

Chemoprotective Potential of *Helianthemum confertum* Against the Loss of Molecular and Functional Integrity of the Liver Cell in Doxorubicin-Treated Rats

Radja Djebbari^{1,2}, Yasmine Chemam¹, Nassima Boubekri^{1,2}, Zohra Lakroun³, Mohammed Kebieche³, Amel Amrani^{1,2}, Fadila Benayache¹, Samir Benayache¹, Djamilia Zama^{1*}

¹Unité de Recherche Valorisation des Ressources Naturelles, Molécules Bioactives, Analyses Physicochimiques et Biologiques (VARENBIOMOL), Université Frères Mentouri Constantine1, Route Ain El Bey, 25000 Constantine, Algérie.

²Département de Biologie Animale ; Faculté des Sciences de la Nature et de la Vie, Université Frères Mentouri Constantine1, Route Ain El Bey, 25000 Constantine, Algérie.

³Laboratory of Molecular and Cell Biology (BMC), University of Jijel, Jijel, Algeria

Received 5th May, 17; Revised 28th June, 17, Accepted 12th July, 17; Available Online 25th July, 2017

ABSTRACT

The objective of the current study was led to reveal the possible protective effects of *n*-butanol extract of *Helianthemum confertum* (*H. confertum*) against doxorubicin (DOX) induced liver damage and its implication on the integrity of liver cells. Adult male rats were randomly divided into groups treated with plant extract (50 mg/kg, 100 mg/kg) for 10 days and/or injected with a single dose of DOX (10 mg/kg). Liver function as well as oxidative stress parameters and histological study were estimated. DOX treated rat's induced hepatic dysfunction revealed by a significant increase in biochemical parameters (serum transaminases, cholesterol and triglycerides) and disturbance in oxidative stress parameters described by an increase in malondialdehyde (MDA) levels, providing information on the loss of cellular integrity. This later elicited histopathological changes in the liver which was confirmed on histological section showing necrotic cells. Although the DOX-treatment reduced significantly the reduced glutathione (GSH) level and the glutathione peroxidase (GPx) activity. The pretreatment of the animals with *n*-butanol extract of *H. confertum* at 50 mg/kg and 100 mg/kg counteracted almost all adverse effects induced by DOX. The results showed a considerable decrease in serum markers of liver function and lipid peroxides. There was significant increase in the GSH level and the activity of antioxidant enzyme (GPx), which allowed the normalization of redox status in liver cells. Data suggest that DOX-induced an oxidative stress in rat's liver and *n*-butanol extract of *H. confertum* exerted antioxidant properties.

Keywords: Doxorubicin, *Helianthemum confertum*, Biochemicals Parameters, Antioxidant, Lipid peroxidation, Hepatoprotective.

INTRODUCTION

Anthracyclines remain an important class of drugs in the treatment of cancer, but also remains a difficult chemotherapeutic agent particular their cardiotoxic effects¹. They are a group of antibiotic that are documented to be effective against many chemo-responsive tumors such as ovarian cancers, breast cancers, and lymphomas^{2,3}.

Doxorubicin is an anthracycline antibiotic frequently used to treat a diversity of cancers as a most effective antitumor. However, the clinical use of DOX is often limited because of its undesirable serious toxic side effects on various organs such as heart and liver. Toxicity induced by doxorubicin has been believed to be mediated through diverse mechanisms as ROS generation, and iron-dependent oxidative damage to biological macromolecules and membrane lipid peroxidation^{4,5,6}.

During DOX therapy, the liver received accumulated and metabolized high concentration of DOX. Hence, it is

expectable that the liver is one of the most affected organs by DOX therapy. Certainly, in many animal experiences, DOX has been revealed to be hepatotoxic^{4,7}.

DOX can generate free radicals through enzymatic and nonenzymatic path ways. It undergoes a one-electron reduction catalyzed by NADPH reductase to yield a semiquinone free radical intermediate that regenerated the parent quinone reacting with O₂. Thus, generates O₂^{•-} and its dismutation produced H₂O₂. These ROS can then be converted into the more potent hydroxyl radical HO[•], which is capable to induced DNA damage. Also, proteins, besides it stimulates lipid peroxidation^{8,9,10}.

Among followed strategies to attenuate DOX toxicity are dosage optimisation, synthesis and use of analogues or combined therapy with antioxidants. The most promising results come from the combination of the drug delivery together with an antioxidant in order to reduce oxidative stress. Many antioxidants have been assayed with very

different results. Among these molecules, metal ions chelators and flavonoids that scavenge reactive oxygen species have been widely studied¹¹.

Actually, the usage of antioxidant in mixture with chemotherapeutic agents is a central subject in cancer treatment to protect against oxidative injuries without attenuating the clinical efficacy and it has been the subject of extensive current debates¹². Although, it is essential to study for complementary and alternative plans against DOX-induced hepatotoxicity. There are favorable preclinical consequences in protecting against DOX-induced toxicity through application of diverse natural compounds¹³.

Helianthemum genus (Cistaceae) contains approximately 110 species¹⁴, some of them are important medicinal plants used in several countries to treat diarrhea^{15,16,17}, gastrointestinal and respiratory diseases¹⁸. This genus is reported to possess different properties, antioxidant and antimicrobial^{19,20,21} anti-inflammatory and anti-ulcer²², antiprotozoal²³, Antioxidant and antibacterial²⁴. Also, some species of this genus have been previously examined for bioactive components like flavonoids^{25,26}, lignans²⁷ and essential oils²⁸. Also, the composition of the methanolic and water extracts from the leaves of plants belonging to the *Helianthemum* genus was strongly related to their medicinal uses^{19,20}.

However, reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism and environmental factors. Sahara species can develop metabolites responses against drought stress and ROS produced by extensive UV exposition. Moreover, to our best knowledge and as a part of our continuing investigation of plants belong to this genus²⁹ and specially this specie³⁰, the present study was designed to investigate the ability of *n*-butanol extract obtained from air-dried aerial parts of endemic Algerian specie (*Helianthemum confertum*) to prevent DOX-induced free radical formation and display hepatoprotective effects after administration of a single dose of DOX to rat.

MATERIALS AND METHODS

Plant material

The plant material was collected from the area of Mogheul in the south-west of Algeria and authenticated by M. Mohamed Benabekhem, director of the nature preservation agency, Bechar³¹. A voucher specimen (HCC0512-MOG-ALG-60) has been deposited at the Herbarium of the research unit (VARENBIOMOL), Université Frères Mentouri, Constantine 1.

Extraction procedure

Air-dried aerial parts (2279 g) of *Helianthemum confertum* Dunal, non Willk. (Cistaceae) were macerated at room temperature with MeOH-H₂O (80:20, v/v) for 72 h, three times. After filtration, the filtrate was concentrated in vacuum (up to 35°C) and dissolved in distilled H₂O (900 mL) under magnetic stirring and then put at the refrigerator for one night to precipitate a maximum of chlorophylls. The resulting solution was filtrated extracted successively with petroleum ether, chloroform, ethyl acetate and *n*-butanol. The organic solutions were dried

with sodium sulfate (Na₂SO₄), filtered using common filter paper and concentrated in vacuum (up to 35°C) to obtain the following extracts: petroleum ether (135 mg), CHCl₃ (1.65 g), EtOAc (7.11 g) and *n*-BuOH (35.07 g).

Animals and treatment

Male wistar albino rats weighing 200-250 g were obtained from Pasteur institute (Algiers, Algeria). Animals were maintained under a daily 12 h light/dark cycle at a constant temperature (24 ± 2 °C) and a free access to food and water. Rats were adapted for 2 weeks before the indicated treatments. All experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals. Also, all experimental procedures were performed between 8–10 a.m. and care was taken to avoid stressful conditions. Rats were housed five per cage and were randomly divided into 6 groups:

Group 1: control rats or the untreated rats: Rats were administered distilled water for 10 consecutive days.

Group 2 and Group 3: HC 50 mg/kg and HC 100mg/kg, animals received by gavage plant extract (50 mg/kg and 100mg/kg respectively) for 10 consecutive days.

Group 4: DOX, rats received a single injection of DOX (10 mg/kg)

Group 5 and Group 6: DOX+50 and DOX+100, rats received orally plant extract (50 mg/kg and 100 mg/kg) for 10 consecutive days and a single injection of DOX.

After treatment the rats were sacrificed, blood samples were drawn from the caudal vena cava, collected in test tubes containing EDTA, and centrifuged to obtain serum for analysis of biochemical parameters. Livers were isolated to measure the levels of antioxidant enzymes, MDA and histopathological studies.

Preparation of tissues

Livers were removed quickly and placed in iced NaCl 0.9% solution, perfused with the same solution to remove blood cells, blotted on filter paper, weighed, and homogenized in ice-cold KCl 1.15% with the addition of 6µL of 250µM butylated hydroxytoluene to prevent the formation of new peroxides during the assay. The homogenization procedure was performed under standardized condition. Homogenates (20%) were centrifuged and the supernatant was kept on ice until assayed.

Malondialdehyde (MDA) measurement

Lipid peroxidation (LPO) was determined by measuring the formation of TBRA_s using the colorimetric method of Uchiyama and Mihara³². 3mL of phosphoric acid (1%) and 1ml of thiobarbituric acid (TBA, 0.67%), aqueous solution were added to 0.5mL of liver homogenate (20%) pipetted into centrifuge tube. The mixture was heated for 45 min in a boiling water bath. Then the mixture was cooled at room temperature, and 4mL of *n*-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was measured at 532 nm. MDA was used as the standard.

Measurement of glutathione

Reduced glutathione (GSH) content in the liver was measured according to the method described by Elman³³ using Elman's reagent. This method is based on the reactive cleavage of 5, 5'-dithiobis-(2-nitrobenzoic acid)

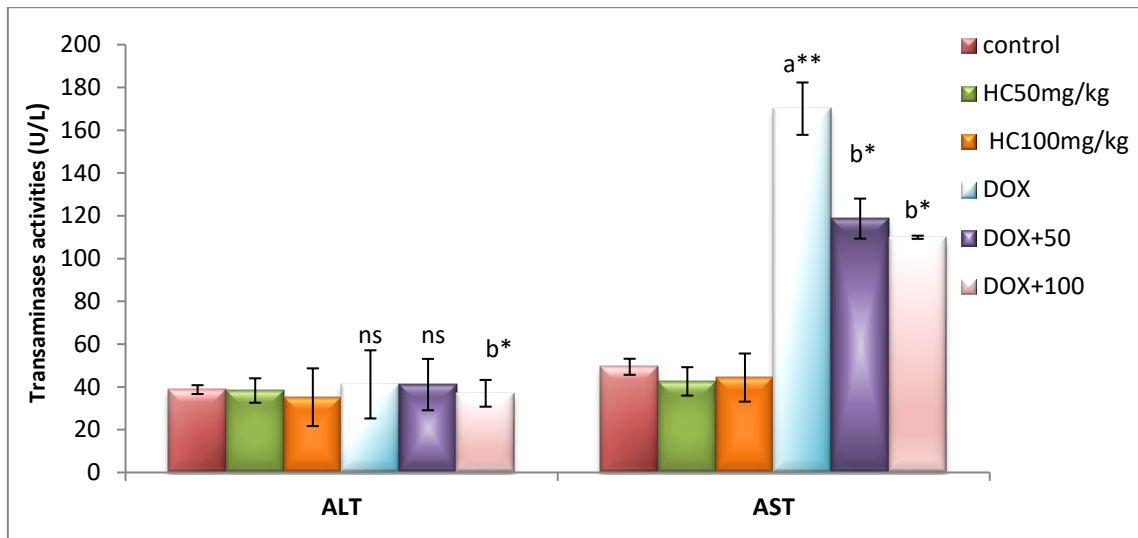


Figure 1: The protective effect of *n*-butanol extract of *H. confertum* (50mg/kg and 100mg/kg) against DOX-induced hepatotoxicity. Effect on serum aspartate transaminase (AST) and alanine transaminase (ALT) levels in rat's liver. Data are reported as means \pm SD. a: group compared to control group, b: group compared to DOX group. (* P <0.05; ** P <0.01).

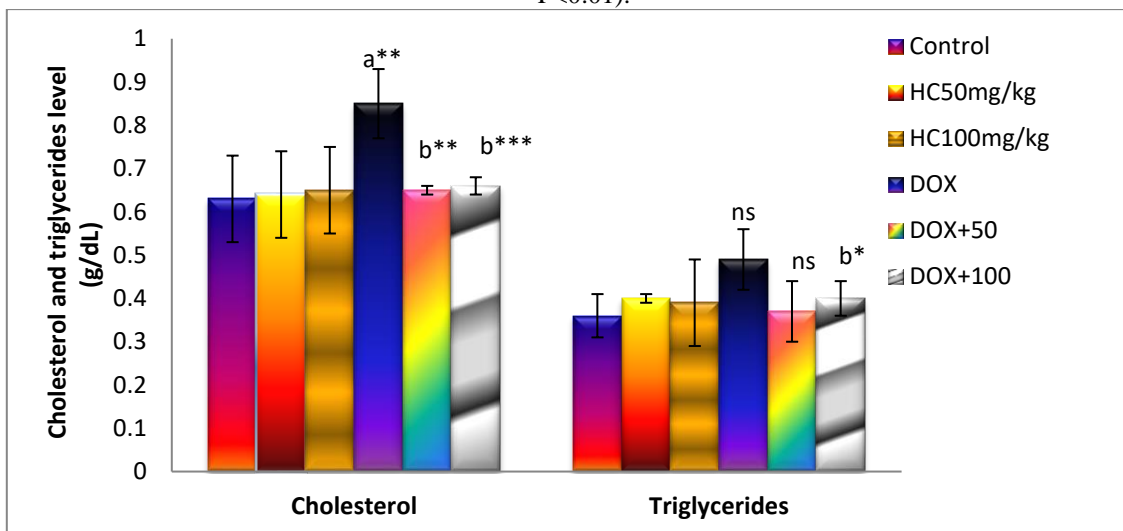


Figure 2: The protective effect of *n*-butanol extract of *H. confertum* (50mg/kg and 100mg/kg) against DOX-induced toxicity. Effect on serum cholesterol and triglycerides levels in rat's liver. Data are reported as means \pm SD. a: group compared to control group, b: group compared to DOX group. (* P <0.05; ** P <0.01).

by sulfhydryl group to yield a yellow color with maximum absorbance at 412 nm against reagent blank.

Evaluation of GPx activity

GPx activity in the liver was measured according to the method described by Flohe and Gunzler³⁴. This method is based on the reduction of H₂O₂ in the medium by GPx in the presence of GSH. Briefly 0.2mL supernatant obtained from tissues, 0.4 ml GSH (0.1 mM), 0.2mL TBS solution (Tris 50mM, NaCl 150mM PH 7.4) were added to the tubes and mixed. After 5 min incubation at 25°C, 0.2mL of H₂O₂ (1.3mM) was added in the mixture. The reaction was stopped after 10 min by addition of 1mL trichloroacetic acid (TCA 1%, w/v), and then the tubes maintained at 0-5°C in an ice bath for 30 min. After centrifugation, 0.48mL supernatant was taken and added to each tube, and then 2.2mL TBS solution and 0.32 mL DTNB (1mM) were added. The optical density was

measured at 412 nm in the spectrophotometer after 5 min. In order to express the antioxidant enzyme (GPx) activities per gram of protein, total protein concentration was determined colorimetrically using the method of Lowry et al³⁵.

Plasma biochemical analysis

The liver marker enzymes, aspartate transaminase (AST) and alanine transaminase (ALT) also, total cholesterol and triglycerides were estimated using commercial kits (Spinreact, SPAIN).

Histopathological examination

For histopathological analysis, fragments of liver were taken and fixed in formal 10% solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were then embedded in paraffin, cut into thick sections (5 μ m) and

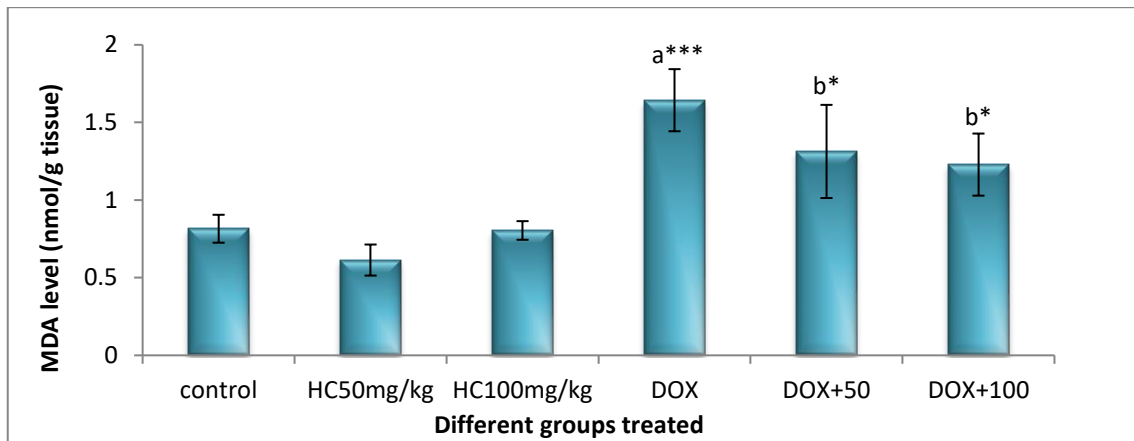


Figure 3: The protective effect of *n*-butanol extract of *H. confertum* (50mg/kg and 100mg/kg) against DOX-induced hepatotoxicity. Effect on lipid peroxidation (TBARs content) in rat's liver. Data are reported as means \pm SD. a: group compared to control group, b: group compared to DOX group. (* $P < 0.05$; ** $P < 0.01$).

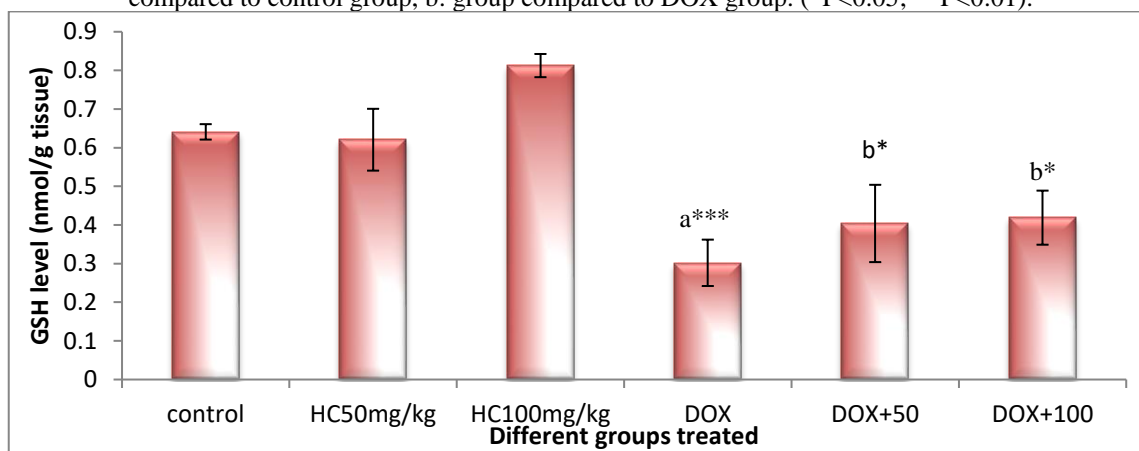


Figure 4: The protective effect of *n*-butanol extract of *H. confertum* (50mg/kg and 100mg/kg) against DOX-induced hepatotoxicity. Effect on GSH level in rat's liver. Data are reported as means \pm SD. a: group compared to control group, b: group compared to DOX group. (* $P < 0.05$; *** $P < 0.001$).

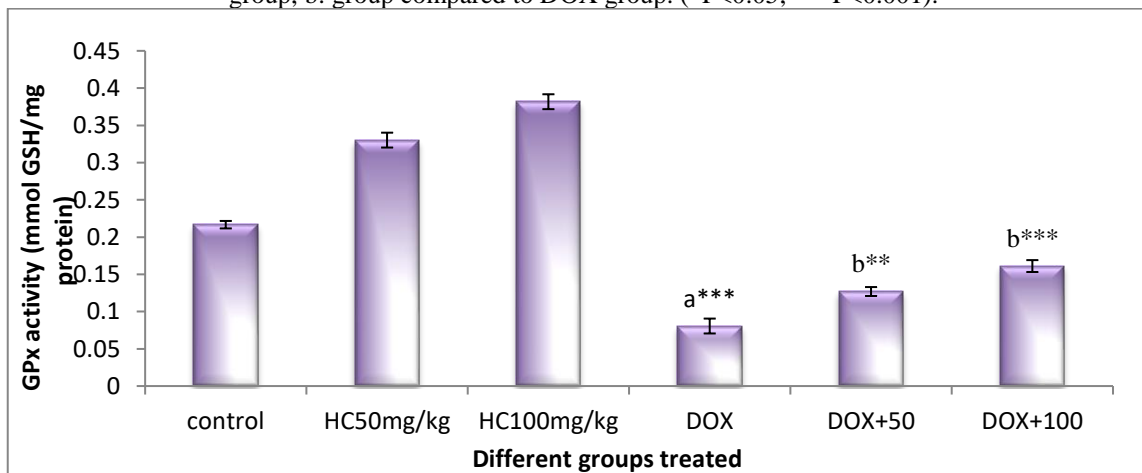


Figure 5: The protective effect of *n*-butanol extract of *H. confertum* (50mg/kg and 100mg/kg) against DOX-induced hepatotoxicity. Effect on the antioxidant enzyme (GPx) activity in rat's liver. Data are reported as means \pm SD. a: group compared to control group, b: group compared to DOX group. (* $P < 0.01$; *** $P < 0.001$).

stained with hematoxylin and eosin and examined microscopically³⁶.

Statistical analysis

Results are expressed as means \pm SD. Assessment of these results was performed using one-way analysis of variance (ANOVA), followed by student's *t*-test for multiple

comparisons using GraphPad Prism 5.01 Retail + 5.02 Update, Version 5. The statistical significance was accepted at a level of $P < 0.05$.

RESULTS

Physical symptoms

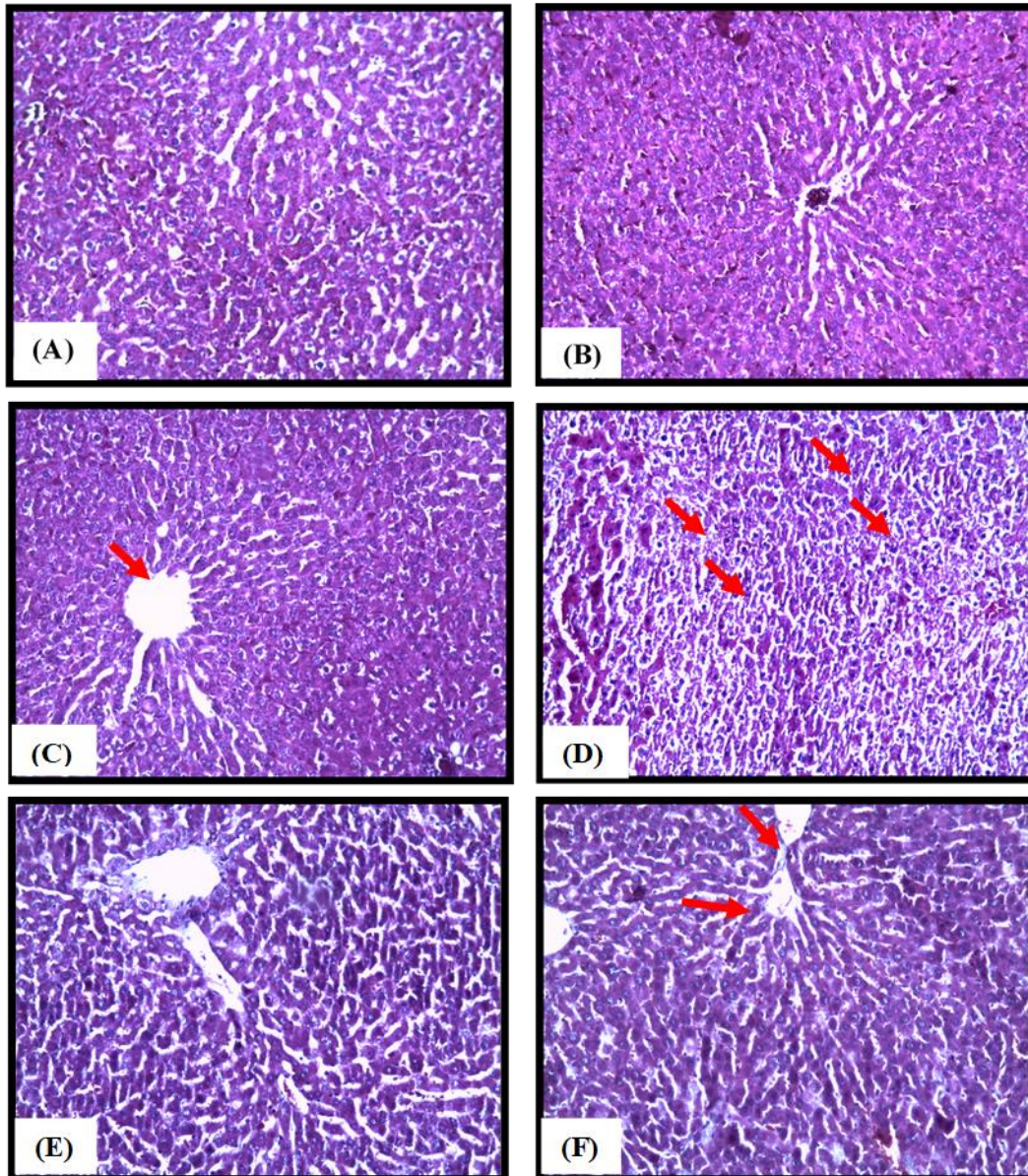


Figure 6: Histopathologic changes affecting the liver of rats ($\times 100$). (A) Normal liver of the control group; (B) and (C) livers of treated group with *n*-butanol extract 100mg/kg and 50 mg/kg showed a histopathological picture that closely approximate that of the control group; (D) liver of DOX treated group showed necrosis and Sinusoidal dilatation; (E) and (F) livers of DOX+ HC50 and DOX+ HC 100 treated groups respectively showed congestion.

All rats survived the experimental period, and no abnormal behavior considered to be related to DOX treatment was observed in any of the treated groups during the experiment.

Effect of DOX and n-butanol extract of H. confertum on serum biochemicals Parameters

The serum marker indicating liver injury AST were significantly increased in the DOX treated group compared to control. DOX treatment caused a significant ($P < 0.01$) increase in the AST activity with the values 170.08 ± 12.24 U/l compared to control values 49.41 ± 3.75 U/l. But, there are no significant increase in ALT activity in DOX treated group with $41,19 \pm 15,93$ U/l compared to control values $38,70 \pm 2,05$ U/l. Also, the pretreatment with both doses of plant extract decreased significantly ($P < 0.05$) the AST activity compared to DOX group. While, the pretreatment

with plant extract at a dose of 100 mg/kg lowered the ALT activity significantly ($P < 0.05$) compared to DOX group (Figure 1).

Also, DOX treatment caused significant increases in total cholesterol ($p < 0.01$) levels compared to the control group. But no significant increase in triglycerides levels was observed. While 50 and 100 mg/kg of *n*-butanol extract administration caused a significant ($p < 0.01$; $P < 0.001$) reduction respectively in total cholesterol levels compared to DOX group. Extract treatment at dose 100mg/kg decrease the level of triglyceride ($p < 0.05$) compared to DOX group (Figure 2).

Effect of DOX and n-butanol extract of H. confertum on lipid peroxidation in rat's liver

The data showed a significant increase ($p < 0.001$) in lipid peroxidation levels in liver rat's tissue after injection of

DOX compared to control. While, both dose (50 and 100 mg/kg) of *n*-butanol extract of *H. confertum* showed a significant decrease ($p < 0.05$) in lipid peroxidation level compared to DOX group (Figure 3).

Effect of DOX and n-butanol extract of H. confertum on liver GSH levels and GPx activity.

DOX treated rats showed a significant decrease ($p < 0.001$) in liver glutathione level and GPx activity compared to control group. While the treatment with both doses of *n*-butanol extract of *H. confertum* showed a significant increase ($p < 0.05$) in GSH level and ($p < 0.01$; $P < 0.001$) in GPx activities with dose 50; 100mg/kg successively (Figures 4, 5).

Effect of DOX and n-butanol extract of H. confertum on liver histology

Photomicrographs of rat's liver (H&E, 100 \times) from (A) control group showing normal hepatic architecture; (B&C) *H. confertum* alone (100 mg/kg and 50 mg/kg) treated group showing normal histology almost similar to the control group. (D) DOX treated group showing distortion of normal architecture and irregularly-shaped hepatocytes, necrosis, dilated and congested sinusoids veins. (E&F) *H. confertum* pre-treated DOX intoxicated group at both doses showing a histological picture comparable to that of the control group with minimal damage of hepatocytes.

DISCUSSION

The clinical use of DOX is limited; because of sever toxic effects of the drug on the body's tissues, including heart, liver, kidneys and nervous system³⁷ which are due to free radicals' generation³⁸.

The liver is highly susceptible to the chain reaction of lipid peroxidation under aerobic conditions³⁹. Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids. Its occurrence in biological membranes caused impaired membrane function and structural integrity, decreased in fluidity, and inactivated of a number of membranes bound enzymes and protein receptors⁴⁰. The result of the present study showed that the administration of a single dose of DOX (10 mg/kg) caused sever acute liver damage in rats. As evidenced by a significant increase in MDA levels and significant reduction of GPx activities, and significant depletion of hepatic GSH in DOX treated rats compared to the control group. GSH is a non enzymatic anti-oxidant defense. It plays a critical role in cellular functions, which included the maintenance of thiols status of proteins, the destruction of H₂O₂, lipid peroxides, free radicals, drug biotransformation and detoxification. Decrease in GSH level might be due to its increased utilization by the hepatocytes in scavenging DOX metabolites⁴⁰.

As well, we found that the cumulative hepatotoxicity of DOX was clearly featured by increase in serum biochemical markers enzymes AST and ALT. ALT enzyme is abundant in cytosol of hepatic parenchymal cells, while, AST is found in cytosol and mitochondria of hepatocytes. It is also distributed in cardiac muscle, pancreas and kidney. Hence, ALT measurement liver is more specific to determine liver damage⁴¹. These results are in agreement with many reports showed that treatment

with DOX caused significant changes in the serum levels of ALT, AST^{42,43} as well as in the levels of MDA, GSH, GPx in liver's tissue of wistar Albinos rats^{44,45,46}.

Polyphenols, flavonoids comprise a family of compounds that scavenge a wide range of free radicals and iron chelating properties, including the most active hydroxyl radical, which may initiate lipid peroxidation and prevent the loss of the lipophilic (α -tocopherol) and hydrophilic (ascorbate) antioxidants, by repairing tocopheryl and ascorbate radicals^{43,45}. Due to their radical-scavenging and iron-chelating properties, flavonoids can be considered as potential protectors against Dox toxicity¹¹.

In the current study, rats treated with *n*-butanol extract before treatment with DOX resulted in decrease in lipid peroxidation. Process as well as increased in glutathione content and glutathione peroxidase activity in liver permitting the prevention of hepatic dysfunction on decreasing the level of serum transaminases, cholesterol and triglyceride following inhibition of their hepatic leakage by preventing lipid peroxidation. So, phenolic compound from *H. confertum* showed antioxidant proprieties. These results are in agreement with many reports showed that treatment with extract of medicinal plant which containing polyphenols and flavonoids have a protective effect against DOX hepatotoxicity^{45,46}.

DOX-induced hepatic injury was further confirmed histopathologically where sinusoidal dilatation, and hepatocytes necrosis were clearly shown. Similar histopathological and biochemical marker alterations have been previously reported in acute DOX-induced hepatotoxicity^{47,48}.

Extract pretreatment of *H. confertum*, significantly ameliorated DOX changes and almost restoring the normal architecture of the liver. DOX- associated abnormalities have been gradually abolished with the various applied doses of *H. confertum*. Optimal effect was reached in animals treated with 100 mg/kg *n*-butanol extract, suggesting a protective role of *H. confertum* against DOX hepatotoxicity.

CONCLUSION

The results of the present study demonstrated that administration of *H. confertum* was markedly efficient in reducing lipid peroxidation and strengthening endogenous antioxidant systems leading to a considerable attenuation of the hepatotoxicity as evidenced by histopathological and biochemical examinations. It was concluded that *H. confertum* extract can be a useful adjuvant along with DOX to possibly reduce or prevent the hepatotoxicity in DOX treated cancer patients.

ACKNOWLEDGMENTS

This work was supported by Algerian Ministry of Education and Scientific Research.

The authors declare that they have no conflict of interest.

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