Attenuation effect of Abnormal Savda Munziq on liver and heart toxicity caused by chemotherapy in mice

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Abstract. Abnormal Savda Munziq (ASMq), an Uighur medicine formula commonly used in the treatment of cancer, has been speculated to possess antioxidative and antiproliferative effects, and to regulate immune activity. The present study was designed to systematically elucidate the toxicity-reducing activity of ASMq in mice undergoing combination chemotherapy with doxorubicin and 5-fluorouracil (5-FU). The mice were divided into normal (saline, 10 ml/kg) and doxorubicin + 5-FU groups (doxorubicin, 2.5 mg/kg; 5-FU, 10 mg/kg on alternate days). In addition, three groups received different doses of ASMq (2, 4 and 8 g/kg), in addition to doxorubicin (2.5 mg/kg) and 5-FU (10 mg/kg) treatment on alternate days. The histology of the heart and liver, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activity, malondialdehyde (MDA) concentrations in heart homogenate, and various biochemical parameters of the liver were evaluated. Compared with the normal control group, ASMq dose-dependently improved a number of variables, including body weight, liver index, transaminase and total protein, and partially normalized liver and cardiac pathology. ASMq restored activities of defense antioxidant enzymes SOD and GSH-Px towards normal levels, and decreased MDA concentration in dose-dependent manner. These results demonstrated that ASMq provides significant protection against doxorubicin + 5-FU combination induced hepatotoxicity and cardiotoxicity. Further studies are required to determine the effects of ASMq against doxorubicin + 5-FU-induced toxicity during chemotherapy *in vivo*.

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Introduction

The radiotherapy and chemotherapy are the primary treatment options for the majority of types of cancer (1). However, a variety of side effects are associated with chemotherapeutic drugs, including immunosuppression, discomfort of the gastrointestinal tract, vomiting and inappetence, which may substantially impact patient health and quality of life (2). The majority of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells, leading to unwanted side effects (3). Therapeutically effective doses of numerous types of anticancer drug may produce irreversible changes in normal tissues (4). Therefore, there is a required for cancer treatments which are able to reduce the harmful side effects of anticancer drugs in normal tissues. Combining herbal medicinal herbs with chemotherapy may improve quality of life, tumor response and performance status, as well as reduce the toxicity of chemotherapy (5). At present, certain medicinal herbs have already attracted a attention due to their low toxicity and purported curative effects.

Doxorubicin (Adriamycin®) is a widely used anthraquinone anticancer drug, with significant dose-limiting cardiac toxicity (6-8). In combination with other anticancer drugs, it is used as first line therapy in malignant lymphoma, sarcomas, cancer of the breast, lung, bladder and various other cancer types (9). 5-Fluorouracil (5-FU), a thymidylate synthetase enzyme inhibitor, is metabolized intracellularly to 5-FU deoxynucleotide (5F-dUMP), which inhibits deoxythymidylic acid synthetase, then prevents deoxyuridylic acid (dUMP) from methylating to deoxythymidine monophosphate (dTMP), which affects DNA synthesis (10). 5-FU is effective against cancers of the digestive system (esophageal, stomach, intestinal cancer, carcinoma of pancreas, liver cancer) and breast cancer (11). Furthermore, 5-FU may be effective in treating cancer of the cervix, ovaries, bladder, head and neck, in addition to chorioepithelioma (11). Clinically doxorubicin and 5-FU (AF) are often combined; however, their use is limited by cardiac and liver toxicity (12).

Abnormal Savda Munziq (ASMq) is an Uighur medicinal herbal preparation that is widely used in the Xinjiang region of China (13). ASMq consists of ten medicinal herbs: *Adiantum capillus-veneris* L. *Alhagi pseudalhagi* (Bieb.) Desv., *Anchusa italica* Retz., *Cordia dichotoma* G.

Forst., *Euphorbia maculata* L., *Foeniculum vulgare* Mill., *Glycyrrhiza glabra* L., *Lavandula angustifolia* Mill., *Melissa officinalis* L., and *Ziziphus jujuba* Mill (14). ASMq has been applied to the prevention and treatment of numerous chronic diseases, including cancer, hypertension, diabetes mellitus and memory dysfunction (15). ASMq has been used as a traditional remedy for the prevention or treatment of digestive cancer (16).

Previous pharmacological and clinical studies have demonstrated that ASMq exhibits antioxidant effects, reducing oxidative damage by free radicals (17), anti-DNA oxidative damage, and inhibiting radiation-induced damage in mice (18-21). In addition, ASMq has been shown to modulate cellular and humoral immunity in a combined stress mouse model, protecting mitochondria and DNA against damage induced by OH in a cell-free system (17,22). Furthermore, ASMq is able to inhibit cancer cell proliferation and viability *in vitro* (15,23,24), and has exhibited anti-tumor properties *in vitro* (15,22-24) and in rats (25). However, its potential protective effects against toxicity induced by doxorubicin and 5-FU have not been systematically evaluated. Therefore, the aim of the present study was to investigate the protective effect of ASMq against doxorubicin- and 5-FU-induced toxicity in mice.

Materials and methods

Chemicals and reagents. ASMq was provided by Qikang Habo pharmaceutical Co., Ltd. (Xinjiang, China). Doxorubicin hydrochloride for injection (adriamycin) was purchased from Pfizer Italia Srl Co., Ltd. (Rome, Italy). 5-FU was purchased from JinYao Amino Acid Co., Ltd. (batch no. 0912302; Tianjin, China). Kits for determining serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, and total protein (TP), were obtained from the Xiamen Jiaxing Biotechnology Co., Ltd. (Xiamen, China; cat. nos. C0010-2, C009-2 and A045-2, respectively). Assay kits used for determination of malondialdehyde (MDA) content, superoxide dismutase (SOD) activity and glutathione peroxidase (GSH-Px) activity were obtained from the Nanjing Jiancheng Institute of Biological Engineering Institute (Nanjing, China; cat. nos. 201304010, 201004010 and 201304010, respectively).

Animals and treatment. A total of 50 Kunming mice (age, 4-6 weeks; weight, 20±2 g), including 25 male and 25 female mice, were supplied by the Experimental Animal Centre of Xinjiang Medical University (Urumqi, China). The present study was approved by the Ethics Committee of the Xinjiang Medical University. Mice were housed in plastic cages at room temperature (22±1˚C) under a 12-h light/dark cycle and provided with rodent chow and water *ad libitum*. A total of 50 mice were randomly divided into five groups (n=10 per group): i) Normal control, orally received saline 0.2 ml/10 g for 14 days, then intraperitoneally injected with 0.4 ml/10 g body weight saline (normal group); ii) doxorubicin + 5-FU toxicity control mice were intraperitoneally injected with doxorubicin (2.5 mg/kg) and 5-FU (10 mg/kg) once in two days (doxorubicin + 5-FU group); and iii-v) three groups of animals were treated with the 2, 4 or 8 g/kg ASMq per day (ASMq.L, ASMq.M and ASMq.H groups, respectively) for 14 days, then intraperitoneally administered doxorubicin (2.5 mg/kg) and 5-FU (10 mg/kg) once in two days (in 0.4 ml/10 g saline). Mice were weighed and sacrificed by cervical dislocation following intraperitoneal injection with 10% chloral hydrate (4 ml/kg; Tianjin Fucheng Chemical Reagents Factory, Tianjin, China). Blood and kidney, spleen, liver and heart tissue samples were collected. Serum was separated for the hematological and biochemical assays. Spleen and body weights were also measured, and the heart, liver, kidney and spleen index was calculated as organ weight divided by body weight. Subsequently, heart, liver, kidney and spleen tissues were fixed in 10% buffered formalin (Sichuan Xilong Chemical Industry Co., Ltd., Chengdu, China) for histopathological analysis. The experiments were performed in accordance with local institutional and governmental regulations on the use of experimental animals.

Biochemical determinations. Serum biochemical markers of hepatic injury ALT, AST and TP were assayed using commercial kits. The activities of AST and ALT are expressed as an international unit (U/l).

Measurement of SOD, MDA and GSH-Px levels in heart homogenate. Heart samples were homogenized in Tris-HCl buffer (5 mM, containing 2 mM EDTA; pH 7.4) resulting in 10% (w/v) liver homogenate. Homogenates were then centrifuged at 191 x g for 10 min at 4˚C and the supernatants were used immediately for the determination of antioxidant status. Activities of SOD and GSH-Px, as well as the level of MDA, as an index of the extent of lipid peroxidation in liver tissue, were determined using commercial kits, according to the manufacturer's instructions. All samples were assayed in triplicate. The content of MDA is expressed in nmol, whereas SOD and GSH-Px activities are expressed as U/mg protein. The protein content of the homogenates was determined using a standard commercial kit (Nanjing Jiancheng Institute of Biological Engineering Institute; cat. no. 20100420).

Histological investigation. After removal, samples of heart and liver tissue were fixed in 10% buffered formalin. Samples were embedded in paraffin and at least four 4-5 μm sections were produced from each heart and liver and stained with hematoxylin and eosin (Beijing SUOLAIBAO Technology, Co., Ltd., Beijing, China). Two changes (2 min xylene treatment each) were performed, and finally tissue sections were mounted with DPX. The slides were observed for histopathological changes and microphotographs were captured using a BX50 microscope system (Olympus Corporation, Tokyo, Japan).

Statistical analysis. All data are presented as the mean ± standard error. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Differences between groups were assessed using Student's t-test and general linear model univariate analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ASMq on heart, liver, kidney and spleen indices. The body weight and heart, liver, kidney and spleen weights were examined on the day of sacrifice, and organ indices were

Table I. Effect of ASMq on body and organ weights of in mice ($\bar{x}$±S).

Group	Left kidney (g)	Heart (g)	Spleen (g)	Liver (g)	Body (g)
Normal	0.218±0.061	0.188±0.037	0.113±0.021	1.718±0.325	34.3±3.17
A + 5-FU	0.107±0.015[a]	0.095±0.019[a]	0.018±0.006[a]	0.673±0.104[a]	17.61±0.85[a]
ASMq.H	0.108±0.013	0.097±0.021	0.026±0.010	0.853±0.109[b]	19.22±1.66[b]
ASMq.M	0.103±0.010	0.095±0.008	0.025±0.007	0.948±0.154[b]	19.15±0.96[b]
ASMq.L	0.098±0.009	0.081±0.014	0.017±0.006	0.673±0.085	18.57±1.17

Values are presented as the mean ± standard error. [a]P<0.05 vs. normal group; [b]P<0.05 vs. A + 5-FU group. ASMq, Abnormal Savda Munziq; A, doxorubicin; 5-FU, 5-fluorouracil.

Table II. Effect of ASMq on A + 5-FU-induced changes in organ/body weight ratios ($\bar{x}$±S).

Group	Kidney/body weight (mg/10 g)	Heart/body weight (mg/10 g)	Spleen/body weight (mg/10 g)	Liver/body weight (mg/10 g)
Normal	63±13	55±7	33±6	497±50
A + 5-FU	61±8	54±10	10±3[a]	383±64[a]
ASMq.H	56±6	50±8	13±5	443±37[b]
ASMq.M	54±3[b]	50±5	13±3[b]	493±62[b]
ASMq.L	53±3[b]	44±8[b]	9±3	363±43

Values are presented as the mean ± standard error. [a]P<0.05 vs. normal group; [b]P<0.05 vs. A + 5-FU group. ASMq, Abnormal Savda Munziq; A, doxorubicin; 5-FU, 5-fluorouracil.

calculated using the ratio of body weight to organ weight (Tables I and II). Doxorubicin + 5-FU treatment significantly reduced mouse body weight compared with the normal group (P<0.05). In addition, treatment with low-, intermediate-, and high-dose ASMq did not significantly increase the heart, liver or kidney weights, as compared with the doxorubicin + 5-FU treatment group (P>0.05). However, there was a significant difference in the spleen and liver indices of the ASMq-treated groups, as compared with the doxorubicin + 5-FU treatment group (P<0.05), thus suggesting that ASMq showed a little therapeutic effects to spleen and liver shrinking during doxorubicin and 5-FU treatment. ASMq can restore the liver and spleen indices proportionately, but it decreases kidney and heart indexes compared with doxorubicin and 5-FU treatment, its reason still unknown (Tables I and II).

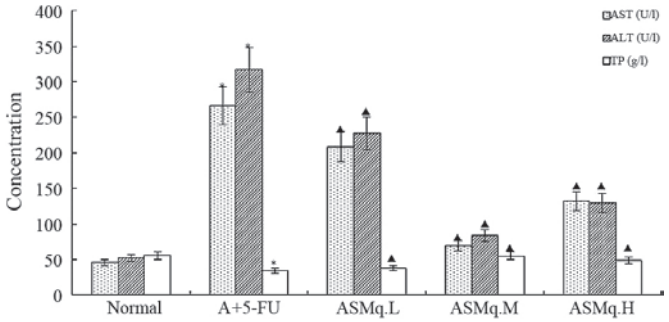

Figure 1. Effect of ASMq on the serum AST, ALT and TP levels in A + 5-FU-treated mice. On day 14, the serum AST, ALT and TP levels were measured using reagent kits. Values are presented as the mean ± standard error. *P<0.05 vs. normal group; ▲P<0.05 vs. A + 5-FU group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TP, total protein; A, doxorubicin; 5-FU, 5-fluorouracil; ASMq, Abnormal Savda Munziq.

ALT, AST and TP concentrations. The concentrations of ALT and AST were significantly increased and the TP level was significantly decreased in the blood of doxorubicin + 5-FU mice compared with normal control mice, indicating the failure of liver function due to doxorubicin + 5-FU-induced hepatotoxicity (Fig. 1) (P<0.05). Treatment with ASMq significantly reduced the levels of ALT and AST (P<0.05). The ASMq.M group was most similar to the normal group. By contrast, a significant increase in TP content (P<0.05) was produced by ASMq treatment as compared to the doxorubicin + 5-FU group (Fig. 1).

Effects of ASMq on heart homogenate SOD activity, GSH-Px and MDA content in doxorubicin + 5-FU mice. As oxidative stress contributes to the development of doxorubicin + 5-FU-induced liver injury, the levels of the antioxidative enzymes SOD and GSH-Px were measured. Levels of SOD and GSH-Px were significantly decreased in the doxorubicin + 5-FU group compared with the normal group (P<0.05) (Fig. 2). Pre-treatment with ASMq significantly increased the SOD and GSH-Px levels as compared with the mice that received doxorubicin + 5-FU treatment. Results showed that the activities of SOD and GSH-Px were significantly increased (P<0.05) by ASMq at the doses of 2, 4 and 8 g/kg (Fig. 2). MDA is an end-product of the breakdown of polyunsaturated fatty acids and related esters, and its formation is an index of lipid peroxidation in numerous organ homogenates (26).

Administration with doxorubicin + 5-FU resulted in a significant increase in MDA concentration when compared with the normal group (P<0.05). However, ASMq significantly reduced MDA in the heart homogenate as compared with doxorubicin + 5-FU-intoxicated group (P<0.05) (Fig. 3).

Histopathological analysis of mouse hearts. As shown in Fig. 4, histological analysis of the heart sections of normal group animals showed normal cells with well-preserved cytoplasm, prominent nucleus and nucleolus. Compared to normal group mice, extensive necrosis and mineralization of cardiomyocytes combined with cardiomyocyte vacuolation and myofibril breakage was observed in the doxorubicin + 5-FU treated group. ASMq.M and ASMq.H mice showed evidence of cardiomyocyte pathological changes, although in the absence of cardiac edema, spotty necrosis, heart vacuolar degeneration and abnormal myocardial interstitial changes (Fig. 4).

Histopathological analysis of mouse livers. Liver histopathology was observed to determine the protective effects of ASMq on doxorubicin + 5-FU mice at the cellular level. Normal mice livers showed limpid central vein and hepatic cells with prominent nuclei and uniform cytoplasm. doxorubicin + 5-FU-treated mice liver sections showed vacuolization of hepatocytes, sinusoidal dilation and congestion, infiltration of cells, loss of cell boundaries and ballooning degeneration, loss of architecture and cell necrosis. The histopathological changes were prominent compared to those of mice in normal group. As demonstrated by the liver histopathological observations, ASMq at all doses showed reduced liver structure damage compared with the doxorubicin + 5-FU group (Fig. 5). Treatment with ASMq reduced the level of hepatic lesions induced by doxorubicin + 5-FU. Photomicrographs indicated reduced damage of liver tissue, on account of the absence of focal or bridging necrosis. Doxorubicin + 5-FU-induced hepatic lesions were most notably reversed by ASMq.M and ASMq.H, which appeared to be comparable to the normal group. These histopathological observations further suggest the hepatoprotective potential of ASMq as an anti-cancer agent *in vitro* (Fig. 5).

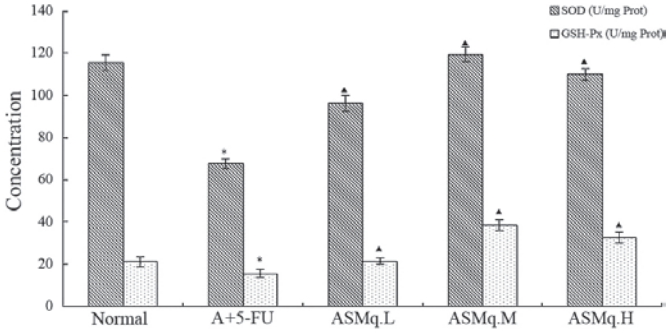

Figure 2. Effect of ASMq on SOD and GSH-Px activity in the heart homogenates of the various groups. Values are presented as the mean ± standard error. *P<0.05 vs. normal group; ▲P<0.05 vs. A + 5-FU group. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; A, doxorubicin; 5-FU, 5-fluorouracil; ASMq, Abnormal Savda Munziq.

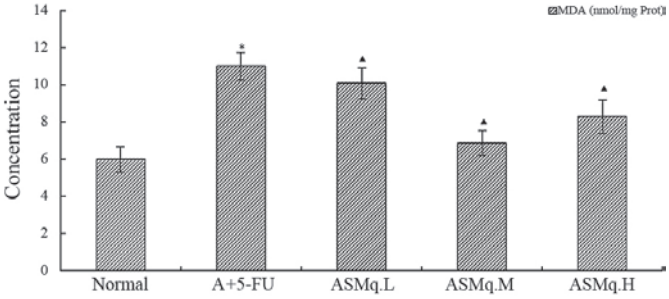

Figure 3. Effect of ASMq on the MDA content in heart homogenate of mice in the various groups. Values are presented as the mean ± standard error. *P<0.05 vs. normal group; ▲P<0.05 vs. A + 5-FU group. MDA, malondialdehyde; A, doxorubicin; 5-FU, 5-fluorouracil; ASMq, Abnormal Savda Munziq.

Discussion

At present, chemotherapy is an crucial modality in the treatment of cancer and in numerous instances may be the single best agent for treatment (27). However, toxic side effects of chemotherapy drugs may be a major limitation to their effective use, in addition to affecting quality of life (11). A key problem associated with cancer chemotherapy are the severe side effects resulting from normal tissue damage, including the heart, liver and spleen (3). Increasing studies have investigated approaches to improve the sensitivity of tumors to chemotherapeutic drugs and to reduce the associated side effects (27). Herbal drugs may offer an alternative to chemotherapeutic compounds, and have been considered to be non-toxic or less toxic, which has led to studies screen herbal drugs for their protective ability of chemotherapy drug toxicity (28,29).

The development of combination chemotherapy produced new evidence of hepatotoxicity, and more instances can be anticipated in the future (30). Combination chemotherapy uses a number of chemotherapeutic agents, each with a different mechanism of action and toxicity profile. Along with the potential for greater tumor kill, however, the possibility for enhanced toxicity occurs (31). Among combination therapies, 5-FU and doxorubicin (sold under the brand name Adriamycin) is the most prevalent combination (32). 5-FU, a pyrimidine analogue, is a cytotoxic agent which is extensively used in different solid tumors such as breast, lung and gastrointestinal tract cancers (33,34). Doxorubicin, an anthracycline glycoside, is commonly used as chemotherapeutic agent in various malignant disorders; however, the possibility of the adverse effect of irreversible cardiomyopathy limits its use (35). The hepatotoxic effects of anticancer drugs may manifest as symptoms other than liver injury, such as necrosis, steatosis, fibrosis, cholestasis and vascular injury (36). 5-FU has been shown to exhibit hepatotoxic effects, such as increased activity of aminotransferases, lactate dehydrogenase and alkaline phosphatase, which indicates hepatic damage (37).

The present results demonstrated that, following administration of doxorubicin, the levels of AST and ALT were elevated and were associated with idiosyncratic drug reactions, including focal infiltration by inflammatory cells and steatosis on liver biopsies. This was considered an idiosyncratic reaction. Chemotherapeutic agents are extensively metabolized in the liver and decrease the antioxidant capacity of the liver, including decreasing glutathione production, which protects

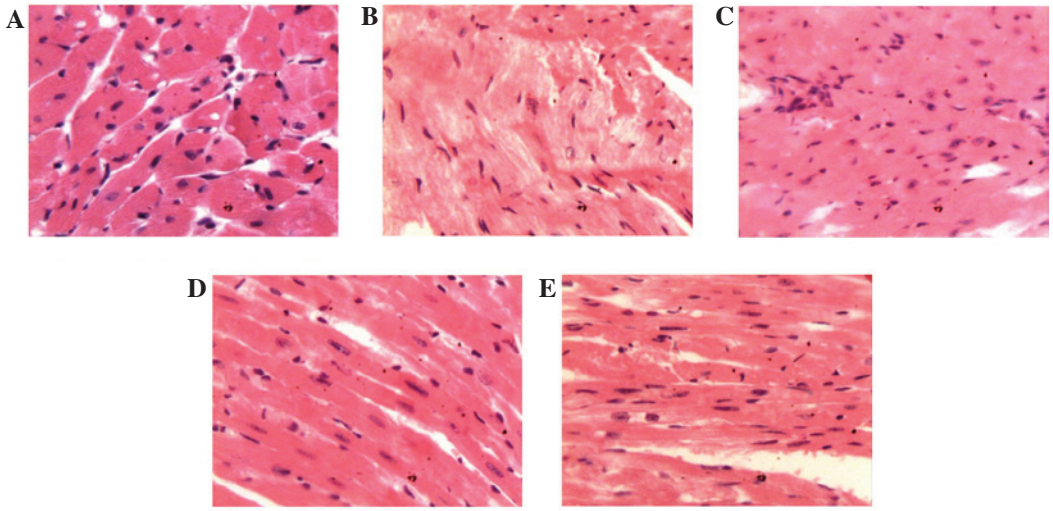

Figure 4. Histopathological features of heart tissues from the various groups (stain, hematoxylin and eosin; magnification, x100). (A) Normal; (B) doxorubicin + 5-FU, (C) ASMq (2 g/kg), (D) ASMq (4 g/kg) and (E) ASMq (8 g/kg) groups. 5-FU, 5-fluorouracil; ASMq, Abnormal Savda Munziq.

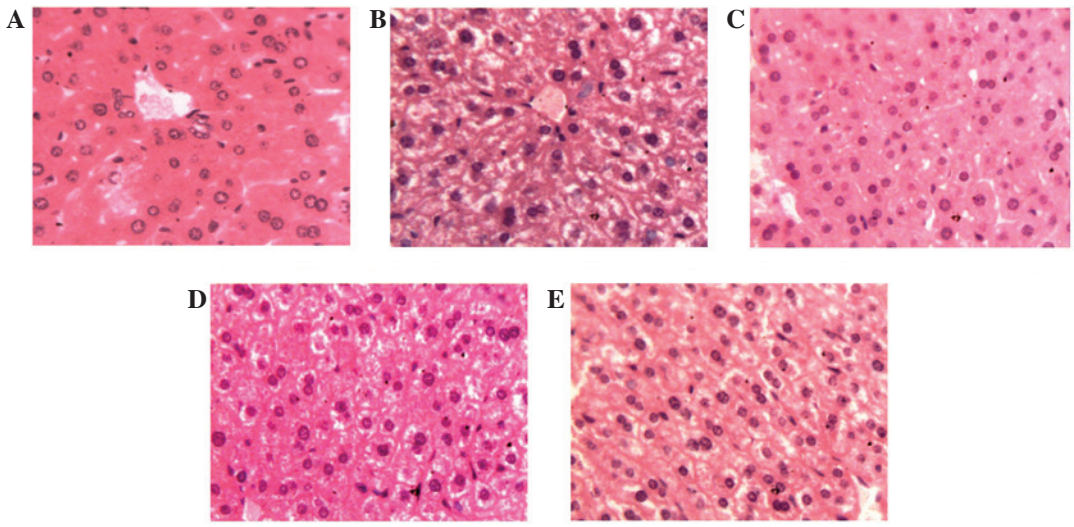

Figure 5. Histopathological features of liver tissues from the various groups (stain, hematoxylin and eosin; magnification, x100). (A) Normal, (B) doxorubicin + 5-FU; (C) ASMq (2 g/kg), (D) ASMq (4 g/kg) and (E) ASMq (8 g/kg) groups. 5-FU, 5-fluorouracil; ASMq, Abnormal Savda Munziq.

against free radical injury (38). Doxorubicin acts via DNA intercalation, alteration of membrane function and free radical formation (39).

In the present study, injection of doxorubicin and 5-FU in mice resulted in decreased liver index and deterioration of hepatic function, as indicated by elevations in ALT and AST and by a significant reduction in total protein. In addition, the examination of liver function correlated with the histopathological changes observed by photomicroscopy. These results are consistent with the previous reports on chemotherapy induced hepatotoxicity (40,41). In the present study it was shown that ASMq protected the liver tissue against damage induced by doxorubicin and 5-FU combination. This protective effect was indicated by a reduction in serum levels of ALT and AST and by a significant elevation in total protein. However, ASMq showed controversial effects to heart and kidney indices, and these require further investigation. The results of the present study indicated that ASMq is able to exert a protective against doxorubicin and 5-FU induced hepatotoxicity. The histological observations supported the results obtained from biochemical analyses.

The clinical application of doxorubicin is complicated by its potential toxicity in heart tissue (15,42,43). The mechanism by which doxorubicin or its metabolites cause chronic cardiomyopathy is not fully understood. Hypotheses regarding the mechanism underlying this cardiac toxicity include perturbation of calcium homeostasis, formation of iron complexes, generation of radical oxygen species, mitochondrial dysfunction and damage to cell membranes (44). The heart is particularly vulnerable to injury from free radicals as it has reduced levels of protective enzymes, such as superoxide dismutase, compared with other tissues (45-47).

The pathophysiology of 5-FU-induced cardiotoxicity is controversial, and conclusions are based on clinical studies and case reports to a greater extent than on experimental evidence (48). Furthermore, 5-FU cardiotoxicity is suspected to be mediated by coronary vasospasm and free radical damage to the myocardium (13,49). Prior studies showed

that the administration of doxorubicin caused an increase in MDA levels, as well as reductions in GSH, SOD and glutathione-S-transferase expression in treated rats compared with a control group. Single injection of doxorubicin increased SOD, MDA, NO and xanthine oxidase and myeloperoxidase expression in kidney tissues in rats at 10 days after administration (50-52). Previous approaches to reduce doxorubicin or 5-FU related toxicities have centred on the use of antioxidants to minimize the generation of reactive oxygen species.

Lipid peroxidation is an crucial mechanism in the pathogenesis of cell damage (53). MDA is the final product of cell membrane lipid peroxidation, and thus its concentration may reflect the intensity of lipid peroxidation (26). SOD and GSH-Px are indicators of resistance to oxidative processes (54). SOD is able to specifically eliminate free oxygen radicals by transforming surplus oxygen free radicals into hydrogen peroxide, which is transformed by catalase and GSH-Px into water, thus reducing free radical-mediated cell damage (54). Furthermore, GSH-Px is an important antioxidase (19-21), and may cause lipid peroxide to change into a alcohol type fatty acid, to complement SOD.

Chemicals such as doxorubicin + 5-FU may induce massive production of free radicals, which accumulate causing cell toxicity and leading to lipid peroxidation of the cell membrane, resulting in cell damage or death (55). The present study found that the administration of doxorubicin and 5-FU was accompanied by signs of oxidative damage, including reduced SOD and GSH-Px activity and increased MDA. These results were consistent with previous studies (56,57), and may be primary to the organ toxicity, or a result of organ toxicity and cell death. ASMq reduced the indicators of oxidative damage, increased SOD and GSH-Px levels to values similar to normal, while partially normalizing the increased levels of MDA. Furthermore, ASMq was found to reverse or oppose the negative effects of doxorubicin + 5-FU on liver function and liver and heart morphology, which was consistent with previous studies (20,21,58-60). In the present study, significant differences were detected in the heart homogenate levels of MDA, GSH-Px and SOD between normal groups and doxorubicin + 5-FU treatment groups. ASMq exhibited a significant effect ($P<0.05$) by increasing levels of SOD, GSH-Px and by reducing MDA levels. However, the histological findings show that of doxorubicin + 5-FU induced a degree of cardiotoxicity. The severity of the histological change was notably reduced in sections from animals treated with ASMq. The most marked effects were observed in the ASMq.M (4 mg/kg) group, with moderate efficacy shown by ASMq.H (8 mg/kg).

Future studies may be warranted involving whole organisms treated with toxic chemotherapy, to assess the capacity of ASMq to reduce the adverse reactions of chemotherapeutics that may be dose- and treatment-limiting. As ASMq also has anti-tumoral effects, it may be useful to monitor the beneficial and toxic effects of this treatment. The presently observed reversal of a number of the signs of toxicity associated with doxorubicin + 5-FU by ASMq are consistent with its known anti-oxidative properties *in vitro*, and warrant further exploratory *in vivo* studies.

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