ANTIOXIDANT APPROACH OF NIGELLA SATIVA, VIT C AND SILYMARIN FOR AMELIORATION OF AZITHROMYCIN INDUCED HEPATOTOXICITY: HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND BIOCHEMICAL STUDY

BY

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ABSTRACT

Background: Azithromycin (AZ) is a broad-spectrum antibiotic however AZ hepatotoxicity limits its therapeutic indications. **The aim of this work** was to clarify the hepatotoxicity of AZ on albino rat and the possible protective effect of antioxidants; Nigella sativa (NG), Vit. C (VC) or Silymarin (SL) against AZ hepatoxicity both histopathologically and biochemically. **Material and methods**: Thirty rats were randomly divided into 5 groups, 6 rats each; Negative control group, AZ group received AZ (200mg/kg/day) orally for 7 days, NG/AZ group received oral NG oil (4ml/kg/day) for 7 days then NG plus AZ for another 7 days, VC/AZ group received oral VC (500 mg/kg/day) for 7 days then VC plus AZ for another 7 days, SL/AZ group received oral SL (200 mg/kg/day) for 7 days then SL plus AZ for another 7 days. The liver specimens of AZ group showed degenerative changes of the hepatic cords. There were dilated central veins with marked portal inflammatory cell infiltration and increase in collagen fibers. Area percentage of Bcl2 immuno-positive cells is significantly decreased while that of Bax is significantly increased. Moreover, AZ group showed significant increase in liver enzymes and oxidative parameters in comparison to control group. Either NG/AZ, VC/AZ or SL/AZ groups showed marked attenuation of liver toxicity both histologically and by normalizing liver enzymes and oxidative parameters. SL/AZ group showed the least hepatic affection. **Conclusion:** This work concludes that administration of either NG, VC or SL before and with AZ attenuates its hepatotoxicity possibly through their liver cytoprotective and antioxidant effects and this was more obvious with SL administration.

Key words: Azithromycin, Oxidative stress, Nigella Sativa, Vit C, Silymarin

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INTRODUCTION

Drug- induced liver disease is considered one of most adverse drug effects accounting for 2% of hospital admissions and 13% of cases of acute liver failure in United States. The manifestations may range from asymptomatic elevation of liver enzymes to fulminant hepatic failure ***(Reuben et al., 2010)***. Their prevention and treatment guidelines still remain inadequate in spite of advancement of pharmacovigilance field. The main cornerstone for pathogenesis of this type of disease is oxidative stress and inflammation ***(Omara et al., 2021)***.

Azithromycin is a semisynthetic macrolide derived from erythromycin, was approved as a potent and generally well-tolerated oral antibiotic. Its popularity rests on its broad spectrum of activity, rapid oral absorption, once-daily administration, tissue penetration, and excellent tolerability ***(Martinez et al., 2015)***.

Recently, Azithromycin usage has risen during the COVID-19 pandemic, according to the ***PTCG (2021)***. Some in vitro investigations have suggested that it has anti-SARS-CoV-2 action. Azithromycin may raise the pH of the Golgi network and recycle endosomes, interfering with SARS-CoV-2 activity and replication within the cell. The medicine may also lower levels of the enzyme furin, which might make it difficult for SARS-CoV-2 to enter cells ***(Touret et al., 2020)***. Furthermore, some individuals with viral respiratory disease may acquire a subsequent bacterial infection or have a bacterial co-infection that azithromycin can cure effectively treat ***(Oliver & Hinks, 2020)***, however, the hepatotoxicity is the most therapeutic limitations of Azithromycin use. Azithromycin has been implicated in variables acute liver failure cases world-wide. The mechanisms for the azithromycin induced hepatotoxicity could be by membrane lipid peroxidation, free radical formation, or

mitochondrial impairment ***(Paulose et al., 2016).***

Hepatic lobule is composed of central vein surrounded by tightly packed cords of hepatocytes in radiating manner separated by thin-walled radiating blood sinusoid. Portal triads are detected at the periphery of the lobules containing branches from hepatic artery, bile duct as well as the portal vein. The microarchitecture of the liver is very similar among mammalian species with the only difference present in the degree of connective tissue development in the portal tracts ***(Kruepunga, 2019)***.

Das (2011) reported that Azithromycin has a long half-life with very high liver concentrations, exceeding the serum levels by 50 folds. This produces destruction of the normal hepatic architecture with inflammatory sequences which could be mild in the form of cholestatic hepatitis or severe iatrogenic hepatitis requiring hepatic transplantation ***(Chandrupatla et al., 2002)***. Furthermore, there were 3 forms of azithromycin-induced liver injury. The first is severe hypersensitivity- related cutaneous reactions with accompanying liver injury, the second is evolution into chronic cholestatic liver injury with vanishing bile duct syndrome, and the last is acute liver failure ***(Melissa et al., 2015)***. Liver injury has been reported within 1–3 weeks after azithromycin initiation and is predominantly hepatocellular in nature ***(Hicks et al., 2013)***. These hepatotoxic effects of azithromycin appeared to be due to the generation of highly reactive free radicals because of oxidative threat caused by the drug which disrupted normal cellular functioning of the liver and kidney. This is marketed by the increased liver enzymes (AST and ALT) and total serum bilirubin concentration which attributed to the liver dysfunction ***(Olayinka & Ore, 2014 and Usadadia et al., 2020)***.

Oxidative stress is a key factor for many inflammatory reactions and has been implicated in aging, immune diseases, neuronal degeneration, and the development and progression of cancer. It has different and not completely understood mechanisms ***(Lenaz, 2012).*** The role of oxidative stress and inflammation is well noted in the pathogenesis of various hepatic diseases with the generation of reactive oxy- gen species (ROS) associated with lipid peroxidation in the liver**.** This led the scientists to continuous evaluation of the hepatoprotective effect of antioxidants in humans and animals ***(Li et al., 2015)***.

Among the antioxidants been evaluated is vitamin C. It potentiates the activities of free radical scavengers thereby preventing microsomal lipid peroxidation and hepatic inflammation ***(Adikwu & Deo, 2013).*** Some scientists had reported the anti-oxidant herbal drugs in the treatment of hepatic disorders. It is considered to be inexpensive and safe ***(Zhang et al., 2013 and Sheetal & Singh, 2008).*** For example**,** Nigella sativa (NS) seeds were documented to have antioxidant, anti-inflammatory, and antihistaminic effects ***(Burits & Bucar, 2000 and Kanter et al., 2006)***. Furthermore, Silymarin has been widely used in the treatment of various liver diseases. Its effect may be associated with the improvement of antioxidant and anti-inflammatory status, as well as the prevention of hepatocyte apoptosis ***(Wang et al., 2018).*** **The aim of this work** was to clarify if Azithromycine has a hepatotoxic effect on adult albino rats and to assess the protective role of antioxidants including Nigella sativa, Vit.C as well as Silymarin against AZ hepatoxicity both histologically and biochemically**.**

MATERIAL AND METHODS

- **Material:**

Drugs and Chemicals:

1. Azithromycin powder (Zithromax for oral suspension) purchased from local pharmacy. It is 600mg powder with 15ml distilled water as a solvent (200mg/5ml). It was manufactured by Hikma Pharma S.A.E, the 6th Of October City, Egypt.
2. Pure Nigella sativa oil (Blackseed oil; pure and natural 100%) was purchased from local pharmacy. It was manufactured by Pharco-Company, Alameria, Alexandria.
3. Silymarin capsules (Legalon 140mg/capsule) were purchased from local pharmacy. It was manufactured by Chemical Industries Development (CID), Giza, Egypt.

4. Vitamin C capsules (500mg/capsule) were purchased from local pharmacy. It was manufactured by SEDICO, 6th OF October City, Egypt.

Animals:

Adult male Wister rats with average weighing 200gm, were used in the present study; they were purchased from the animal house at faculty of medicine, Zagazig University. Rats were housed in plastic cages for at least one week prior to the experiments to be acclimatized to laboratory condition under controlled environmental conditions; constant temperature (25 ± 2° C), humidity (60 ±10 %), and alternating 12 h light-dark cycles. Standard pellet diet and water were allowed ad libitum. Animal use and procedures were in harmony with the National Institutes of Health Strategies for caring and using laboratory animals (NIH Publications No. 8023, revised 1978).

Experimental Design:

Thirty rats were randomly divided into 5 groups, 6 rats each, based on a power analysis (power = 0.8, α = 0.05)

Group I (Negative control group): Consisted of 6 rats did not receive any solvent or drug for 14 days to determine the basic values of tested parameters

GroupII (AZ group): Consisted of 6 rats each of them received AZ at a daily dose of 200mg/kg (about 1ml of the AZ oral suspension) given orally via a nasogastric tube for 7 days. The dose of azithromycin was chosen according to the previous dose–response studies of ***Singh et al. (2016).***

Group III (NG/AZ group): Consisted of 6 rats each rat received Nigella Stiva pure oil in a daily oral dose of (4ml/kg) for 7 days then NG(4 mL/kg) plus AZ (200mg/kg) orally for another 7 days. The dose of nigella stiva oil was chosen according to the previous dose–response studies of ***El-kader (2019).***

Group IV (VC/AZ group): Consisted of 6 rats each rat received vit C (500 mg/kg) dissolved in water for 7 days then Vit C (500 mg/kg) plus AZ (200mg/kg) orally for another 7 days. The doses of vit C was chosen according to the previous dose–response studies of ***Zhong et al (2017).***

Group V (SL/AZ group)**:** Consisted of 6 rats each rat received oral silymarin (200 mg/kg) dissolved in water for 7 days then silymarin (200 mg/kg) plus AZ (200mg/kg) orally for another 7 days. The doses of silymarin were chosen according to the previous dose–response studies of ***Wang et al. (2018).***

At the end of experiment all rats were sacrificed and the left lobes of liver were dissected then divided into parts for histological and immunohistochemical preparation. Also the blood samples were collected through cardiac puncture for analysis of liver enzymes (ALT, and AST) in all groups.

- **Methods:**

Histological examination:

Hepatic tissues in all groups for each rat were collected, washed with saline and the gastric wall samplings were put in 10% buffered formalin on behalf of fixation, handled in machine of tissue processing and paraffin embedded. Designed for histological assessments, slides of the hepatic sections of each group were set at a 5 µm thickness and stained with either hematoxylin and eosin (H&E) or Mallory trichome stain ***(Suvarna et al., 2019).***

Immunohistochemical examination:

Five µm sections of the liver tissue were immunohistochemically stained to estimate immunoexpression of Bcl2 (antiapoptotic protein) and Bax (proapoptotic protein). Concisely, the paraffin-embedded liver sections were dewaxed and rehydrated. Then the sections were incubated with a monoclonal antibody against Bcl2 and Bax (Dako, Carpinteria California, USA); Diaminobenzidine (DAB) was used to demonstrate the immune reaction. The sections were noticed for the brownish coloration of DAB reaction and once visualized it was immediately washed off. Counterstaining with Mayer's hematoxylin was done. Negative controls were prepared by excluding the primary antibodies ***Buchwalow & Böcker (2010).*** The cells were considered positive if showing a brownish coloration of DAB reaction compared to the negative control.

Morphometric study:

Immunohistochemically quantification was conducted by using image analysis software (Image J, 1.46a, NIH, USA). Ten non-overlapping randomly selected fields from each slide were measured at a magnification of 400 for quantitative evaluation of mean area percentage of Bcl2 and Bax immunostaining reaction [calculated as the area of positive immunohistochemical reaction *100/total area].

Laboratory investigations:

Estimation of tissue biochemical parameters:

Measurement of lipid peroxidation

The extent of lipid peroxidation in the liver was quantified using the ***Ohkawa et al. (1979)*** technique. The quantity of malondialdehyde (MDA) was determined using a Schimadzu spectrophotometer and a reaction with thiobarbituric acid at 532 nm (Japan). The values were calculated using the molar extinction co-efficient of chromophore (1.56 × 105 M−1 cm−1) and expressed as percentage of control.

Estimation of reduced glutathione levels:

The amount of reduced glutathione was calculated using Ellman's (1959) technique. 1 ml supernatant was precipitated with 1 ml of 4% sulphosalicylic acid and cold digested for 1 h at 48 °C. After that, the samples were centrifuged at 1200g for 15 minutes at 4 °C. 2.7 ml phosphate buffer (0.1 mmol/l, pH 8) and 0.2 ml 5,5′dithio-bis (2-nitrobenzoic acid) (DTNB) were added to 1 ml of the obtained supernatant. Using a Schimadzu spectrophotometer, the yellow color generated was measured at 412 nm (Japan). Results were calculated using molar extinction co-efficient of the chromophore (1.36 × 104 (mol/l)−1cm−1) and expressed as percentage of control.

Estimation of serum biochemical parameters:

Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), were obtained from SPINREACT Company (UAA Ctra, Santa Coloma, Spain), All other reagents used were of analytical grade from (Sigma-Aldrich, USA).

Biochemical parameters including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated according to manufacturer's instructions of the commercial enzymatic biochemical diagnostic kits; SPINREACT® Rat GPT (ALT)-LQ., SPINREACT®Rat GOT (AST)-LQ. and SPINREACT®Rat (ALP)-LQ.

Protein determination of plasma and all fractions was estimated by the method of ***Lowry et al. (1951)*** using bovine serum albumin as standard.

Statistical analysis:

Quantitative data were assessed with mathematical set SPSS version 22, IBM, Armonk, NY, United States of America. All records were expressed as mean values ± standard deviation (SD). The criterion for statistical significance was significant at the P value < 0.05 for the collected data, highly significant at P value < 0.001. Analysis was done by to one way analysis (ANOVA) to evaluate significant differences between treatment groups, Group differences were calculated by either t-test for immunohistochemical analysis or post hoc analysis using Tukey test for biochemical analysis ***(Dawson and Trapp, 2004).***

RESULTS

Histological results:

H&E Sections: The haematoxylin and eosin-stained sections of livers from the negative control group (Group I) revealed that each hepatic lobule was composed of central vein surrounded by tightly packed cords of hepatocytes in radiating manner separated by thin-walled radiating blood sinusoid. The hepatocytes in each hepatic cord were polygonal in shape with acidophilic cytoplasm and large rounded vesicular nuclei with prominent nucleoli. Binucleated cells were also observed. The portal tracts was detected at the periphery of the lobules containing a portal vein with a thin wall and large lumen and a bile duct with its characteristic single cuboidal cell lining with dark, rounded nuclei **(Fig.1; GI: A, B).** The hepatic lobules of the Group II (AZ group) showed disorganization of the hepatic cords with ill-defined borders of the hepatocytes which showed rarified nuclei, and vacuolated cytoplasm. There were dilated

central veins, blood sinusoids and marked inflammatory cell infiltration around central vein. The portal tracts showed increase in the wall thickness of the portal vein which appeared dilated, congested, and surrounded by inflammatory cell infiltration. Presence of more than one bile duct (bile duct proliferation) is detected **(Fig.1; GII: A, B).** In Group III (NG/AZ group) receiving the nigella sativa oil, the hepatic cords retained their radiating organization however they were separated by wide sinusoids. Many binucleated cells were observed. The central vein was thin walled but remained dilated. No congestion or inflammatory cell infiltration appeared around the central vein. In their portal tracts, the portal vein appeared less dilated and congested with minimal inflammatory cell infiltration around it **(Fig.1; GIII: A, B).** In Group VI (VC/AZ group), the hepatic cords appeared normal and radiating around slightly dilated and congested central vein. However, some hepatic lobules showed intracellular vacuolations. The hepatic sinusoids between the hepatic cords appeared normal. The portal triad appeared normal with minimal inflammatory cell infiltration beside the portal vein **(Fig.1; GIV: A, B).** In Group V (SL/AZ group), the central vein appeared slightly dilated but not congested and surrounded by normal hepatic cords containing numerous binucleated cells appeared. The portal triad including the portal vein and bile duct appeared normal with no inflammatory cell infiltration **(Fig.1; GV: A, B).** This cytoprotective effect was more among the V group administrated silymarin compared to the cytoprotective effect of nigella stiva and vit C in group III and IV respectively.

Mallory trichome sections:

The Mallory trichrome stained sections of livers from the negative control group (Group I) revealed few collagen fibers around the central veins and in the portal tracts **(Fig.2; GI: A, B).** The hepatic lobules of the group II (AZ group) showed thick collagen fibers deposition around the central veins and very thick corrugated collagen bundles in the portal tracts were seen surrounding the portal vein, hepatic artery and bile duct **(Fig.2; GII: A, B).** Group III (NG/AZ group), the central veins within the hepatic lobules appeared normal with scanty fine collagen fibers around them. However, the portal tract showed thick collagen fibers deposition around the portal vein (Fig.2; GIII: A&B). In Group IV (VC/AZ group), some central veins appeared normal with few collagen fibers around them, and others appeared dilated with thick collagen fibers deposition around them. The portal tracts showed moderate collagen fibers around the portal vein and bile duct (Fig.2; GIV: A&B). Meanwhile, the central veins within the hepatic lobules in Group V (SL/AZ group) showed few collagen fibers around dilated central veins and scanty collagen fibers in the portal tracts surrounding the portal veins **(Fig.2; GV: A, B).** Statistical analysis of morphometric results of the periportal fibrosis showed highly significant ($P<0.001$) increase in the area percentage of Mallory's Trichrome positive staining in portal region of group II as compared with control group. While, the hepatic lobules in groups III, IV &V showed highly significant ($P<0.001$) decrease of area percentage of Mallory's Trichrome positive staining in the portal region as compared with group II. On the other hand, groups III, IV &V showed a non-significant ($P>0.05$) difference in Mallory's Trichrome positive staining in the portal region as compared to the control group **(Table 1).**

Immunohistochemical results:

Bcl2 immunohistochemical staining

Immunostained sections from negative control group (Group I) revealed moderate immunoreactivity of Bcl2 in the cytoplasm of the hepatic cells **(Fig.3; GI: A).** Bcl2 immunoreactivity in hepatic lobules of group II appeared very weak when compared with control rats **(Fig.3; GII: A).** Stronger immunostaining reaction for Bcl2 was detected in the group III, IV and V (**Fig.3; GIII: A, GIV: A, GV: A).** Statistical analysis of morphometric results showed highly significant ($P<0.001$) decrease in the area percentage of Bcl2 positive immunoreaction in hepatic lobules of group II compared with that of control group. Meanwhile, rats in groups III, IV and V demonstrated highly significant ($P<0.001$) increase in the both parameters as compared with rats in group II. On the other hand, groups III, IV and V showed a non-

significant (P>0.05) difference in immunoreactivity as compared to the control group **(Table 2).**

Bax immunohistochemical staining

Immunostained sections from the control groups (Group I) revealed faint immunoreaction for Bax in the cytoplasm of hepatic cells **(Fig.3; GI: B).** Meanwhile, Group II revealed strong positive reaction in most of the hepatic cells **(Fig.3; GII: B).** On the other hand, sections from groups III, IV &V showed very weak reaction for Bax in the cytoplasm of hepatic cells **(Fig.3; GIII: B, GIV: B, GV: B).**

Statistical analysis of morphometric results showed highly significant (P<0.001) increase in the area percentage of Bax positive immunoreaction in hepatic lobules of group II as compared with control group. While, the hepatic lobules in groups III, IV and V showed highly significant (P<0.001) decrease of area percentage of Bax positive immunoreaction as compared with group II. On the other hand, groups III, IV and V showed a non-significant (P>0.05) difference in immunoreactivity as compared to the control group **(Table 3).**

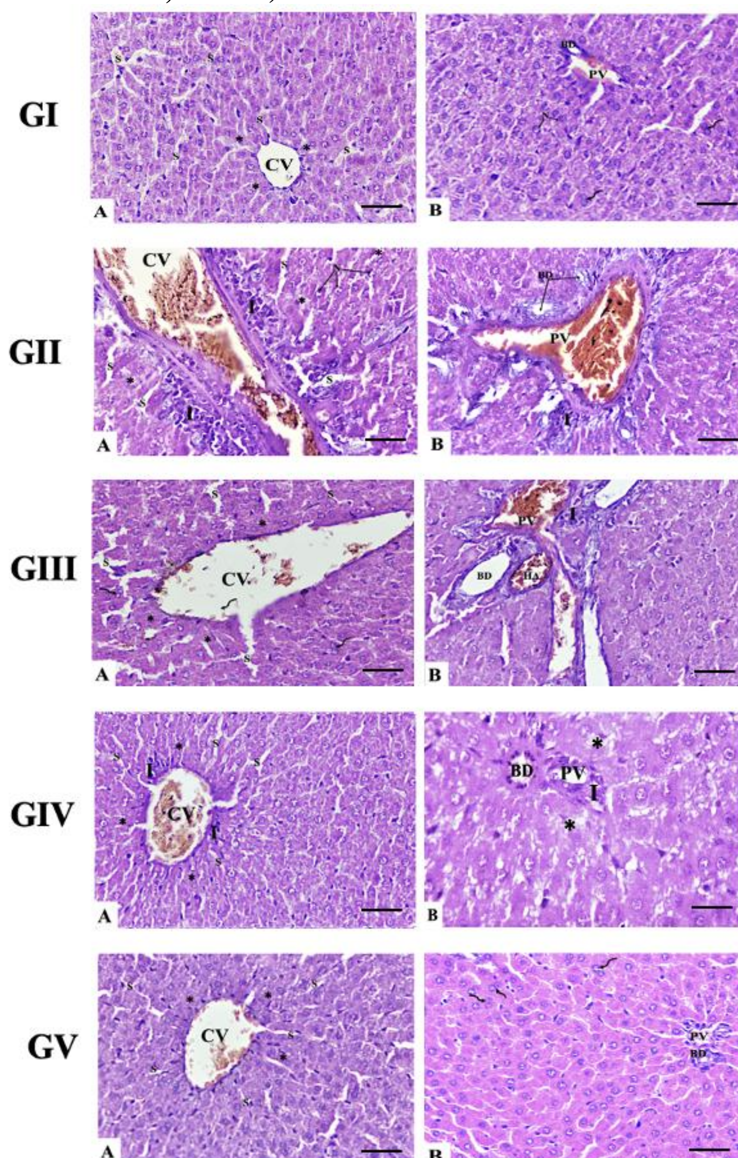

Fig.1: *Photomicrographs of sections of hepatic lobules of albino rats in different groups showing that in the negative control group (GI) the hepatic lobule is composed of central vein (CV) surrounded by tightly packed cords of hepatocytes (*) in radiating manner separated by thin-walled radiating blood sinusoid (S). Polygonal hepatocytes with acidophilic cytoplasm and large rounded vesicular nuclei with prominent nucleoli (N). Binucleated cells were also observed (curved arrows). The portal tracts is detected at the periphery of the lobules containing a portal vein (PV) with a thin wall and large lumen and a bile duct (BD) with its characteristic single cuboidal cell lining with dark, rounded nuclei (GI: A&B). The hepatic lobules of the AZ group (GII) showed disorganization of the hepatic cords with ill-defined borders of the hepatocytes which showed rarified nuclei (N), and vacuolated cytoplasm (*). Dilated central veins (CV), blood sinusoids (S) and marked inflammatory cell infiltration (I) around central vein. Increase in the wall thickness of the portal vein (PV) which appears dilated, congested, and surrounded by inflammatory cell infiltration (I). Presence of more than one bile duct (BD) is detected (GII: A&B). In Group III (GIII), the hepatic cords (*) retain their radiating organization however they are separated by wide sinusoids (S). Many binucleated cells are observed (curved arrows). The central vein (CV) is thin walled but remained dilated. In their portal tracts, the portal vein (PV) appears less dilated and congested with minimal inflammatory cell infiltration (I) around it (GIII: A&B). In Group VI (GIV), the hepatic cords appeared normal and radiating around slightly dilated and congested central vein (CV). However, some hepatic lobules showed intracellular vacuolations (*). The hepatic sinusoids (S) between the hepatic cords appeared normal. The portal triad appears normal with minimal inflammatory cell infiltration (I) beside the portal vein (PV) (GIV: A&B). In Group V (GV), the central vein (CV) appears slightly dilated but not congested and surrounded by normal hepatic cords (*) containing numerous binucleated cells (curved arrows). The portal triad including the portal vein (PV) and bile duct (BD) appeared normal (GV: A&B)* ***(Hx.& E. A, B X400)***

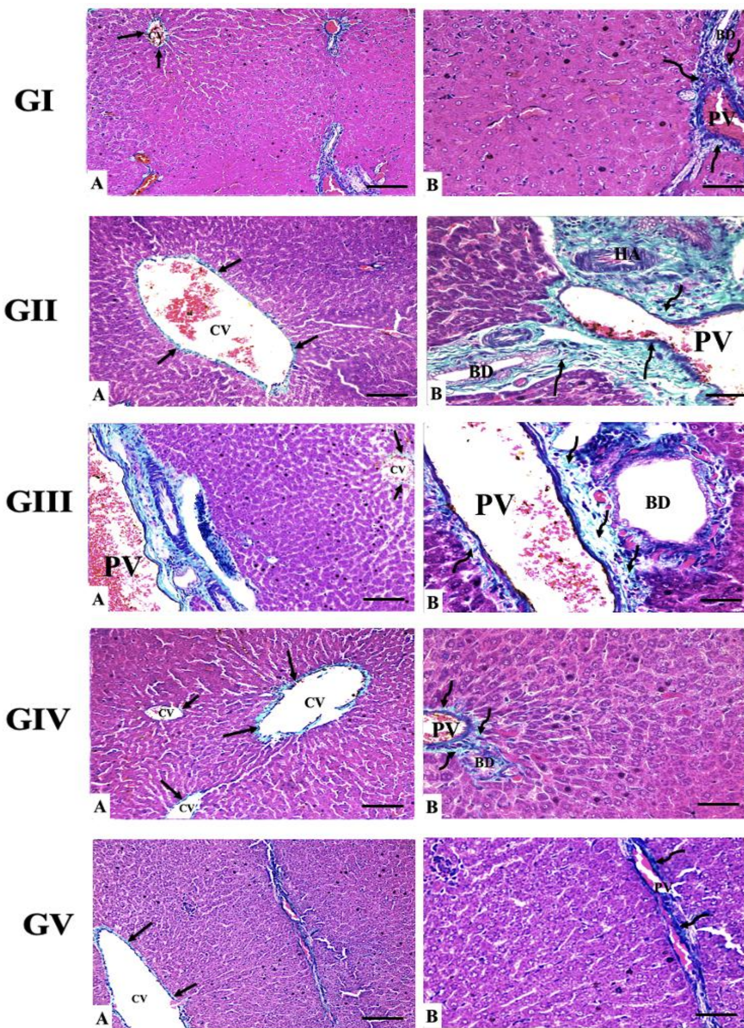

Fig.2: *Photomicrographs of sections of hepatic lobules of albino rats in different groups showing few collagen fibers (arrows) around the central veins (CV) and the portal veins (PV) of the group I (GI: A&B). The hepatic lobules of the group II (GII) show thick collagen fibers deposition around the central veins (CV) and very thick corrugated collagen bundles in the portal tracts surrounding the portal vein (PV), hepatic artery (HA) and bile duct (BD) (GII: A&B). In Group III (GIII), the central veins (CV) within the hepatic lobules appear normal with scanty fine collagen fibers (arrows) around them. However, the portal tract showed thick collagen fibers deposition (arrows) around the portal vein (GIII: A&B). In Group IV (VC/AZ group), some central veins (CV) appeared normal with few collagen fibers around them, and others appeared dilated with thick collagen fibers deposition (arrows) around them. The portal tracts showed moderate collagen fibers around the portal vein (PV) and bile duct (BD) (GIV: A&B). Meanwhile, the central veins within the hepatic lobules in Group V (SL/AZ group) showed few collagen fibers (arrows) around dilated central veins (CV) and scanty collagen fibers in the portal tracts surrounding the portal veins (PV) (GV: A &B)* ***(Mallory Trichrome A X 200 & BX400)***

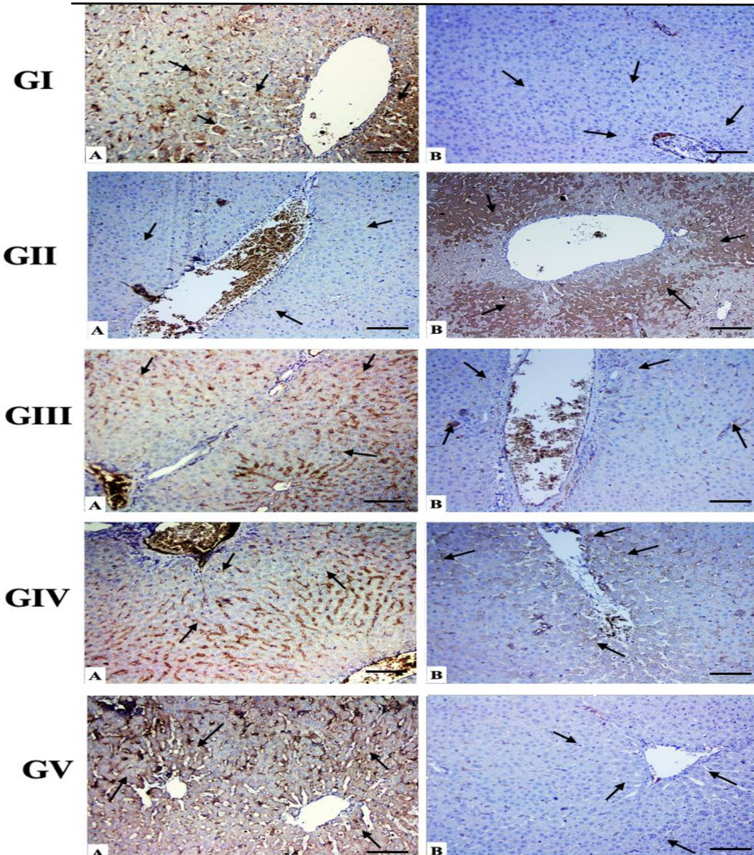

Fig.3: *Photomicrographs of sections of hepatic lobules of albino rats in different groups stained with either Bcl2 (A) or Bax (B) immunostaining showing moderate immunoreactivity of Bcl2 (arrows) in the cytoplasm of the hepatic cells in group I, very weak Bcl2 immunoreactivity in hepatic lobules of group II and Stronger immunostaining reaction for Bcl2 was detected in the group III, IV & V. Bax Immunostained sections from group I reveals faint immunoreactivity (arrows) for Bax in the cytoplasm of hepatic cells. Meanwhile, group II revealed strong positive reaction in most of the hepatic cells. On the other hand, sections from groups III, IV &V showed very weak reaction for Bax in the cytoplasm of hepatic cells* ***(Bcl2 AX200 & Bax BX200).***

Table (1): Mallory's trichrome positive staining percentage area expression and their statistical comparison in different groups. SD=standard deviation, P-value > 0.05 means no significant difference and P-value ≤ 0.001means highly significant difference (**)

Groups (n=6)	Mallory's Trichrome percentage area		ANOVA	
	Range	Mean ± SD	F	P-value
Negative control (Distilled water for 14 days)	1.872 - 4.534	3.185 ± 0.783	2554.49	< 0.001**
AZ (AZ for 7 days)	42.366 - 46.794	44.537 ± 1.901		
NG/AZ (NG for 7 days then NG+AZ for another 7 days)	2.878 – 5.532	3.667 ± 0.776		
VC/AZ (VC for 7 days then VC+AZ for another 7 days)	1.424 – 3.782	2.621 ± 0.809		
SL/AZ (SL for 7 days then SL+AZ for another 7 days)	1.197- 4.761	3.233 ± 1.102		

t-Test

Negative control & AZ	Negative control & NG/AZ	Negative control & VC/AZ	Negative control & SL/AZ	AZ & NG/AZ	AZ & VC/AZ	AZ & SL/AZ
<0.001 **	0.184	0.130	0.912	<0.001 **	<0.001 **	<0.001 **

Table (2): Bcl2 immunostaining percentage area expression and their statistical comparison in different groups. SD=standard deviation, P-value > 0.05 means no significant difference and P-value ≤ 0.001means highly significant difference (**)

Groups (n=6)	Bcl2 percentage area		ANOVA	
	Range	Mean ± SD	F	P-value
Negative control (Distilled water for 14 days)	21.756 - 26.391	24.305 ± 1.796	128.887	< 0.001**
AZ (AZ for 7 days)	2.231 - 5.211	3.329 ± 0.909		
NG/AZ (NG for 7 days then NG+AZ for another 7 days)	17.902 - 26.581	22.404 ± 3.596		
VC/AZ (VC for 7 days then VC+AZ for another 7 days)	19.234 - 25.931	22.8 ± 2.553		
SL/AZ (SL for 7 days then SL+AZ for another 7 days)	19.221- 26.198	23.194 ± 2.694		

t-Test

Negative control & AZ	Negative control & NG/AZ	Negative control & VC/AZ	Negative control & SL/AZ	AZ & NG/AZ	AZ & VC/AZ	AZ & SL/AZ
<0.001 **	0.152	0.145	0.292	<0.001 **	<0.001 **	<0.001 **

Table (3): Bax immunostaining percentage area expression and their statistical comparison in different groups. SD=standard deviation, P-value > 0.05 means no significant difference and P-value ≤ 0.001means highly significant difference (**)

Groups (n=6)	Bax percentage area		ANOVA	
	Range	Mean ± SD	F	P-value
Negative control (Distilled water for 14 days)	0.492 - 3.391	1.699 ± 0.968	524.78	< 0.001**
AZ (AZ for 7 days)	15.581 - 18.909	17.354 ± 1.154		
NG/AZ (NG for 7 days then NG+AZ for another 7 days)	0.909 - 3.778	2.224 ± 1.081		
VC/AZ (VC for 7 days then VC+AZ for another 7 days)	0.945 - 3.427	2.287 ± 0.935		
SL/AZ (SL for 7 days then SL+AZ for another 7 days)	0.505 – 2.221	1.156 ± 0.541		

t-Test

Negative control & AZ	Negative control & NG/AZ	Negative control & VC/AZ	Negative control & SL/AZ	AZ & NG/AZ	AZ & VC/AZ	AZ & SL/AZ
<0.001 **	0.268	0.184	0.138	<0.001 **	<0.001 **	<0.001 **

Laboratory results:

Oral treatment with AZ in group II showed significant increase oxidative damage indicating increase of lipidperoxidation in the liver of the rat as compared to control group. In group III, IV or V, oral administration of either NG or VC or SL significantly ($p<0.05$) attenuated the increased level of the lipid peroxidation in comparison to AZ treated rats. However, there was still significant difference ($p<0.05$) between control group and group III, IV or V suggesting partial hepatoprotective potential regarding level of lipid peroxidation **(Fig. 4).**

In group II (AZ group), administration of AZ significantly reduced GSH in the liver as compared with the control group. In group III, IV or V, oral administration of either NG or VC or SL significantly ($p<0.05$) restored reduced GSH as compared with AZ group. However, there was still significant difference ($p<0.05$) between control group and group III, IV or V suggesting partial hepatoprotective potential regarding level of lipid peroxidation **(Fig. 5).**

Oral administration of AZ in group II, significantly ($p<0.05$) increased the levels of liver enzymes (ALT, AST and ALP) when compared to the control group, however, either oral administration of NG, VC or SL in groups III, IV or V significantly ($p<0.05$) attenuated the liver enzymes (ALT, AST and ALP) in comparison to AZ group, suggesting hepatoprotective potential. There was no significant difference ($p> 0.05$) between control group and group III, IV or V regarding ALT, AST, and ALP levels **(Table 4).**

Oral administration of AZ in group I significantly ($p<0.05$) decreased protein and Albumin as compared to control group. However, either oral administration of NG or VC or SL in group III, IV or V, significantly ($p<0.05$) restored protein and albumin in comparison to AZ group, suggesting hepatoprotective potential. There was no significant difference ($p>0.05$) between control group and group III, IV or V regarding protein and albumin levels **(Table 5).**

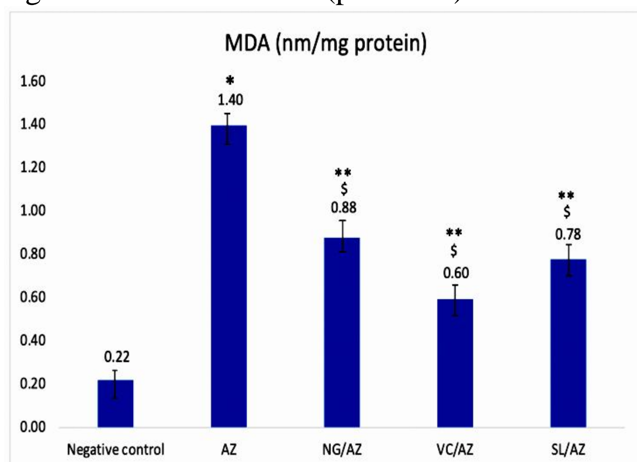

Fig.4: *Statistical comparison between tested groups regarding (MDA) by one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test ANOVA: analysis of variance. Data are expressed as mean ± SEM, n = 6. SEM: standard error of the mean; (*): P-value < 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test. (**): P-value < 0.05, significantly different from AZ group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test ($): P-value < 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.*

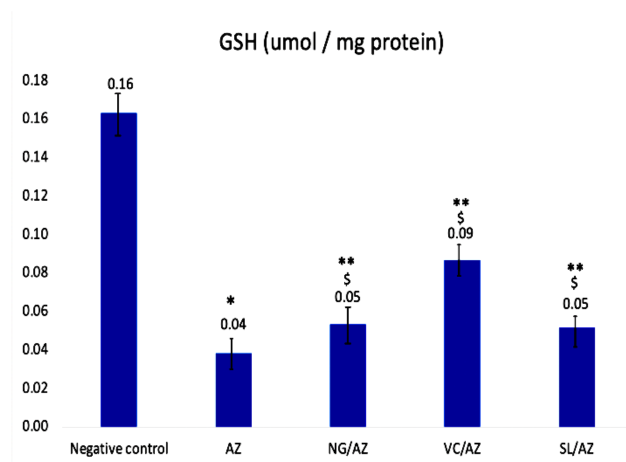

Fig.5: *Statistical comparison between tested groups regarding (GSH) by one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test ANOVA: analysis of variance. Data are expressed as mean ± SEM, n = 6, SEM: standard error of the mean; (*): P-value < 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test. (**): P-value < 0.05, significantly different from AZ group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test ($): P-value < 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.*

Table (4): Statistical comparison between tested groups regarding (liver enzymes) by one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.
ANOVA: analysis of variance. Data are expressed as mean ± SEM, n = 6.

Groups (n=6)	ALT (iu/L)	AST (iu/L)	ALP (iu/L)
Negative control (Distilled water for 14 days)	29.00±1.3	32.17±1.2	142.17±4.9
AZ (AZ for 7 days)	99.17±3.9*	136.17±4.7*	395.83±13.7*
NG/AZ (NG for 7 days then NG+AZ for another 7 days)	45.67±1.5**$	71.50±5.4**$	136.83±7.4**$
VC/AZ (VC for 7 days then VC+AZ for another 7 days)	69.83±1.5**$	96.67±6.2**$	148.17±9**$
SL/AZ (SL for 7 days then SL+AZ for another 7 days)	59.83±2.7**$	104.50±4**$	148.67±4.9**$

SEM: Standard error of the mean;
(*): P-value ≤ 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.
(**): P-value ≤ 0.001, highly significant difference from AZ group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.
($): P-value ≥ 0.05, not significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.

Table (5): Statistical comparison between tested groups regarding (total proteins, albumin) by one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test
ANOVA: analysis of variance. Data are expressed as mean ± SEM, n = 6.

Groups (n=6)	PROTEIN (gm/dl)	ALBUMIN (gm/dl)
Negative control (Distilled water for 14 days)	7.38±0.17	4.47±.08
AZ (AZ for 7 days)	4.00±0.13*	2.58± 0.11*
NG/AZ (NG for 7 days then NG+AZ for another 7 days)	7.43±0.25**$	4.55±0.1**$
VC/AZ (VC for 7 days then VC+AZ for another 7 days)	7.27±0.14**$	4.37±0.19**$
SL/AZ (SL for 7 days then SL+AZ for another 7 days)	7.62±0.21**$	4.40±0.14**$

SEM: standard error of the mean;
(*): P-value < 0.05, significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.
(**): P-value < 0.05, significantly different from AZ group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test
($): P-value ≥ 0.05, not significantly different from control group using one-way ANOVA followed by Tukey -Kramer multiple comparisons post hoc test.

DISCUSSION

Azithromycin is frequently prescribed for the treatment of many infectious conditions. It's characterized by being lipophilic and so easily distributed in body fluids and tissues exerting its antibacterial action. However, many studies suggest its cytotoxicity on the heart, liver and kidney *(**Alti et al., 2015 and Usadadia et al., 2020**)*. The aim of this work was to evaluate if Azithromycin has a hepatotoxic effect on adult albino rats and to assess the protective role of antioxidants including Vit. C, Nigella sativa as well as Silymarin against AZ hepatoxicity both histologically and biochemically.

In this study, administration of Azithromycin showed disorganization of the hepatic cords with ill-defined borders of the hepatocytes which showed rarified nuclei, and vacuolated cytoplasm. There were dilated central veins, blood sinusoids and marked inflammatory cell infiltration around central veins. The portal tracts showed increase in the wall thickness of the portal vein which appeared dilated, congested, and surrounded

by inflammatory cell infiltration. These results are in agreement with ***Abo-Samak et al. (2000)*** whose results showed degenerative changes of liver, cellular infiltrations and cytoplasmic vaculation, with variable degrees and extents. Furthermore, ***Usadadia et al (2020)*** proved by histopathological examination of the liver that treatment with azithromycin caused severe sinusoidal hemorrhages and congestion, disruption of hepatic cords, inflammatory cells infiltration, vacuolar degeneration, and fatty changes

The liver is a vital organ in the body, having a high rate of metabolism. It is very sensitive to oxidative stress and free radical damage. So, oxidative stress is one of the primary causes of liver injury that depletes the antioxidant enzymes sources and decreases the ability of cells in functioning against injury ***(Muriel, 2009)***. The hepatotoxic effects of azithromycin appeared due to the generation of highly reactive free radicals because of oxidative threat caused by the drug which disrupted normal cellular functioning of the liver and kidney ***(Olayinka and Ore, 2014)***.

Aadministration of either Nigella Sativa, Vit C or Silymarin before the Azithromycin provides a cytoprotective effect to the hepatic lobules. This is proved in this work by histological examination of the hepatic tissues in group III, IV & V which showed that the hepatic cords retained their radiating organization around slightly dilated and congested central vein. Many binucleated cells were observed. No or minimal inflammatory cell infiltration appeared around the central vein or in the portal tracts. the portal vein appeared less dilated and congested with minimal inflammatory cell infiltration around it. This cytoprotective effect was more among the V group administrated silymarin compared to the cytoprotective effect of Nigella Stiva and vit C in group III and IV respectively. These results are explained by ***Mollazadeh & Hosseinzadeh (2014), Adikwu & Deo (2013) and Wang et al. (2018)*** who assessed the hepatoprotective effects of Nigella stiva, Vit C and Silymarin respectively and found it to be due to the antioxidant properties of these substances preventing the production of free radicals, scavenging free radicals or enhancement in hepatocytes antioxidant defense.

In this study, liver specimens from different groups were stained with Mallory trichrome stain which colour the collagen fibers. The hepatic lobules of the group II (AZ group) showed thick collagen fibers deposition around the central veins and very thick corrugated collagen bundles in the portal tracts. Meanwhile, the central veins within the hepatic lobules in Group III, IV & V showed few collagen fibers around dilated central veins and scanty collagen fibers in the portal tracts surrounding the portal veins. The amount of collagen fibers was estimated based on the measuring the percentage area of Mallory trichrome stained cells. Statistical analysis of morphometric results of the periportal fibrosis showed highly significant increase in the area percentage of Mallory’s Trichrome positive staining in portal region of group II as compared with control group. While, the hepatic lobules in groups III, IV &V showed highly significant decrease of area percentage of Mallory’s Trichrome positive staining in the portal region as compared with group II. These results were explained by ***Lo (2017)*** who reported that during chronic hepatic injury, stellate hepatocytes are activated and transformed into a myofibroblast-like phenotype. The main causes of stimulating these cells include tissue inflammation, cytokine production from injured parenchymal cells, and technically, thick collagen fibers have been targeted as the main source of fibrosis.

The immunohistochemical results of this work measure the area percentage of the Bcl2 and Bax immunopositively staining cells in all groups and showed highly significant decrease in the area percentage of Bcl2 positive immunoreaction and highly significant increase in the area percentage of Bax positive immunoreaction in hepatic lobules of group II compared with that of control group. Meanwhile, rats in groups III, IV &V demonstrated highly significant increase in the area percentage of Bcl2 positive immunoreaction and highly

significant decrease in the area percentage of Bax positive immunoreaction as compared with rats in group II. ***Hikita & Takehara (2017)*** explained that hepatocyte apoptosis is regulated by a fine balance of between anti-apoptotic Bcl-2 family proteins and BH3-only proteins. A lack of Bcl-2 family proteins alters this balance which induces Bak/Bax activation resulting in mitochondrial outer membrane permeability (MOMP). Once MOMP occurs, apoptosis is initiated by the activation of downstream caspases.

Hepatotoxic drugs have been shown to cause damage to the liver cell membranes. This either increases the permeability of cell membrane or the cell wall ruptures which makes marker enzymes like AST, ALT and ALP leak into serum and show increased activities ***(Kumar et al., 2004)***. In the present study, AZ-induced liver damage was confirmed by elevated levels of ALT, AST, and ALP in serum of rats which attributed to the liver dysfunction. These results are in consistence with previous reports for drug induced liver toxicity (***Chandrupatla et al., 2002; Baciewicz et al., 2005; Das, 2011; Olayinka and Ore, 2014 and Martinez et al., 2015***). However, ***Paulose et al., (2016)*** reported liver ALT values were significantly decreased in azithromycin treated group, indicating the hepatotoxic potential of the antibiotic at 30mg/kg body weight because once the cell death happened, the source of enzyme declined.

In current work a significant decrease in plasma level of total protein and albumin in AZ treated rats was observed. This may be as a result of releasing total protein and albumin from the cytoplasm into the blood quickly after cellular destruction and a reduction in forming hepatic protein ***(Salem et al., 2015)***.

A significant reduction in the activities of GSH in the liver of azithromycin treated rats was observed in this work. This may be due to the damaging effects of free radicals possibly generated by the action of the drug. The level of reduced GSH is a measure of non-enzymic antioxidant and cellular redox status of cells in higher animals. Azithromycin may alter the expression and activities of antioxidant enzymes as a result of toxic metabolites generated during their biotransformation and the hepatotoxic effects of azithromycin appear to progress through generation of free radicals and ROS ***(Olayinka & Ore, 2014 and Atli et al., 2015)***.

A significant increase in the level of lipid peroxidation was observed in rats treated with azithromycin that may be related to the reduction in the enzymic and non enzymic antioxidant systems, leading to accelerated oxidation of lipids. The thiobarbituric acid assay is a satisfactory means of assessing lipid peroxidation through assay of MDA in biological samples. The elevated level of (MDA) in the liver is a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation systems ***(Berger and Chioléro, 2007)***. The accumulation of lipid peroxidation products observed in this study agrees with previous study on certain macrolide antibiotics ***(Salvemini, and Cuzzocrea, 2002)***.

In current work, there was a decrease in the liver marker enzymes such as AST, ALT& ALP as well as an increase in albumin and total protein reversing them toward the normal values in nigella sativa and azithromycin–treated rats compared to Azithromycin-treated rats. Moreover, there was an increase in GSH and decrease in lipid peroxidation levels in NG/AZ group compared to AZ group. These results agreed with that of ***Krishnan and Muthukrishnan, (2012)*** who reported that AST, ALT and ALP elevated enzymatic levels were significantly returned toward normal levels by the 10 % aqueous extract of N. sativa. This protective effect of NG against AZ-induced liver damage was explained by ***Adam et al. (2016)*** to be due to suppression of an increase in intra-cellular ROS which cause pro-oxidative state in the cellular environment and results in oxidative damage by either apoptosis or necrosis and can induce mitogen activated proteinkinase activation.

Burits, M. and Bucar (2000) also assessed the antioxidant properties of *N. Sativa* and found that it has a potent O^2 scavenger activity through inhibition of iron-dependent lipid peroxidation. With this characteristic, N. Sativa can decrease

oxidative stress and increase antioxidant defense in the body. Decrease in malondialdehyde and other biomarkers of oxidative stress in parallel with increase in total thiol content and glutathione level are the results of Nigella Sativa treatment. ***Mollazadeh & Hosseinzadeh (2014)*** added that the content of glutathione in the liver is known to have key functions in cellular protective mechanisms. Depletion in total thiol content caused by oxidative stress can result in protein inactivation, protein oxidation, lipid peroxidation, perturbation in calcium homeostasis and resultful loss of cell viability.

Vitamin C is hydrophilic and is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxidative damage. It is also reported to be an excellent source of electrons and, therefore, can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity ***(Krishnamoorthy & Sangeetha, 2008 and El-Shitany & El-Desoky, 2016)***. The results of present study agree with earlier report which suggests that Vit. C may play a role in the prevention of hepatic cellular injury by its antioxidant capacity ***(Ganesh et al., 2012)***.

In current work there was a decrease in the liver marker enzymes such as AST, ALT, ALP and increase in albumin and total protein revesing them toward the normal values in Vit C and azithromycin–treated rats as compared to Azithromycin-treated rats. Moreover, there was an increase in GSH and decrease in lipidperoxidation levels in VC/AZ group compared to AZ group. This may be due to the antioxidant effect of Vit. C, which is reported to protect the liver from damage. Vit. C may protect lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals, thus may prevent certain types of hepatic cellular damage ***(Uboh et al., 2012)***.

Silymarin has hepatoprotective properties and is used in treatment of various liver diseases ***(Elmowafy et al., 2013)***. Various studies indicate that silymarin exhibits strong antioxidant activity and shows protective effects against hepatic toxicity induced by a wide variety of agents by inhibiting lipid peroxidation ***(Simeonova et al., 2013 and Freitag et al., 2015)***. Higher total phenolic content of silymarin has been known to contribute to the antioxidant activity of extracts while antioxidant activity has also been linked to the hepatoprotective effect of some extracts ***(Yuan et al., 2014 and Gu et al., 2014)***. These findings corroborate with the results of this study on the ability of silymarin to exert a hepatoprotective activity and this was shown in the decrease in the liver marker enzymes such as AST, ALT, ALP and increase in albumin and total protein reversing them toward the normal values in silymarin and azithromycin–treated rats compared to Azithromycin-treated rats. Furthermore, the increase in GSH and decrease in lipidperoxidation levels in silymarin and azithromycin –treated rats compared to Azithromycin-treated rats reveals the better recovery of hepatic cells from the oxidative stress induced by azithromycin toxicity.

CONCLUSION

This work concludes that administration of either Nigella Sativa, Vit C or Silymarin before and with AZ reduces its hepatotoxic effect both histologically and biochemically and this protective effect was more obvious with administration of silymarin more than Nigella Sativa or Vit C.

RECOMMRNDATIONS

The authors recommend that if it's indicated to give Azithromycin drug for any reason, it's better to give concomitant Nigella Sativa, Vit C or Silymarin to protect the liver and reduce its hepatotoxic effect. Further studies should be conducted on the human to confirm the efficacy of antioxidants; Nigella Sativa, Vitamin C or Silymarin as curative and protective agents against Azithromycin induced hepatotoxicity.

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نهج مضادات الأكسدة في حبة البركة، فيتامين ج وسيليمارين لتحسين التسمم الكبدي الذي يسببه أزيثروميسين: دراسة نسيجية وكيميائية مناعية وكيميائية حيوية

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مقدمة البحث: أزيثروميسين هو مضاد حيوي قوي واسع المجال، ولكن استخدامه يولد شوارد حرة شديدة التفاعل والتي لديها تأثير سام للكبد. **الهدف من البحث:** توضح هذه الدراسة ما إذا كان للأزيثروميسين تأثير سام للكبد على الفئران البيضاء البالغة وتقيم دور مضادات الأكسدة بما في ذلك حبة البركة و فيتامين ج وكذلك السيليمارين في تحسين حالة الكبد من الناحيتين النسيجية والكيميائية الحيوية. **طريقة البحث:** تم تقسيم ثلاثين جرذا عشوائيا إلى خمس مجموعات، ست فئران لكل مجموعة. لم تتلق مجموعة المراقبة السلبية أي دواء لمدة ١٤ يومًا بينما تلقت مجموعة الأزيثروميسين بجرعة ٢٠٠ مجم/كجم/اليوم بالفم لمدة ٧ أيام وتلقت مجموعة حبة البركة/أزيثروميسين زيت حبة البركة بجرعة ٤ مل/كجم/اليوم لمدة ٧ أيام ثم زيت حبة البركة بجرعة ٤ مل/كجم بالإضافة للأزيثروميسين بجرعة ٢٠٠ مجم/كجم يوميا بالفم لمدة ٧ أيام أخرى وقد تلقت مجموعة فيتامين ج/أزيثروميسين فيتامين سي بجرعة ٥٠٠ مل/كجم/اليوم لمدة ٧ أيام ثم فيتامين ج بجرعة ٥٠٠ مل/كجم بالإضافة للأزيثروميسين بجرعة ٢٠٠ مجم/كجم يوميا بالفم لمدة ٧ أيام أخرى،تلقت مجموعة سليمارين/أزيثروميسين السليمارين بجرعة ٢٠٠ مل/كجم/اليوم لمدة ٧ أيام ثم السليمارين بجرعة ٢٠٠ مل/كجم بالإضافة للأزيثروميسين بجرعة ٢٠٠ مجم/كجم يوميا بالفم لمدة ٧ أيام أخرى. **النتائج:** أظهرت عينات الكبد من مجموعة الأزيثروميسين عدم انتظام الحبال الكبدية والتي بدت خلاياها ذات نوى متهتكة وسيتوبلازم مفرغ. كما ظهر توسع في الأوردة المركزية وتسلل ملحوظ للخلايا الالتهابية وزيادة ألياف الكولاجين حول الاوردة البابية وقد انخفضت في عينات هذه المجموعة النسبة الإيجابية لصبغة Bcl2 بشكل ملحوظ بينما زادت النسبة الإيجابية لصبغة Bax بشكل ملحوظ كما أظهرت جميع النتائج المختبرية تأثر الكبد في مجموعة الأزيثروميسين. وقد أظهرت هذه النتائج تحسنًا ملحوظًا في المجموعات التي تتلقى حبة البركة أو فيتامين ج أو سيليمارين وكانت المجموعة التي تتلقى السيليمارين هي الأقل تأثراً. **الخلاصة والتوصيات**: يخلص هذا العمل إلى أن إعطاء حبة البركة أو فيتامين ج أو سيليمارين قبل ومع الأزيثروميسين يقلل من تأثيره الضار على الكبد وكان هذا التأثير الوقائي أكثر وضوحًا مع إعطاء السيليمارين أكثر من حبة البركة أو فيتامين ج. لذا يوصي المؤلفون بأنه إذا تمإعطاء عقار أزيثروميسين لأي سبب من الأسباب، فمن الأفضل إعطاء حبة البركة أو فيتامين ج أو سيليمارين قبل ومع الأزيثروميسين لحماية الكبد وتقليل تأثيره السام للكبد كما يجب إجراء مزيد من الدراسات على الإنسان لتأكيد فعالية مضادات الأكسدة؛ حبة البركة ، فيتامين ج أو سيليمارين كعوامل علاجية ووقائية ضد السمية الكبدية التي يسببها أزيثروميسين.