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Hepatoprotective Effect of *Crocus sativus* on Amiodarone-Induced Liver Toxicity

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Authors' contributions

This work was carried out in collaboration among all authors. Author NS and RA designed the study, performed research work. Author YM wrote the protocol. Authors MA, SHK and SK managed the materials. Authors HR and SAR collected all data, performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this study was to investigate the hepatoprotective effect of aqueous and ethanolic extract of *Crocus sativus* in amiodarone-induced hepatotoxicity in rabbits.
Methods: Study was divided into 2 major groups; acute group and prophylactic group and randomized control trial was used. Acute group was further divided into five sub groups (n=5) i.e. control group (group I), amiodarone group (group II), saffron group (group III), amiodarone + aqueous saffron group (group IV), and amiodarone + ethanolic saffron group (group V). Hepatotoxicity was induced by intraperitoneal administration of 200 mg/kg amiodarone solution thrice a day in group II, IV and V. Aqueous extract of saffron (100 mg/kg) was administered intraperitoneally once a day in group III and group IV. Ethanolic extract of saffron (100 mg/kg) was given to group V. However saffron extracts were administered half hour before the amiodarone first dose in both the groups. Aqueous extract of saffron (100 mg/kg) was given to rabbits for seven

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days as prophylactic therapy and then 200 mg/kg amiodarone solution was injected thrice a day in order to observe the hepatoprotective effect of *Crocus sativus*. After the experimental period, blood samples were collected for the evaluation of biochemical parameters. Histopathological examination was performed also.

Results: Study showed that both aqueous and ethanloic extract of *Crocus sativus* significantly decreased serum ALT and AST enzyme activities and significant results were obtained when compared to the amiodarone group.

Conclusion: Based on the results it was concluded that addition of *Crocus sativus* to the treatment protocol of patients maintained on amiodarone for long time period can be recommended to prevent the liver injury.

Keywords: Crocus sativus; hepatoprotective; amiodarone; prophylactic; histopathological.

1. INTRODUCTION

Liver is the largest gland and second largest organ in the body. Various important functions of the body are regulated by the liver such as, metabolic, secretory and excretory functions, immunological functions, vascular, storage and synthesis functions, hemolytic and hematopoietic functions [1].

According to an estimate about 1000 drugs have been reported to cause liver injury however damage may be predictable or unpedictabe [2]. Liver enzymes are involved in biotransformation reactions which result in the formation of various active drug metabolites that may lead to depletion of glutathione. All of these effects damage the liver by directly affecting the organelles or indirectly via inhibition or activation of transcription factors, signalling kinases and gene expression profiles. This intracellular stress may lead to hepatocyte death by apoptosis or necrosis [2]. Hypersensitivity reactions are also produced by some drugs. Endothelial or kupffer cells are primary target for some drugs rather than hepatocytes [3].

In every culture herbal plants have been used as a source of folk medicine since early days. Four hundred medicinal plants were suggested by Hippocrates; father of medicine with the saying, “let food be your medicine or let medicine be your food”. Saffron uses dates back to ancient Egypt and Rome where it was used for spice, aroma and as colouring agent. It was also used as folkoric remedy against various diseases [4].

Crocus sativus L. commonly known as saffron has been traditionally used in Indian Ayurvedic medicine to cure different diseased conditions like cramps, asthma, bronchospasm, menstruation disorders and pain. Saffron has been widely used as stimulant, aphrodisiac, antidepressant, anti- anxiety, anti-cancer, anti-parkinsonism, anti-hypertensive, anti-atherogenic, anti-tussive, anti- diabetic, anti-ulcer, anti-inflammatory, anti-arrhythmic, cardio protective, hepatoprotective and as aphrodisiac agent [5,6].

The beneficial and protective effect of *Crocus sativus* is because of the crocin, crocetin and safranal on different enzymes of antioxidant defense system [7].

Amiodarone is a distinctively effective anti-arrhythmic and anti-fibrillatory agent. However intravenous administration of amiodarone may cause hepatotoxicity with distinct pattern of enzyme disturbances as compared to liver damage from oral amiodarone [8].

In the present investigation, the hepatoprotective effect of *Crocus sativus* was observed by liver injury induced by Amiodarone hydrochloride which is an iodinated benzofuran compound that is the structural analogue of thyroid hormone and approximately contains 37% iodine by weight [8].

2. MATERIALS AND METHODS

2.1 Experimental Animals

Male rabbits of local strain (*Oryctolagus cuniculus*) weighing between 1.5-2.5 kg were purchased from the market and housed in the iron cages in the animal house of University College of Pharmacy, University of Punjab, Lahore. Animals were kept at 12 hr light and dark cycle at 25℃ and were provided with fresh green fodder and water ad libitum throughout the experiment. Animals were acclimatized for a period of 1 week. All the rabbits were kept in the same room under a constant temperature (24±1° C). The study protocol was approved by the animal ethical committee of University College of

Pharmacy, University of Punjab, Lahore and AEC/UCP/1016/4313 voucher number was obtained.

2.2 Chemicals

Amiodarone 200 mg tablets of Sanofi Aventis were purchased from the local pharmacy. Liquid paraffin, Normal saline of immunasol, Formalin, methanol and ethanol were purchased from MERCK and gifted by University College of Pharmacy. Estimation of marker enzymes for liver functions such as ALT, AST, ALP, Bilirubin, Total protein, Albumin and uric acid was investigated using kits supplied by Cresent Diagnostics, Saudi Arabia. LDH was estimated by using kit supplied by Human, Germany and sodium was estimated by using kit supplied by Global in vitro LLP, UK. All the chemicals and reagents used in the experiment were of analytical grade.

2.3 Plant Material

Petals and dried red stigmas of *Crocus sativus* (Saffron) were purchased from the local Supermarket of Pakistan and a sample of saffron petals and stigmas were deposited at the Herbarium of the Government College University, Lahore and voucher number was obtained. Samples were then stored in the zip locked plastic bags and protected from humidity.

2.4 Preparation of *Crocus sativus* Aqueous and Ethanolic Extract

Dried red stigmas of *Crocus sativus* were accurately weighed (100 mg/kg body weight of animal) [4] and then macerated separately into the pre-warmed normal saline and into the analytical grade ethanol solution (37±2℃) for 24 hours. The sample was filtered and residue was again soaked in the fresh solutions. After third maceration the crude drug was fully exhausted. Then the extract was evaporated on 40℃ and the dose was prepared according to the weight of animal at the time of administration.

2.5 Toxicity Induction

Toxicity was induced by injecting 200 mg/kg amiodarone solution intraperitoneally three times a day [9]. Solution was prepared by using 1 portion of high grade ethanol and 3 portions of distilled water. Blood was collected for baseline data of study before induction of toxicity.

2.6 Experimental Design and Treatment Protocol

Study was divided into acute and prophylactic group.

2.6.1 Acute group

Acute group was further divided into five sub groups having n=5. Baseline evaluation was obtained before drug administration and after 24, 48 and 72 hours of drug administration.

Group I (Control group): Hydroalcoholic solution (2 ml; 25%) was administered intraperitoneally three times a day.

Group II (Amiodarone group): Amiodarone solution (200 mg/kg) was freshly prepared and administered intraperitoneally three times a day for three days.

Group III (Saffron group): Aqueous extract of saffron (100 mg/kg) was administered intraperitoneally once a day for three days.

Group IV (Amiodarone + aqueous saffron group): Amiodarone solution (200 mg/kg) was injected intraperitoneally three times a day for three days. Aqueous extract of saffron (100 mg/kg) was administered half hour before the first dose of amiodarone.

Group V (Amiodarone + ethanolic saffron group): Amiodarone solution (200 mg/kg) was injected intraperitoneally three times a day for three days. Ethanolic extract of saffron (100 mg/kg) was administered half hour before the first dose of amiodarone.

2.6.2 Prophylactic group

Prophylactic group was further divided into two subgroups containing five rabbits in each group.

Group I: Normal saline (5 ml) was administered once a day intraperitoneally for seven days. Blood sample was obtained on seventh day from marginal ear vein and 200 mg/kg amiodarone solution was injected intraperitoneally to the rabbits three times a day for three days. Blood was obtained after 24, 48 and 72 hours after first amiodarone administration.

Group II: Aqueous extract of saffron (100 mg/kg) was freshly prepared and administered once a day to the rabbits for seven days. Blood was obtained and tested

for all the parameters and then 200 mg/kg amiodarone solution was injected intraperitoneally three times a day for three days. Blood was obtained after 24, 48 and 72 hours from the administration of first amiodarone dose.

2.7 Biochemical Parameters Evaluation

Serum was collected and ALT, AST, ALP, Bilirubin, Total protein, Albumin and uric acid was investigated by using Crescent Diagnostics Kits. LDH was estimated by using Human, Germany kit and sodium was estimated by using Global *in vitro* LLP, UK kit.

2.8 Histopathological Analysis

After biochemical and hematological tests, liver was removed carefully for histological evaluation and was fixed in 38% formalin. Tissue was then processed in different percentages of ethanol. Xylene was added to make the tissue hard. Tissues were then embedded in paraffin wax for 6hrs and blocks were then created for microtoming. Tissues slices were then fixed with the help of gelatin. Slides were then stored at 58℃ for 10-12 hrs in the oven. After drying slides were then stained with hematoxylin and eosin, covered with the cover slip and sealed with wax. The slides were then examined.

2.9 Statistical Analysis

Values were expressed as mean±SE which were then compared statistically by applying one way analysis of variance (ANOVA) using post hoc Tukey's test in acute group. In prophylactic group values were compared by applying student T test using SPSS Statistics 13.0. P value was then calculated from student T-Table [10].

$P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Acute Group Study

Comparison of ALT, AST and ALP activities in different groups is shown in Fig. 1. Administration of 200 mg/kg Amiodarone three times a day induced significant change in marker enzymes. Elevation of serum ALT and AST activities were obtained in group II when compared with the control group as shown in Table 1. However administration of aqueous extract of saffron (100 mg/kg) to rabbits in group III give insignificant ALT and AST enzyme activities when compared to the control group. Aqueous and ethanolic extract (100 mg/kg) of saffron when given to group IV and V significantly decreased the ALT and AST enzyme activities. However insignificant difference was observed in ALP and in bilirubin levels in all the groups.

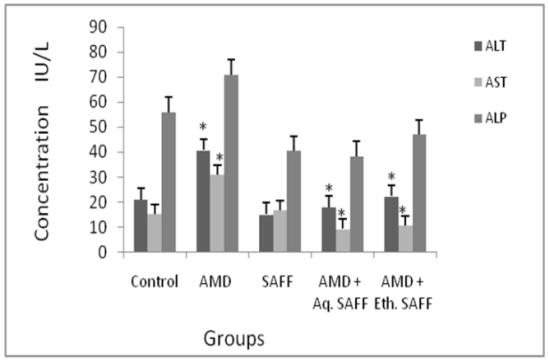

Fig. 1. Changes in ALT, AST, ALP activities in different groups of acute study

Elevated serum LDH activity in group II when compared with the control group was observed as shown in Table 1.

Saffron alone when given to group III did not show any increase in LDH activity. Aqueous and ethanolic extract of saffron when given to group IV and V along with amiodarone significantly decreased LDH activity which showed the protective effect of saffron on maintaining the normal LDH activity.

Comparison of LDH activity in different groups of acute study is shown in Fig. 2.

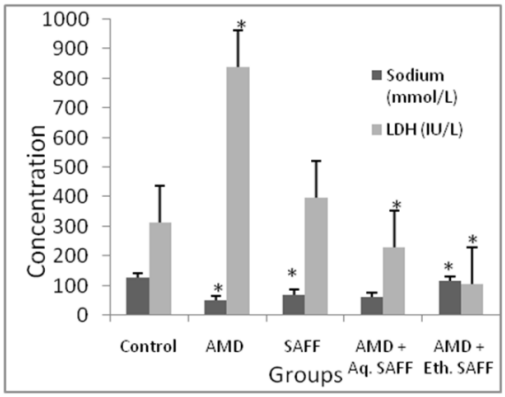

Fig. 2. Changes in sodium level and LDH activity in different groups of acute study

Amiodarone induced hyponatremia as highly significant value was obtained by comparing amiodarone group with the control group. Aqueous extract of saffron also show significant decreased in sodium level when compared with the control group as shown in Table 2. Ethanolic extract of saffron in 100 mg/kg dose when given to group III did not induce hyponatremia.

Albumin decreased significantly in group II when compared with the control group as shown in Table 2. Both aqueous and ethanolic extract of saffron significantly increased the albumin synthesis when given to group IV and V respectively. However insignificant results were obtained with Protein and uric acid. Comparison of Protein, albumin, bilirubin and uric acid are shown in Fig. 3.

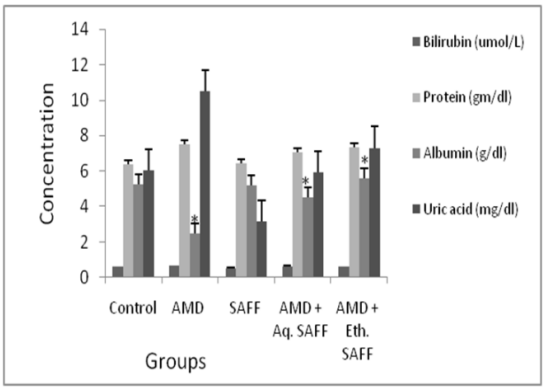

Fig. 3. Changes in bilirubin, protein, albumin and uric acid in different groups of acute study

3.2 Prophylactic Group Study

Pretreatment with the aqueous extract of saffron showed protective effect on all the parameters performed in the study except the serum sodium level.

ALT, AST, LDH, albumin and uric acid values were decreased significantly when compared with the amiodarone group as shown in Tables 3 and 4. Comparisons of all the biochemical parameters of prophylactics study are shown in Figs. 4, 5 and 6.

Histopathological observation showed that the normal architecture of the rabbits liver was completely changed with the amiodarone administration and histopathological slides of the liver of AMD group (Figs. 9 and 10) showed variable findings like dilated vascular channels, presence of fibrosis and fibroblasts, widening of portal tract, periportal inflammation, coagulation and periportal necrosis, ballooning degeneration of hepatocytes, lymphocyte infiltration and little steatosis when compared to the livers of control group and saffron group. Histopathologic examination of liver confirmed the hepatotoxic effect of amiodarone and hepatoprotective effect of saffron which is significantly correlated with its antioxidant properties.

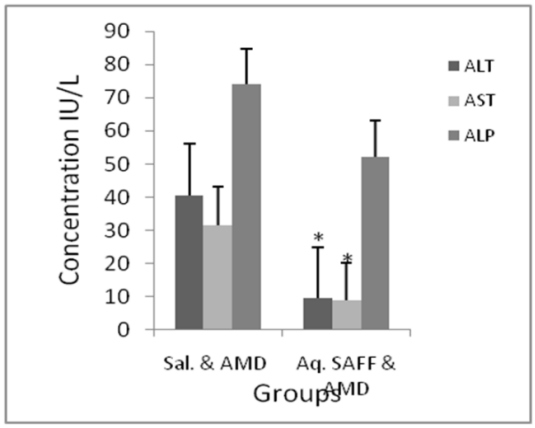

Fig. 4. Changes in ALT, AST and ALP activities in different groups of prophylactic study

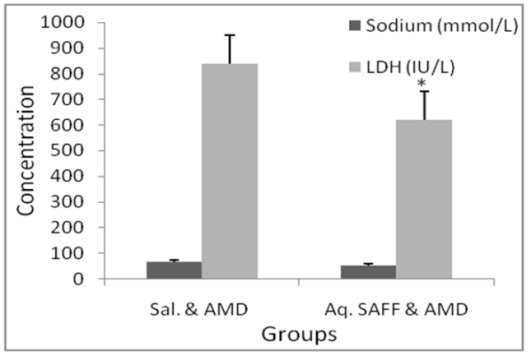

Fig. 5. Changes in sodium level and LDH activity in different groups of prophylactic study

4. DISCUSSION

Amiodarone [2-Butyl-3-(3',5'-diiodo-4'α-diethylaminoethoxybenzoyl)-benzofuran] is class III antiarrhythmic drug and has been used for diseases of heart since 1960s in tachyarrhythmias and in refractory angina [11].

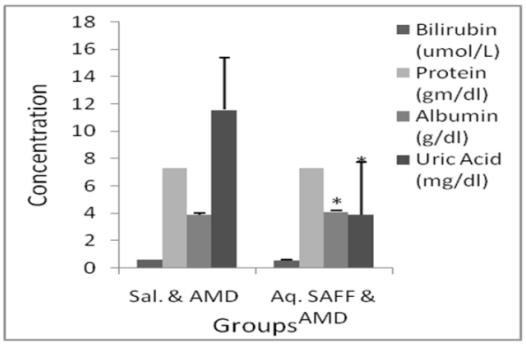

Fig. 6. Changes in bilirubin, protein, albumin and uric acid levels in different groups of prophylactic study

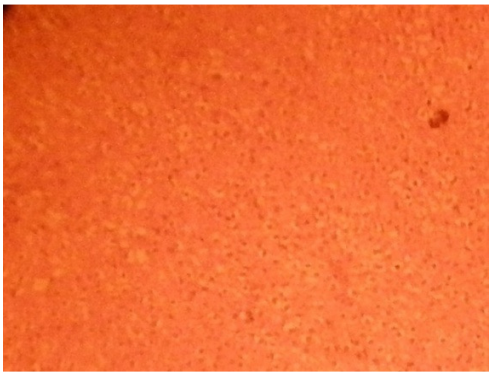

Fig. 7. Hematoxylin and eosin staining of liver of control group ×10

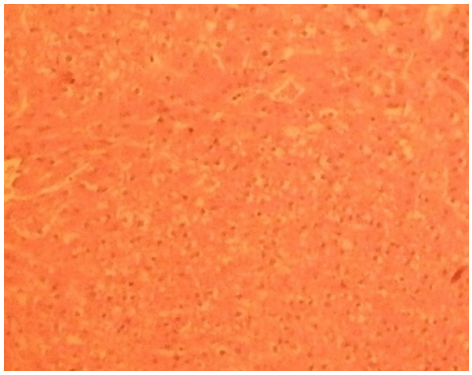

Fig. 8. Hematoxylin and eosin staining of liver of control group ×10

In the present investigation, liver toxicity was induced by intraperitoneal administration of 200 mg/kg amiodarone solution thrice a day. Amiodarone is a lipophilic drug with $T_{1/2}$ of 35-110 days and large Volume of Distribution so accumulates in the lipid rich reservoirs which may induce injury to the liver tissues and altered the permeability of membranes by the formation of ROS and oxidative stress lead to the leakage of enzymes from the cells [12]. Therefore elevation of serum ALT and AST enzyme activities in group II indicates damage to the liver as shown in Table 4. Chronic liver injury from amiodarone may lead to lesions of varying nature which may progress to steatosis and non-alcoholic steatohepatitis (NASH) thus lead to fibrosis and cirrhosis of liver [12]. N-desmethylamiodarone is the major active metabolite of amiodarone is as active as parent drug but elimination half life and volume of distribution is longer than the parent drug. Thus amiodarone damaging effect on liver may persist up to a year even after drug discontinuation [13].

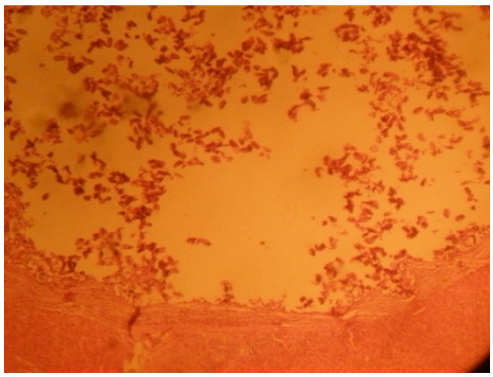

Fig. 9. Hematoxylin and eosin staining of liver of amiodarone group ×10

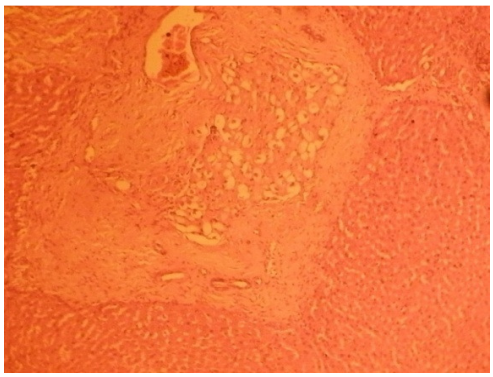

Fig. 10. Hematoxylin and eosin staining of liver amiodarone group ×10

Oxidative stress is also induced by amiodarone due to the depletion of GSH; which activates the magnesium dependent neutral sphingomyelinase (N-SMase). N-SMase then increases the cellular ceramide level which then followed by the protein kinase/c-jun kinase (SAPK/JNK) signaling pathway activation and ultimately it lead to apoptosis [14].

Table 1. Comparison of effects of *Crocus sativus* on biochemical parameters alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bilirubin and lactate dehydrogenase (LDH) after amiodarone induced acute hepatotoxicity

Group no.	Groups	ALT (IU/L) Mean±SE	AST (IU/L) Mean±SE	ALP (IU/L) Mean±SE	Bilirubin (umol/L) Mean±SE	LDH ((IU/L)) Mean±SE
I	Control group	20.92±3.01	15..35± 2.86	56.00 ±3.66	0.61±0.032	314.02±17.09
II	AMD group	40.72±4.54 *	31.07±5.33 +*	71.52±17.73	0.65±0.13	836.90 ±12.77 *
III	SAFF group	15.10±1.84	16.86±2.05	40.45±4.32	0.53±0.053	397.20± 81.60
IV	AMD + Aq. SAFF group	18.04±0.94 *	9.29±2.03 *	38.33±2.43	0.64±0.067	228.95 ±16.03 *
V	AMD + Eth. SAFF group	22.29±6.78 *	10.59±2.93 *	46.92±12.23	0.60±0.042	104.13 ±24.94 *#

1) * represents comparison of AMD group with control, AMD + aq. SAFF group and AMD + Eth. SAFF group.
2) # represents comparison of SAFF group with control, AMD + aq. SAFF group and AMD + Eth. SAFF group.
3) * and # represents P<0.05.
4) + represents value after 72 hours of drug administration.
5) For T test, oneway ANOVA was applied followed by a Tukey's test post hoc.

Table 2. Comparison of effects of *Crocus sativus* on biochemical parameters (protein, albumin, sodium and uric acid) after amiodarone induced acute hepatotoxicity

Group no.	Groups	Protein (g/dl) Mean±SE	Albumin (g/dl) Mean±SE	Sodium (mmol/L) Mean±SE	Uric acid (mg/ dl) Mean±SE
I	Control group	6.39±0.36	5.24±0.13	127.00±4.52	6.02±0.88
II	AMD group	7.49±0.34	2.47±0.54*	50.97±20.69*	10.51± 1.02
III	SAFF group	6.46±0.53	5.20±0.23	70.39±11.11*	3.18±0.44
IV	AMD + Aq. SAFF group	7.08±0.10	4.52±0.30*	59.85±8.77	5.93±2.19
V	AMD + Eth. SAFF group	7.35±0.192	5.60±0.28*	115.66±18.87*#	7.31±2.40

1) * represents comparison of AMD group with control, AMD + aq. SAFF group and AMD + Eth. SAFF group.
2) # represents comparison of SAFF group with control, AMD + aq. SAFF group and AMD + Eth. SAFF group.
3) * and # represents P<0.05.
4) For T test, oneway ANOVA was applied using post hoc Tukey's test.

Table 3. Comparison of effects of *Crocus sativus* on biochemical parameters alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bilirubin and lactate dehydrogenase (LDH) after amiodarone induced hepatotoxicity in prophylactic group

Group no.	Group name	ALT (IU/L) Mean±SE	AST (IU/L) Mean±SE	ALP (IU/L) Mean±SE	BILIRUBIN (umol/L) Mean±SE	LDH (IU/L) Mean±SE
I	Sal. and AMD group	40.52±3.49	31.67±5.01[+]	73.98±11.07	0.65±0.08	840.70±13.26[a]
II	Aq. Saff and AMD group	9.51±2.58*	8.81± 0.923*	52.26±3.69	0.56±0.025	620.66±90.53*

1) * represents comparison of AMD group with AMD + aq. SAFF group.
2) + value after 72 hours of drug administration.
3) For P value:* represents P<0.05
4) For T test, oneway ANOVA was applied using post hoc Tukey's test.

Table 4. Comparison of effects of *Crocus sativus* on biochemical parameters (protein, albumin, sodium and uric acid) after amiodarone induced hepatotoxicity in prophylactic group

Group no.	Group name	Protein (gm/dl) Mean±SEM	Albumin (g/dl) Mean±SEM	Sodium (mmol/L) Mean±SEM	Uric acid (mg/dl) Mean±SEM
I	AMD group	7.30±0.17	3.92±0.26	65.86±6.18	11.57±1.32
II	AMD + Aq. SAFF Group	7.34±0.290	4.12±0.156*	51.39±7.48	3.91±0.69*

1) * represents comparison of AMD group with AMD + aq. SAFF group.
2) For P value:* represents P<0.05
3) For T test, oneway ANOVA was applied using post hoc Tukey's test.

Fig. 11. Hematoxylin and eosin staining of liver of saffron group ×10

Hepatoprotective effect of aqueous and ethanolic extracts of C*rocus sativus* was evaluated in the study. When 100 mg/kg aqueous extract of saffron was given to rabbits in group III it preserves the permeability of lipid membranes and liver physiology. When compared to the control, P value was insignificant for ALT and AST enzyme activities. Aqueous and ethanolic extract of saffron (100 mg/kg) when given to group IV and V significantly decreased the ALT and AST enzyme activities by radial scavenging activity, inhibition of free radical chain reactions and by decreasing oxidative stress. Decreased aminotransferaes activity indicate that saffron inhibits further liver injury.

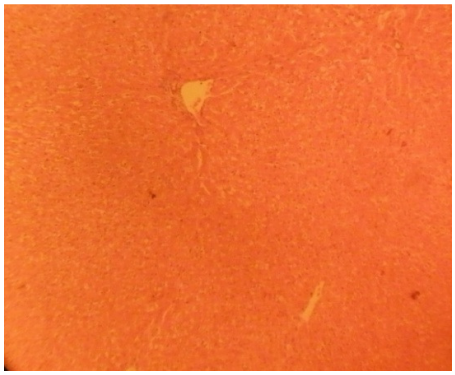

Fig. 12. Hematoxylin and eosin staining of liver of saffron group ×10

Crocus sativus L. (or saffron) is a herb of *Iridaceae* family, protects hepatocytes by inhibiting lipid peroxidation and cell proliferation. It also inhibits the mechanism of apoptosis by increasing GSH antioxidant non protein during oxidative stress as it contains antioxidant

compounds crocin and safranl. Also saffron replenish the thiol pool and increases the level of glutathione, glutathione S transferase, glutathione peroxidase, super oxide dismutase and catalase [4,14]. Safranal is major constituent of saffron obtained by degradation of picrocrocin possess radical scavenging and antioxidant activity. Crocin and Crocetin are caretenoid glycosides and soluble in both water and ethanol; restore SOD activity and inhibit fatty acids enzymatic peroxidation [15,16].

Elevated serum LDH level in group II with highly significant value when compared with the control group was observed as shown in Table 4 because of increased LDH transcription under anaerobic condition as production of LDH can be used as hypoxic marker [17]. However saffron did not divert the cells towards the hypoxic conditions but aqueous and ethanolic extract of saffron when given to group III and IV along with amiodarone significant decreased LDH enzyme activity which showed the protective effect of saffron on maintaining the normal LDH enzyme activity as it prevent the hepatocytes from structural damage by reduction of lipid peroxidation [18].

Amiodarone induced hyponatremia because amiodarone may suppress the secretion of anti-diuretic hormone. It may also cause the mutation of aquaporins regulating vasopressin receptos (V2). This decreases the water permeability of distal tubule and collecting duct epithelial cells in the kidney and increases the excretion of water along with electrolytes like sodium and thus lead to hyponatremia by amiodarone [19]. However aqueous extract of saffron show vasodilatory and spironolactone like diuretic effect [20,21]. Ethanolic extract of saffron in 100 mg/kg dose when given to group III provide defense against hyponatremia. This may be because some constituent necessary for the diuretic properties of saffron may absent in ethanolic extract and may present in aqueous extract.

Reduced synthesis of albumin may be the result of injury to hepatocytes, malnutrition, malabsorption or due to the decreased concentration of albumin mRNA in the liver. In drug induced liver injury various intracellular signaling mediators are released leading to endoplasmic reticulum (ER) stress, dysfunctional ER and decreased albumin synthesis [22,23]. Crocetin in the saffron stigmas prevent the ER stress by preventing the formation of intracellular signaling mediators and maintains the integrity of ERs responsible for albumin synthesis so both extracts increased the albumin synthesis and both of them are equally potent as saffron protects the hepatocytes from damage [24].

Amiodarone administration may lead to tubular alteration with the partial loss of brush boarder microvilli and necrotic tubular epithelium which may lead to the decrease uric acid excretion [25].

Pretreatment with the aqueous extract of saffron in prophylactic group showed protective effect on all the parameters performed in the study except the serum sodium level. ALT, AST, LDH, albumin and uric acid activities were decreased significantly when compared with the amiodarone group as shown in Tables 3 and 4. Reduction in all these parameters indicates that saffron preserve the liver integrity and hepatocytes functioning.

Histopathologic examination of liver further confirmed the hepatotoxic effect of amiodarone and hepatoprotective effect of saffron which is significantly correlated with its antioxidant properties.

5. CONCLUSION

Amiodarone induced biochemical and histological changes by interrupting the balance between oxidant and antioxidant system which is protected by concomitant or pretreatment with the saline or ethanolic extract of saffron by radical scavenging properties. In this study it was concluded that both aqueous and ethanolic extract of saffron can be used for their hepatoprotective properties however ethanolic extract is more potent.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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