^e	486.67±6.01 ^b	579.67±34.98 ^a	315±2.89 ^d	416.67±4.41 ^c

(5FU) administration on oxidative stress markers.

Values conveyed as mean ± SE, different alphabets expressed as statistical differences as p<0.05. 5-FU: fluorouracil, AP: *Azolla pinnata*, APN: *Azolla pinnata* nanosized bilosomes, 5-FU + AP: fluorouracil + *Azolla pinnata*, 5-FU + APN: fluorouracil + *Azolla pinnata* nanosized bilosomes, MDA: malondialdehyde, NO: nitric oxide, CAT: catalase, SOD: super oxide dismutase, GSH: reduced glutathione, TAC: total antioxidant capacity.

4. DISCUSSION

Chemotherapy is efficient anticancer therapy that causes damage to other organs [53]. Fluorouracil is a more prevalent effective anticancer drug but unfortunately, it has side effects such as hepatotoxicity and nephrotoxicity [54]. It also causes oxidative stress and apoptosis [55].

The goal of this investigation was using of *Azolla pinnata* to ameliorate hepatorenal toxicity induced by 5-FU.

Results of present investigation showed that the ability of AP and APN to ameliorate oxidative stress and hepatorenal injury that caused by injected 5-FU. Results of group treated with APN were better than group treated with AP.

The obtained results showed that the bile salt (SDCh) exhibited synergistic effect on EE % (as the concentration of SDCh increased, the EE % increased). SDCh increase the EE% as its concentration increased because SDCh have surface active behaviour and entrapped into the bilayer membrane of the bilosomal vesicles. Also, SDCh is known to increase the flexibility of bilayer membrane and increase the AP solubility in the lipid bilayer membrane of the constructed bilosomal vesicles hence increased the EE % of AP. The higher concentration of bile salt (BILO-4) could result in fluidizing the lipid bilayers of the vesicles causing leakage of the AP with reduction in the EE %. Also, the reduction in the EE could be attributed to the enhanced AP solubility in the dispersion medium (distilled water) due to the formation of mixed micelles.

The obtained results revealed that the nanosized bilosomes showed significantly higher AP amount released ($p < 0.05$) than free AP suspension. The obtained results showed that the bile salt (SDCh) exhibited a positive effect on the AP release. The increases in SDCh concentration resulted in increased AP release [29].

Previous researches reported that the bile salts as SDCh give an elastic nature to the bilosomal vesicles and act as permeability enhancers which could be attributed to the surface negative charge which has a membrane-destabilizing effect that increase the fluidity of the rabbit intestinal membrane [30] and thus enhance the AP diffusion and penetration across it. Also, the vesicles negative

charge could be promoted AP uptake through intestinal M-cells and enhance the AP absorption via the intestinal lymphatic transport pathways. Furthermore, the higher permeation of AP in the case of BILO-3 formulation could be due to nanosized bilosomal vesicle, high encapsulation in the lipid matrix, and the presence of nonionic surfactant (Span 60). The nonionic surfactant opens the tight junction of intestine due to more hydrostatic pressure [29] resulting in enhanced AP permeation.

Results of this investigation indicated that rats received 5-FU had increased ALT and AST in serum so liver damage. It induced liver damage with increased liver functions [56].

Results of the present study indicated that rats which received 5-FU had exceeded ALT, AST, urea, creatinine and uric acid in serum so liver and renal damage. It induced liver and kidney damage with increasing liver and kidney functions [35,57,58].

Significant reduction in ALT, AST, urea and creatinine in treated AP and APN groups and this agreed with [7,36].

5-FU caused oxidative stress through significant reduction in CAT, SOD and GSH and increasing significantly MDA and NO in rats injected with 5-FU which were such as [59,60] that referred to 5-FU group showed decrease in SOD, GSH and CAT with increase in NO and MDA which agreed also with [35] that mentioned that 5-FU caused elevation in MDA and decrease in SOD, GSH and CAT.

Azolla pinnata contains several compounds that give it an antioxidant, anti-inflammatory and hepatoprotective activity, such as phenols, flavonoids, alkaloids, amino acids, minerals, vitamins, saponins, fatty acids, triterpenoids and tannins [19].

AP and APN treated groups showed reduction of oxidative stress induced by 5-FU as significant increase in CAT, SOD, GSH and TAC and decrease significantly MDA and NO in rats treated with AP or APN and 5-FU simultaneously when compared to 5-FU group and these results in the same line with [7,36].

Plants have less side effects even with frequent administering [61,62]. Flavonoids and polyphenols in chemical structure of plants and algae which are

responsible for antioxidant and anti-inflammatory effects [63]. *Azolla pinnata* has pharmacological activities such as anticaries, antimicrobial, anti-inflammatory and antioxidants. It is an inexpensive drug with less side effects [20].

Histopathological hepatic and renal inspection illustrated that normal structure in control, solvent, AP and APN groups. 5-FU group had deteriorations as sever diffuse vacuolar degeneration and coagulative necrosis of hepatocytes, also there was dilatation of hepatic sinusoids that was in the same line with [32,35,64,65]. Although 5-FU is used in treatment of cancers, it has severed potent health problems [66].

The present investigation agreed with [67] that stated that 5-FU caused liver necrosis so allowed out of ALT and AST into serum with high levels.

5-FU group had deteriorations as glomerular vacuolation, sever hydropic degeneration of renal tubular epithelium, renal tubular necrosis and desquamation of epithelium lining forming cellular cast in the tubular lumen which agreed with [17,32,35].

AP and 5-FU treated group stated that diffuse vacuolar degeneration of hepatocytes and dilatation of hepatic sinusoids, sever diffuse hydropic degeneration of renal tubular epithelium with moderate renal tubular necrosis and desquamation of epithelium and APN and 5-FU treated group stated that nearly normal hepatocytes with slight dilatation of hepatic sinusoids, showed nearly normal renal tissue with slight vacuolation of renal tubular epithelium and this agreed with [7].

5. CONCLUSIONS

In conclusion, receiving of 5-FU, anticancer drug, that caused renal and hepatic damage which were confirmed by elevation of oxidative stress and histopathological changes. Simultaneous administration of AP or APN with 5-FU decreased these influences.

By reducing oxidative stress that was increased by 5-FU, treatment by AP or APN reduced oxidative stress.

The current investigation suggests that AP and APN have an ameliorating effect against hepatorenal toxicity that was caused by 5-FU in rats. May be due to antioxidant, antiapoptotic and anti-inflammatory effects of *Azolla pinnata*.

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