Original article:

SCIENTIFIC VALIDATION OF CARDIOPROTECTIVE ATTRIBUTE BY STANDARDIZED EXTRACT OF *BOMBYX MORI* AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY IN MURINE MODEL

Masood S. Khan[1], Mhaveer Singh[1], Mohammad A. Khan[2], D. S. Arya[3], Sayeed Ahmad[1]*

[1] Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, India-110062

[2] Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, India-110062

[3] Department of Pharmacology, All India Institute of Medical Sciences (AIIMS), New Delhi

* Corresponding author: Dr. Sayeed Ahmad, Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard (Hamdard University) Hamdard Nagar, New Delhi – 110062, India; Tel: +91 9891374647, E-mail: sahmad_jh@yahoo.co.in

ABSTRACT

Doxorubicin (DOX) is an excellent antineoplastic agent used for the treatment of hematological and solid malignancies. The aqueous extract of *Bombyx mori* (BMAE) contains amino acids and some flavonoids with obvious cardioprotective effect. The aim of this study was to investigate the possible protective effect of BMAE against DOX-induced cardiotoxicity and its underlying mechanisms on murine model. The metabolic profiling of BMAE was carried out by Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) and the amino acid profiling by HPLC method using fluorescence detector (HPLC-FLD)**.** The biochemical parameter like caspase-3, tumor necrosis factor–alpha (TNF—α), interleukin -6 (IL-6), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) and malondialdehyde (MDA) were studied. Tissue damage was further evaluated by histopathological studies. The metabolic profiling of BMAE exhibited presence of quercetin 7-*O*-β-D-glucoside, kaempferol 7-*O*-β-D-glucopyranoside, coumaric acid glucoside, 2-hydroxy-nonadecanoic acid and 9,12-dihydroxy stearic acid as important constituents. The amino acid profile by HPLC-FLD showed presence of 17 amino acids. The BMAE showed prominent free radical scavenging activity when assessed by the H_2O_2 and super-oxide method. The results of present investigation showed protection against DOX-induced oxidative stress (lipid peroxidation), by reverting activities of apoptotic markers (caspase-3 and TNF-α), cardiac markers (CK-MB and LDH activities) as well as pro-inflammatory marker IL-6 followed by oral administration of BMAE. In addition, results of histopathology also supported well the above results. It was observed that BMAE protects DOX-induced cardiotoxicity by virtue of its antioxidants possibly by flavonoids and amino acids.

Keywords: *Bombyx mori,* doxorubicin, amino acids, cardioprotective, antioxidants

INTRODUCTION

Doxorubicin (DOX), is a naturally occurring anthracycline and widely used for the treatment of a range of human malignancies including hematomas and solid tumors (El-baky et al., 2010). The use of doxorubicin has been associated with severe toxic effects and most dangerous one is the dose-dependent cardiotoxicity, leading to cardiomyopathy and eventually congestive heart failure. Cardiotoxicity includes a wide range of cardiac effects from small changes in blood pressure and arrhythmias to cardiomyopathy (Schimmel et al., 2004).

Acute DOX cardiotoxicity occurs within 2-3 days of its administration. Studies have shown that DOX causes cardiotoxicity through mechanisms other than those mediating its antitumor effect (Khan et al., 2014). However, it appears that the induction of an oxidative stress within myocardial tissue constitutes a common denominator (Vergely et al., 2007). It is usually arbitrated through lipid peroxidation and inhibition of long fatty acid oxidation in cardiac tissues. The oxidative stress, lipid peroxidation and mitochondrial dysfunction have been associated with DOX-induced cardiomyopathy (Nohl et al., 1998).

DOX generates reactive oxygen species (ROS) via several mechanisms (Malisza and Hasinoff, 1995) and can directly form complexes with ferrous ion within the cell. These complexes are pertinent to generate ROS in the presence or the absence of reducing components (Vergely et al., 2007). The investigations showed that animals treated with compounds of antioxidant potential protects heart against DOX induced toxicity (Nazeyrollas et al., 1999; Liu et al., 2002). Unani System of Medicine offers a number of cardioprotective and cardiotonic single and compound drugs which have been mentioned by ancient renowned Unani physicians in classical literatures including *Khameera Abresham Sada, Khameera Gaozabansada, Khameera Abresham Uood Mastagi Wala* etc (Ahmad et al., 2010). Scientific studies have proven the cardioprotective effect of some Unani compound formulations such as *Khameera Abresham Hakeem Arshad Wala* (KAHAW) (Goyal et al., 2010), *Khamira Abresham Uood Mastigi Wala* (KAUM), *Khamira Abresham Sada* (KAS) etc (Kabeer, 1951). which are used by Unani Physicians since ancient times.

Abresham, a cocoon of *Bombyx mori* (silkworm) is the main ingredient of KAHAW, KAUM and KAS. Therefore, it was thought worthwhile to carry out scientific validation of cardioprotective potential of Abresham. The two main constituents of abresham are fibroin (70 %) and sericin (25 %). The non sericin components consist mainly of carbohydrates, salt, waxes (Mondal et al., 2007), and some flavonoids derivatives. The main flavonoids are quercetin glycosides (quercetin 5-O-beta-D-glucoside, quercetin 7-O-beta-D-glucoside, and quercetin 4-O-beta-D-glucoside) with two kaempferol glycosides (kaempferol 5-O-beta-D-glucoside and kaempferol 7-O-beta-D-glucoside) (Kurioka and Yamazaki, 2002). Sericin and fibroin are rich sources of amino acids (Schroeder et al., 1955), which can play an important role in myocardial protection (Julia et al., 1990), since shielding effect of amino acids has already been reported to significantly improve the recovery of cardiac function (Shug et al., 1994). However, flavonoids act as scavengers of reactive oxygen species (Haenen et al., 1993). Mahmood and colleagues (2013) have suggested the potent antioxidative activity of *B. mori* cocoon is mainly due to rich source of free amino acids and flavonoid compounds present in them (Kurioka and Yamazaki, 2002).

Most of the previous studies are either on alcoholic extract or on crude *B. mori,* whereas, according to official Unani Pharmacopoeia and other literature of traditional system of medicine aqueous decoction is used in most of the Unani formulations. Therefore, the purpose of the present study was to elucidate the potential of standardized *B. mori* aqueous extract (BMAE) against DOX induced cardiomyopathy in rats.

MATERIALS AND METHODS

Experimental animals

Adult male wistar albino rats (10-12 week old), having body weight of 150-200 g, were used. Animals were acclimatized and housed under controlled conditions of illumination (12 h light/dark cycles) with temperature of 20-25 °C and relative humidity of 30 ± 5 %. Standard pellet diet (Ashirwad Rat Feed, Chandigarh, India) and water *ad libitum* were provided to the animals throughout the study period. The study was approved by the Institutional Animal Ethics Committee (CPCSEA registration no 173/CPCSEA) of Jamia Hamdard, New Delhi.

Drugs and chemicals

B. mori was procured from the local drug market and identified by Dr. A. Ahmad, Department of Ilmul Advia, Faculty of Medicine, Hamdard University, New Delhi. A voucher specimen was deposited in the herbarium of the Bioactive Natural Product Laboratory (Specimen no-54/BM/BNPL/2012). DOX was obtained as Adrim injection (Dabur Pharmaceuticals, India) whereas desferrioxamine as Desferal injection (Novartis Pharmaceuticals, Switzerland). All the other chemicals used were of analytical grade.

Preparation of extract

The aqueous extract of *Bombyx mori* (BMAE) was prepared similar to the method used for development of traditional formulations. In brief, the cocoons were sliced into small pieces and 200 g of it was placed in round bottom flask of 5 L capacity. The extraction was carried out using reflux condenser and double distilled water (DDW) (3 L) as solvent for two hours on water bath. It was kept aside overnight with occasional shaking and press filtered using muslin cloth. The residue left was washed with fresh solvent (DDW). The extract and washings were pooled and concentrated in rota-vapour, followed by drying in lyophilizer (extractive value 5.66 % w/w) to get free flowing pow-

der. It was stored in cool place (4 °C) till analysis and bioactivity.

Metabolic profiling of extract by using UPLC-MS

The freeze dried powdered of BMAE was dissolved in LCMS grade acetonitrile and water (100 µg mL^{-1}) and filtered. The constituents were analyzed on a Water's ACQUITY UPLC^{TM} system (Serial No# F09 UPB 920M; Model Code# UPB; Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Serial No# JAA 272; SYNAPT; WATERS, Manchester, UK). Chromatographic separation was performed on a Water's ACQUITY UPLCTM BEH C18 (100.0 mm x 2.1 mm; 1.7 µm) column at 40 ± 5 °C. The UPLC analysis was done using water: acetonitrile (90:10; v/v), as solvent system in gradient elution. The flow rate of the mobile phase was kept at 0.35 mL min^{-1} and 10 µL of sample solution was injected in each run. The total chromatographic run time was 30 min. The column and auto-sampler were maintained at 40 ± 5 and 25 ± 5 °C, respectively whereas the pressure of the system was set to 15000 psi.

Determination of free amino acids by using HPLC-FLD

Determination of amino acids as their respective 6-aminoquinolyl-**N**-hydroxysuccinimidyl carbamate (AQC) derivatives were successfully applied for assessing free amino acid levels in BMAE based on the **Waters AccQ.Tag™** method for high performance liquid chromatography (HPLC) (Kabelová et al., 2009).

Determination of in vitro anti-oxidant potential

Superoxide radical scavenging and hydrogen peroxide scavenging potentials of BMAE were determined as per the methods reported by Amir and colleagues (2011) in the concentration range of 5.0-35 µg mL^{-1} using ascorbic acid as standard.

In vivo cardioprotective activity

Animals and dosing schedule

The animals were housed in polypropylene cages (size 40 x 25 x 16 cm) in groups of six rats per cage. These were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory animal diet and water ad libitum. After acclimatization rats were randomly assigned into five groups of six animals each. Group 1 (vehicle control), rats were treated with 0.5 % carboxy methyl cellulose (CMC) in normal saline (2 mL/Kg/p.o./day) for 30 days. Group 2 (pathogenic control; DOX), received 0.5 % CMC in normal saline (2 mL/Kg/p.o./day) for 30 days along with doxorubicin (20 mg/Kg, single i.p. injection) on 31st day. Group 3 (BMAE 30) animals were administered with BMAE (30 mg/Kg/p.o./day) for 30 days and DOX (20 mg/Kg, single i.p. injection) on 31st day. Similarly, group 4 (BMAE 60) animals were administered with BMAE (60 mg/Kg/p.o./day) for 30 days and DOX (20 mg/Kg, single i.p. injection) on 31st day. Group 5 (DFX) was given desferrioxamine (50 mg/Kg/i.v. injection /day) for 30 days with DOX (20 mg/Kg, single i.p. injection) on 31st day (Ayla et al., 2011; Sharma et al., 2011). At the end of treatment (i.e. 31st day), animals were fasted overnight (12 h) and blood samples were collected from retro-orbital plexus under mild ether anesthesia for separation of serum. Further, animals were sacrificed by cervical dislocation and heart was excised, which was stored at -80 °C for histopathology and biochemical estimation.

Biochemical estimation

CK-MB and LDH activity (Reckon Diagnostics Pvt. Ltd, India) (Lum and Gambino, 1974), IL-6 (Ray Biotech) (Helle et al., 1991), TNF-α (e-Bioscience, Inc., USA) (Lehmann et al., 2008) and protein (Lowry et al., 1951) were measured in serum as per standard methods. Similarly, caspase-3 was measured (Bio Vision, Inc., USA) (Jaeschke et al., 1998). Lipid peroxidation (Iqbal et al., 2008) and protein (Lowry et al., 1951) were estimated in heart tissue homogenate as per the standard protocol.

Histopathological examination

The heart tissues were fixed in 10 % formalin and sections were prepared with thickness up to 5.0 μm. These were stained with hematoxylin and eosin for histopathological evaluation of heart sections and were carried out by a pathologist unaware of groups (Belur et al., 1990).

Statistical analysis

The data is expressed as mean ± SD. Statistical differences between means were determined by one-way analysis of variance (ANOVA), followed by Dunnett's t test. The values of $P<0.05$ were considered as significant.

RESULTS

Quality control of BMAE

The extraction of *B. mori* as per the traditional method resulted in 5.66 % w/w of BMAE, which was further subjected to the quality control analysis. The metabolic profiling of BMAE by UPLC-qTOF-MS followed by tentative structure assignment using m/z and literature showed identification and separation of twenty eight constituents out of which quercetin 7-*O*-β-D-glucoside, kaempferol 7-*O*-β-D-glucopyranoside, coumaric acid glucoside, 2-hydroxy-nonadecanoic acid, 9,12-dihydroxy stearic acid were present in highest amount as compared to other constituents (Table 1). The amino acid profiling by HPLC-FLD revealed presence of 17 amino acids v.i.z. proline, cysteine, methionine, aspartic acid, serine, alanine, lysine, leucine, histidine, threonine, glycine, arginine, tyrosine, valine, isoleucine and phenylalanine (Figure 1). The content of cysteine, methionine and proline were found highest, whereas isoleucine and tyrosine were found lowest (Table 2).

Table 1: Metabolites tentatively identified with their m/z ratio and literature in the *Bombyx mori* [UPLC/qTOF/MS study]

Bombyx mori	m/z	Chemical formula	Molecular weight	Chemical name
S. No	Rt			
1	3.77	281.14	$C_{15}H_{22}O_5$	282.14	Oxyhumulinic acid
2	4.44	537.31	$C_{30}H_{42}N_4O_5$	538.31	PheLeuPhe Ile
3	4.96	497.44	$C_{34}H_{58}O_2$	498.44	Myristoleyl arachidonate
4	5.41	521.33	$C_{26}H_{50}O_{10}$	522.34	Polysorbate 20
5	5.8	187.14	$C_9H_{20}N_2O_2$	188.15	7,8-Diaminononanoate
6	6.39	329.23	$C_{14}H_{18}O_9$	330.23	Vanilloyl glucose
7	6.53	329.22	$C_{14}H_{18}O_9$	330.23	Vanilloyl glucose
8	7.46	663.42	$C_{35}H_{60}N_4O_6S$	664.42	Prostaglandin F2α-biotin
9	7.92	447.38	$C_{21}H_{22}O_{11}$	448.38	Kaempferol 7-*O*-β -D-glucopyranoside
10	8.58	1233.81	$C_{63}H_{114}N_2O_{21}$	1234.79	Ganglioside
11	9.6	329.32	$C_{14}H_{18}O_9$	330.23	Vanilloyl glucose
12	10.01	375.28	Unknown		
13	11.03	463.37	$C_{18}H_{38}N_6O_4$	464.37	Quercetin 7-*O*-β-D-glucoside
14	12.09	236.17	$C_{13}H_{19}NO_3$	237.17	3-O-Methylrimiterol
15	12.53	580.47	Unknown		
16	13.53	383.27	$C_{17}H_{32}N_6O_4$	384.24	Arg Pro Leu
17	14.64	313.32	$C_{19}H_{38}O_3$	314.28	2-hydroxy-nonadecanoic acid
18	16.25	315.33	$C_{18}H_{36}O_4$	316.26	9,12-dihydroxy stearic acid
19	17.12	311.25	$C_{13}H_{12}O_9.$	312.25	Caffeoyl tartrate
20	17.49	295.3	$C_{20}H_{40}O$	296.30	Phytol
21	18.14	293.29	$C_{15}H_{22}N_2O_4$	294.29	Sinapoyl putrescine
22	18.68	297.32	$C_{20}H_{42}O$	298.3236	Phytanol
23	19.04	566.48	$C_{24}H_{45}N_{11}O_5$	567.36	Lys His Lys Arg
24	21.27	277.28	Unknown		
25	21.47	227.26	Unknown		
26	22.05	279.3	Unknown		
27	22.78	281.33	Unknown		
28	23.07	325.28	$C_{15}H_{18}O_8$	326.29	Coumaric acid glucoside

*A list of putative identifications was retrieved from HMDB, Metlin, MMCD, and LipidMaps databases.
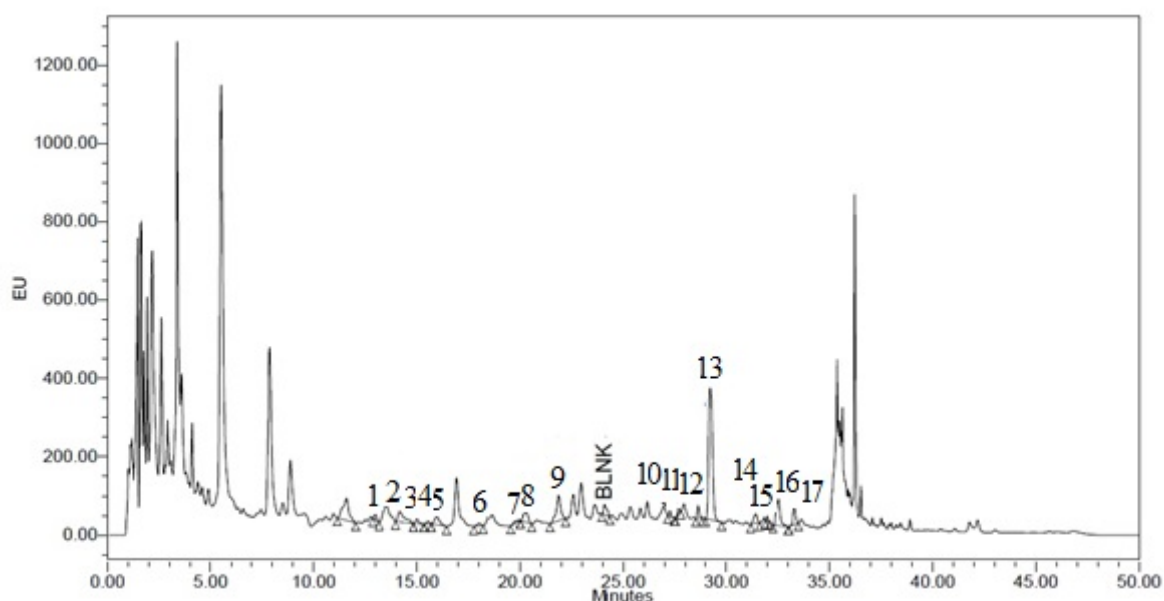
Figure 1: Separation of different aminoacids from BMAE using HPLC-FLD at excitation wavelength- 250 nm emission wavelength- 395 nm. (1: aspartic acid, 2: serine, 3: glutamic acid, 4: glycine, 5: histidine, 6: arginine, 7: threonine, 8: alanine, 9: proline, 10: cystiene, 11: tyrosine, 1: valine, 13: methionine, 14: lysine, 15: isoleucine, 16: leucine, 17: phenyl alanine)

Table 2: Analysis of amino acids in BMAE using HPLC-FLD

S. No.	Amino acid	Retention time	Content (Mean ± SD) ng/g
1	Aspartic acid	12.896	95.37±0.53
2	Serine	13.648	99.54±0.68
3	Glutamic acid	14.464	23.26±0.18
4	Glycine	14.876	5.64±0.08
5	Histidine	15.556	29.76±0.34
6	Arginine	19.438	8.34±0.02
7	Threonine	19.882	26.04±0.16
8	Alanine	19.991	46.88±0.65
9	Proline	21.376	131.5±1.91
10	Cystiene	27.269	477.90±2.89
11	Tyrosine	27.690	4.73±0.007
12	Valine	28.636	9.97±0.069
13	Methionine	29.213	243.08±1.29
14	Ly sine	31.415	41.10±0.28
15	Isoleucine	31.978	2.42±0.01
16	Leucine	32.525	28.58±0.31
17	Phenyl alanine	33.287	7.58±0.06

The BMAE showed a concentration-dependent antioxidant activity by inhibiting super oxide radical with an IC_{50} value of 30.6 μg mL^{-1}, whereas IC_{50} value of ascorbic acid was found to be 14.9 μg mL^{-1}(Figure 2B). Similarly, results of hydrogen peroxide scavenging activity for BMAE showed dose dependent activity between 5.0 - 35 μg mL^{-1} with IC_{50} value at 14.05 μg mL^{-1} and ascorbic acid at 6.77 μg mL^{-1}(Figure 2A).
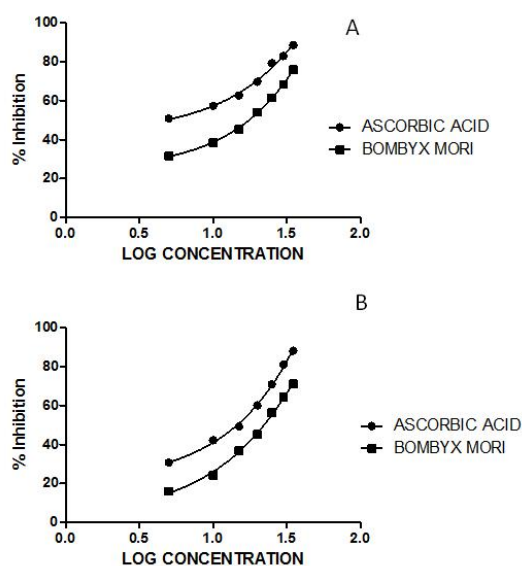
Figure 2: Dose response curve showing log concentration (µg/ml) vs % inhibition of H_2O_2 scavenging assay (A) and superoxide scavenging assay (B) for *Bombyx mori* aqueous extract.

significant reversal (P<0.01) to DOX toxicity of TNF-alpha, IL-6 as well as caspase-3 levels (Table 4).

Table 3: Effect of BMAE (30 mg/kg and 60 mg/kg) on serum lactate dehydrogenase and creatine kinase-MB levels against doxorubicin-induced cardiotoxicity in rats

Group	LDH (U/L)	CK-MB (U/L)
CONTROL	845.09±57	624.56±64
DOX	1914.19±62[#]	2133.29±86[#]
DFX	964.69±27[**]	698.96±40[**]
DOX+BMAE30	1477±57[**]	1461.80±47[**]
DOX+BMAE60	1350.4±27[**]	1316.77±29[**]

Each value is represented as mean ± SD. No. of animals (n) = 6. [#]P<0.01 when toxic control compared with control, **P<0.01 vs toxic control. One way ANOVA followed by Dunnett's t-test. CK-MB: creatine kinase-MB; DOX: doxorubicin; LDH: lactate dehydrogenase; DFX: desferrioxamine
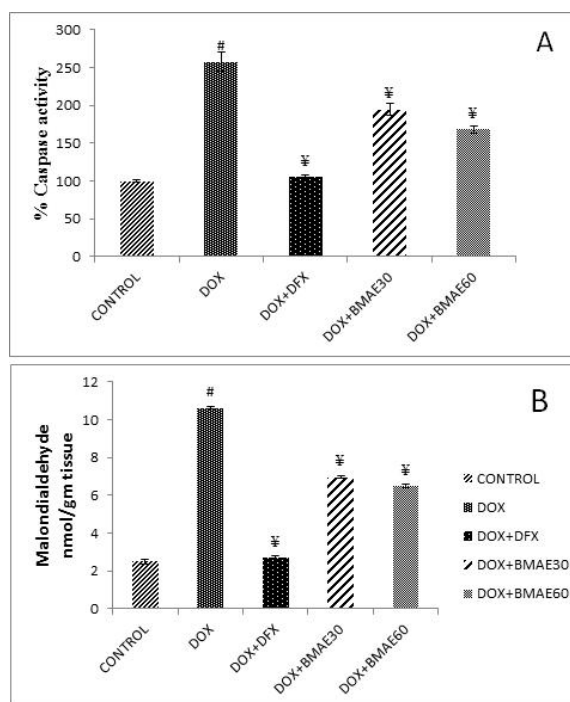
Biochemical estimation

There was marked elevation in the mean serum CK-MB level of DOX treated group as compared to control group (P<0.01 vs CNT). Treatment with BMAE and DFX showed significant reduction (P<0.01 vs DOX) in the CK-MB levels when compared to toxicant group (Table 3). Results of LDH estimation showed significant (P<0.01 vs CNT) elevation in the mean serum LDH level of DOX treated group in comparison to control group. Whereas, groups treated with BMAE and DFX showed significant reduction (P<0.01 vs DOX) in mean serum LDH level as compared to toxicant group (Table 2). The mean tissue MDA and caspase-3 levels (nmol mg^{-1} of protein) of DOX treated group were found significantly higher (P<0.01vs CNT), which were significantly reversed after treatment with BMAE and DFX (P<0.01 vs DOX) (Figure 3). DOX treated group also showed significant elevation (P<0.01 vs CNT) in serum TNF-alpha, IL-6 and tissue caspase-3 levels as compared to control group. Whereas, DFX and BMAE treated groups at 30 and 60 mg kg^{-1} showed

Figure 3: Effect of BMAE on cardiac caspase-3(**A**) and cardiac malondialdehyde (**B**) on doxorubicin (DOX)-induced cardiotoxicity in rats. Values are mean ± SD (*n* = 6). [#]P<0.01 when toxic control compared with control, [¥]P<0.01 vs toxic control. One way ANOVA followed by Dunnett's t-test.

Table 4: Effect of BMAE (30 mg/kg and 60 mg/kg) on serum tumor necrosis factor-α, interleukin-6 levels against doxorubicin-induced cardiotoxicity in rats

Group	TNF-α (pg/mL)	Interleukin-6 (pg/mL)
CONTROL	0.010±0.0003	82.25±0.95
DOX	0.061±0.001[#]	429.25±8.26[#]
DFX	0.025±0.0007[**]	146.5±4.04[**]
DOX+BMAE30	0.041±0.001[**]	252.5±10.53[**]
DOX+BMAE60	0.040±0.0005[**]	227.25±11.67[**]

Each value is represented as mean ± SD. No. of animals (n) = 6. [#]$P<0.01$ when toxic control compared with control, **$P<0.01$ vs toxic control. One way ANOVA followed by Dunnett's t-test. TNF-α: Tumor necrosis factor; IL-6: Interleukin-6; DOX: doxorubicin; DFX: desferrioxamine

Histopathology

The cardiac tissue samples belonging to CNT, DOX, BMAE and DFX treated groups were examined with special reference to myocardial fiber integrity and histological evidence of DOX induced cardiac damage. CNT group showed normal myocardial structure, whereas DOX treated group showed disarray of myocardial cells with small and large vacuolar myopathy. However, there was no evidence of necrosis of the myocardium. The heart samples from the BMAE treated group at 30 and 60 mg Kg^{-1} day^{-1} as well as STD group showed a normal myocardium with no evidence of vacuolar myopathy (Figure 4).

DISCUSSION

Doxorubicin (DOX) is an excellent antineoplastic agent for treating several types of hematological and solid malignancies (Shah et al., 2012; Pathan et al., 2012). Previously, DOX related cardiotoxicities are well documented; DOX is metabolically reduced to highly reactive free radicals, which generates superoxide and hydrogen peroxide. These highly toxic free radicals cause lipid peroxidation, inhibition of long chain fatty acids (Khan et al., 2014; Nohl et al., 1998) and cause damage to cellular components.

The aqueous extract/decoction of *Bombyx mori* has been used since long in the Unani System of Medicine as a part of many of such formulations for the treatment of heart diseases. Some of the studies have already been reported earlier on alcoholic extract and on cocoon as such (crude) for its hypolipidemic potential (Mahmood et al., 2013), cardioprotective activity against DOX induced cardiotoxicity and isoprenaline induced myocardial necrosis (Kumar et al., 2013; Nazmi et al., 2013). However, effect of *B. mori* aqueous extract in prevention of cardiac toxicity is yet to be established. Hence, we envisaged to evaluate the cardioprotective potential of the standardized aqueous extract (freeze dried and powdered) for scientific validation of its use in traditional formulations as a major cardioprotective agent.

In the present investigation DOX produced a significant cardiotoxicity at the acute dose of (20 mg/Kg/i.p./single dose) in male Wistar rats as evident by increased levels of serum LDH and CK-MB whereas reduced MDA and caspase-3 levels in cardiac tissue. The results were further supported by histopathological studies of cardiac tissue, which is consistent with previous work (Ayla et al., 2011; Mokni et al., 2012) in which significant acute cardiotoxicity was reported in rats 24 h after DOX treatment i.e. 20 mg/Kg/i.p./single dose. In the present study, pretreatment with *B. mori* aqueous extract (BMAE) at a dose of 30 and 60 mg/Kg significantly suppressed cardiac toxicity induced by DOX. The protective effect of BMAE against DOX-induced cardiovascular damage is due to its prominent anti-oxidant activity.

Increased level of different enzymes is possibly a reflection that the DOX treatment induces cardiac tissue damage, whereas LDH and CK-MB are relatively specific for myocardial damage (Yagmurca et al., 2003). In the present study, marked elevation in the activities of LDH and CK-MB in the serum
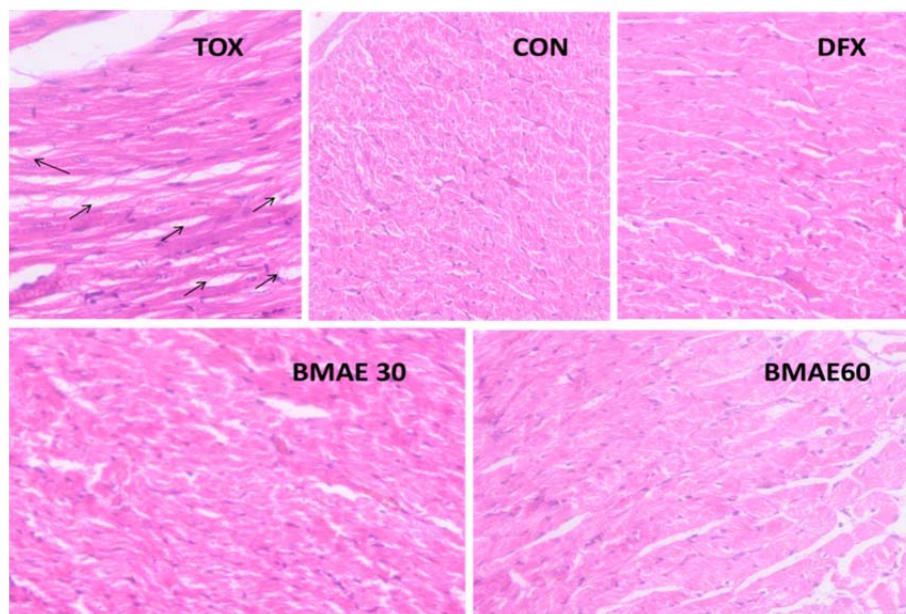
Figure 4: Effect of BMAE on cardiac tissue against doxorubicin (DOX)-induced cardiotoxicity in rats. Representative photographs of cardiac tissues are stained with hematoxylin and eosin, ×100. (CON): A representative section from control group showing unremarkable changes. (DOX): Sections from DOX-treated group showing myocardial degeneration (arrow in DOX) and perinuclear vacuolization. Histological improvement of lesions is seen in both BMAE 30 group (3) and BMAE 60 group (4) as well as in DFX group (5).

of doxorubicin intoxicated rats were observed ($P<0.01$), which are in agreement with Abdel-Sattar and colleagues (2012). Shah and colleagues (2012) who reported significant increase in serum LDH and CK-MB level after a single dose of doxorubicin 20 mg/Kg. The rise in serum LDH and CK-MB level suggests an increased leakage of this enzyme from cardiac tissues into the systemic circulation after DOX treatment. However, treatment of rats with BMAE (30 and 60 mg/Kg), LDH and CK-MB level was decreased to a level near to that of the control group. This protective effect could be attributed due to the antioxidant property and presence of various amino acids and flavonoids in *B. mori*. Significant increase in MDA content (an index of lipid peroxidation) in cardiac tissues, of DOX induced rats has been observed, which correlates with the previous studies (Fard et al., 2010; Hadi et al., 2012; Abdel-Wahab et al., 2003). The pretreatment with BMAE decrease the oxidative stress and lowers the level of MDA in cardiac tissues indicating decrease in oxidative stress and lipid peroxidation.

Free radicals may directly disrupt lipid membranes and they may also mediate the activation of genes for some pro-inflammatory cytokines (TNF-α, IL-1, IL-6) (Schreck and Baeuerle, 1991). In our study, rise in the levels of inflammatory cytokine (TNF-α, IL-6) ($P<0.01$) was found in DOX treated group as compared to control group which are in agreement with that of previously reported work (Elbaky et al., 2010; Bien et al., 2007; Moore et al., 2001). Pretreatment with BMAE, significantly lowered ($P<0.01$) the levels of TNF-α and IL-6, which may be due to its antioxidant property.

DOX treatment increased the production of free radicals and activates the caspase-3, a major effector caspase that plays a critical role in the apoptotic cascade. In the present investigation increase in the caspase activity in cardiac tissue of DOX treated rats was observed which supports the results of Yang and colleagues (2006), Ueno and colleagues (2006), who reported increase in caspase-3 activity in DOX induced cardio-toxicity. Pretreatment with BMAE before DOX admin-

istration lowers the cardiac caspase-3 levels by inhibiting apoptosis.

The results of present investigations validated scientifically its use in various Unani formulations such as Khamira Abresham Hakim Arshadwala, Khamira Abresham Sada etc. which is in agreement with earlier reports on Khamira's for cardioprotection (Goyal et al., 2010).

Histological observations further confirm the DOX induced disarray of myocardial fibers and vacuolization. These deleterious changes seem resistent on pretreatment with BMAE. These observations provide convincing microscopic evidences regarding the cardioprotective potential of BMAE. Parameters investigated here indicated that aqueous extract of BM is potent in alleviating DOX induced myocardial damage and amelioration of heart vacuolation. The observed results have been attributed to its free radicals scavenging capability, and to regulate the generation of inflammatory mediators.

CONCLUSION

On the basis of these findings it can be concluded that aqueous extract of BM has cardioprotective potential. This may be due to high content of amino acid and flavonoid in BMAE. The mechanism of cardioprotection may involve reduction in apoptotic factor (caspase-3 and TNF-alpha), oxidative stress (MDA), cardiac enzyme activity (CK-MB and LDH) as well as proinflammatory (interleukin-6) marker.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Abdel-Sattar E, El-Gayed SH, Shehata I, Ashour OM, Nagy AA, Mohamadin AM. Antioxidant and cardioprotective activity of Stachys schimperi Vatke against doxorubicin-induced cardiotoxicity. Bull Fac Sci Cairo Univ 2012;50:41-7.

Abdel-Wahab MH, El-Mahdy MA, Abd-Ellah MF, Helal GK, Khalifa F, Hamada FMA. Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. Pharmacol Res 2003;48:461–5.

Ahmad S, Rehman S, Ahmad AM, Siddiqui KM, Shaukat S, Khan MS et al. Khamiras, a natural cardiac tonic: An overview. J Pharm Bioallied Sci 2010;2:93.

Amir M, Khan A, Mujeeb M, Ahmad A, Usmani S, Akhtar M. Phytochemical analysis and in vitro antioxidant activity of Zingiber offcinale. Free Radic Antioxid 2011;1:75-81.

Ayla S, Seckin I, Tanriverdi G, Cengiz M, Eser M, Soner BC et al. Doxorubicin induced nephrotoxicity: protective effect of nicotinamide. Int J Cell Biol 2011;2011:390238.

Belur B, Kandaswamy N, Mukherjee KL. Laboratory technology – A procedure manual for routine diagnostic tests. Delhi: Tata McGraw Hill Co., 1990.

Bien S, Riad A, Ritter CA, Gratz M, Olshausen F, Westermann D et al. The endothelin receptor blocker bosentan inhibits doxorubicin-induced cardiomyopathy. Cancer Res 2007;67:10428-35.

Elbaky NAA, Ali AA, Ahmed RA. Cardioprotective effect of simvastatin on doxorubicin-induced oxidative cardiotoxicity in rats. J Basic Appl Sci 2010;6:29-38.

Fard MH, Naseh G, Bodhankar SL, Dikshit M. Cardioprotective effect of Lagenaria siceraria (Molina) Standley (Cucurbitaceae) fruit juice on doxorubicin induced cardiotoxicity in rats. Am J Pharmacol Toxicol 2010;5:103-8.

Goyal S, Siddiqui MK, Siddiqui KM, Arora S, Mittal R, Joshi S et al. Cardioprotective effect of 'Khamira Abresham Hakim Arshad Wala', a unani formulation in isoproterenol-induced myocardial necrosis in rats. Exp Toxicol Pathol 2010;62:61-74.

Hadi N, Yousif NG, Al-Amran FG, Huntei NK, Mohammad BI, Ali SJ. Vitamin E and telmisartan attenuates doxorubicin induced cardiac injury in rat through down regulation of inflammatory response. BMC Cardiovasc Disord 2012;12:63.

Haenen GRMM, Janssen FP, Bast A. The antioxidant properties of five (O-_hydroxyethyl) rutosides of the flavonoid mixture. Venoruton Phlebology 1993;1 (Suppl):10–7.

Helle M, Boeije L, Groot ED, Vos AD, Aarden L. Sensitive ELISA for interleukin-6: Detection of IL-6 in biological fluids: synovial fluids and sera. J Immunol Meth 1991;138:47-56.

Iqbal M, Dubey K, Anwer T, Ashish A, Pillai KK. Protective effects of telmisartan against acute doxorubicin-induced cardiotoxicity in rats. Pharmacol Rep 2008;60:382-90.

Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase 3 (CPP32)-like proteases is essential for TNF- α-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol 1998;160:3480-6.

Julia P, Young HH, Buckberg GD, Kofsky ER, Bugyi HI. Studies of myocardial protection in the immature heart. II. Evidence for importance of amino acid metabolism in tolerance to ischemia. J Thorac Cardiovasc Surg 1990;100:888-95.

Kabeer U. Bayaaz-e-Kabeer, 15th ed. (pp 66-8). New Delhi: Daftarul Miyah, 1951.

Kabelová I, Dvorakova M, Cizkova H, Dostálek P, Melzoch K. Determination of free amino acids in cheeses from the Czech market. Czech J Food Sci 2009;27:143-50.

Khan MA, Singh M, Khan MS, Ahmad W, Najmi AK, Ahmad S. Alternative approach for mitigation of doxorubicin-induced cardiotoxicity using herbal agents. Curr Clin Pharm 2014;9:1-10.

Kumar SR, Hussain SH, Mahmood T, Ahsan F. Evaluation of cardioprotective effect of silk cocoon (Abresham) on isoprenaline-induced myocardial infarction in rats. Avicenna J Phytomed 2013;3:216-23.

Kurioka A, Yamazaki M. Purification and identification of flavonoids from the yellow green cocoon shell (Sasamayu) of the silkworm, *Bombyx mori*. Biosci Biotechnol Biochem 2002;66:1396.

Lehmann GL, Carreras FI, Soria LR, Gradilone SA, Marinelli RA. LPS induces the TNF-alpha-mediated down regulation of rat liver aquaporin-8: role in sepsis-associated cholestasis. Am J Physiol Gastrointest Liver Physiol 2008;294:G567-75.

Liu X, Chen Z, Chua CC, Ma YS, Youngberg GA, Hamdy R et al. Melatonin as an effective protector against doxorubicin-induced cardiotoxicity. Am J Physiol Heart Circ Physiol 2002;283:H254–63.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

Lum G, Gambino SR. A comparison of serum versus heparinized plasma for routine chemistry tests. Am J Clin Pathol 1974;61:108-13.

Mahmood T, Siddiqui HH, Dixit R, Hussain MS. Effect of Bombyx mori L. cocoon (Abresham) and its polyherbal formulations against high fat diet induced dyslipidemia, obesity and other related ailments: Comparative study with Atorvastatin and Orlistat. J Nat Pharmaceut 2013;4:86-92.

Malisza KL, Hasinoff BB. Production of hydroxyl radical by iron (III)–anthraquinone complexes through self-reduction and through reductive activation by the xanthine oxidase/hypoxanthine system. Arch Biochem Biophys 1995;321:51–60.

Mokni M, Hamlaoui-Guesmi S, Amri M, Marzouki L, Limam F, Aouani E. Grape seed and skin extract protects against acute chemotherapy toxicity induced by doxorubicin in rat heart. Cardiovasc Toxicol 2012;12: 158-65.

Mondal M, Trivedy K, Kumar SN. The silk proteins, sericin and fibroin in silkworm, Bombyx mori Linn. - a review. Caspian J Env Sci 2007;5:63-76.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

Nazeyrollas P, Prévost A, Baccard N, Manot L, Devillier P, Millart H. Effects of amifostine on perfused isolated rat heart and on acute doxorubicin induced cardiotoxicity. Cancer Chemother Pharmacol 1999;43:227–32.

Nazmi AS, Ahmad SJ, Rashikh A, Akhtar M, Pillai KK, Najmi AK. Protective effects of Bombyx mori, quercetin and benazepril against doxorubicin induced cardiotoxicity and nephrotoxicity. J Saudi Chem Soc 2013; http://dx.doi.org/10.1016/j.jscs.2013.04.001.

Nohl H, Gille L, Stanick K. The exogenous NADH dehydrogenase of heart mitochondria is the key enzyme responsible for selective cardiotoxicity of anthracyclines. Z Naturforsch C 1998;53:279–85.

Pathan RA, Bhandari U, Javed S, Nag TC. Anti-apoptotic potential of gymnemic acid phospholipid complex pretreatment in Wistar rats with experimental cardiomyopathy. Indian J Exp Biol 2012;50: 117-27.

Schimmel KJM, Richel DJ, van den Brink RB, Guchelaar HJ. Cardiotoxicity of cytotoxic drugs. Cancer Treat Rev 2004;30:181-91.

Schreck R, Baeuerle PA. A role for oxygen radicals as second messengers. Trends Cell Biol 1991;1:39-42.

Schroeder WA, Kay LM, Lewis B, Munger N. The amino acid composition of Bombyx mori silk fibroin and of Tussah silk fibroin. J Am Chem Soc 1955;77:3908–13.

Shah SL, Mali VR, Zambare GN, Bodhankar SL. Cardioprotective activity of methanol extract of fruit of Trichosanthes cucumerina on doxorubicin-induced cardiotoxicity in wistar rats. Toxicol Int 2012;19:167-72.

Sharma H, Pathan RA, Kumar V, Javed S, Bhandari U. Anti-apoptotic potential of rosuvastatin pretreatment in murine model of cardiomyopathy. Int J Cardiol 2011;150:193-200.

Shug AL, Madsen D, Dobbie R, Paulson DJ. Protection of mitochondrial and heart function by amino acids after ischemia and cardioplegia. Life Sci 1994;54:567-77; erratum in: Life Sci 1994;54:2109.

Ueno M, Kakinuma Y, Yuhki K, Murakoshi N, Iemitsu M, Miyauchi T et al. Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. J Pharm Sci 2006;101:151-8.

Vergely C, Delemasure S, Cottin Y, Rochette L. Preventing the cardiotoxic effects of anthracyclines: from basic concepts to clinical data. Heart Metab 2007;35:1-7.

Yagmurca M, Fadillioglu E, Erdogan H, Ucar M, Sogut S, Irmak MK. Erdosteine prevents doxorubicin-induced cardiotoxicity in rats. Pharmacol Res 2003;48:377-82.

Yang S, Thor AD, Edgerton S, Yang X. Caspase-3 mediated feedback activation of apical caspases in doxorubicin and TNF-α induced apoptosis. Apoptosis 2006;11:1987-97.