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Ameliorative Activities of Morus and Jamun against Cr Induced Andro-Hepatic Anomalies

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Chromium (Cr) is a vital micronutrient for sugar metabolism while its unauthorized use may agonize health fatalities. This study was conducted about the novelty about exposure of hexavalent chromium (Cr^{+6}) histopatholgical potentials along with Mulberry Fruit Extract and Jamun Fruit Extract to define the ameliorative aptitude. The histological finding in Cr exposure specify; cessation of testicular seminiferous tubules (ST), annihilation of smooth muscles in basement membrane, Leydig's cell and spermatids. The ST have halted spermatogenesis, with irregular boundary and dead spermatogonia and Leydig's cells are disorganized. The sperm head

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cross sectional area (CSA), tail length and middle piece diameter ($p \leq 0.001$) significantly reduced while there is significant elevation of hepatocytes nuclei size, central vein CSA and mean width of Sinusoidal Spaces as compared to control. The protuberant marks of steatosis, fibrosis, dehydration and atrophy were nullified by designated fruit extracts and their possible bio-chelating effects was obvious due to the presence of anthocyanin, β-sitosterol and phytochemicals. The given results specify that hexavalent Cr induce andro-hepatic anomalies when it is freely used without scientific authentication. Cr^{+6} as food additives in coloring rice and local sweets should be prohibited while bio-products of Morus and Jamun can be recommended and sponsored for traditional medicines.

Keywords: Chromium; steatosis; fibrosis; dehydration and atrophy.

LIST OF ABBREVIATIONS

Abbreviations	Abbreviations details
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMPK	amp-activated protein kinase
AS	attached sperm
AST	aspartate aminotransferase
BUN	Blood Urea Nitrogen
CH	club headed sperm
CSA	cross sectional area
C.V	central vein
Cr	Chromium
Cr-M	chromium+ Mulberry
Cr-J	chromium+Jambul
Cr-MJ	chromium+ Mulberry +Jambul
CRT	Creatine
DS	dislocated sperm
FSH	follicle stimulating hormone
GLU	glucose (sugar)
Hb	Hemoglobin
HDL	high density lipoprotein
He.C	Hepatocytes
H.N	hepatocytic nucleus
JFE	Jambul Fruit Extract
K.C	kupffer cells
LDL	low density lipoprotein
MCV	mean cell volume
MFE	Mulberry Fruit Extract
P. SPER	primary spermatocytes
PBH	parrot beak headed sperm
RBC	erythrocyte count
ROS	reactive oxygen species
S. GONIA	Spermatogonia
SGOT	serum glutamic oxalacetic transaminase
SGPT	serum glutamic pyruvate transaminase
S.S	sinusoidal spaces
ST	seminiferous tubules
TA	total albumin
TG	Triglyceride
TP	total protein
VLDL	very low density lipoprotein
UA	Uric Acid
WBC	white blood cell count

1. INTRODUCTION

The unhygienic heavy metals contaminated water frequently used to irrigate crops and bio-products of such crops cause inevitable anomalies [1]. Metals such as lead, mercury, chromium, arsenic, copper, cadmium, and iron in contaminated water and food supplements generate reactive oxygen species [2,3], affect immune system and body organs such as testes, kidneys and liver [4]. The adequate amount of chromium (Cr) as food supplement enhance endocrine system and ethological activities [5] but their ridiculous use may cause severe injuries on androgen receptors lead hepatic, renal and hematologic anomalies [6]. The elevated androgen block endogenous androgens of Leydig cells [7] and effect testicular seminiferous tubules and hepatocytes which fight against reactive oxygen species (ROS) in liver [8,9].

Many herbs are investigated as source of antioxidants as free radical's scavenger [27] and ameliorative competency against heavy metals [28,29,30] Antioxidant may be synthesized in the body or obtained in the diet; have capability of β-carotene-linoleate and β-sitosterol with reducing power, superoxide, nitric oxide-scavenging capacity and ferrous ion chelating potency [31] and their anthocyanin control lipid peroxidation [32]. The flavonoids and polyphenols in *Trianthema triquetra* [33], *Pisonia aculeata* [34], *Benincasa hispida* and Castor oil [35] are very beneficial in scavenging the free radicals [36] by regulating aspartate aminotransferase, alanine aminotransferase, total serum bilirubin and malondialdehyde in hepatocytes [37].

Mulberry antioxidants with cytotoxic protective activity reduce edema [38], also have ameliorative capability against hyperlipidemia, lipogenesis and fatty acid oxidation [39]. *Morus nigra* have anti-cancerous, anti-inflammatory, antibacterial and anti-fungal, antimicrobial and radical rummaging activities [40]. Their flavonoids quercetin protects from lipid peroxidation [41]. Pharmacologically *Syzigium cumini* fruit extracts protect the cultured human peripheral blood lymphocytes from DNA damage [42] also recommended to treat human breast cancer, repair hepatocyte from hydrogen peroxide injuries and gamma-irradiations [43]. Their anthocyanins are anti-spasmodic [44] can ameliorate hepatic enzymes to moderate serum ALT and AST against methylmercury [45]. The flavonoids in litchi (*Litchi chinensis*) are effective against cancer [46], *Viola odorata* for asthma [47], red clover for hot flashes/menopausal symptoms [48], green tea for the risk of prostate problems [49], cactus (*Opuntia ficus-indica*) cladodes on methotrexate-induced oxidative

Table. 1. Plants phytochemical compounds used for bio-chelation

Plant	Compound	Bio-chelation	References
Fruit extract	Anthocyanin	Fe	[10,11]
Mulberry extracts	phenolics, β-sitosterol and anthocyanins	CCl_4 and heavy metals	[12,13]
Morus alba	Phytochemicals	CCl_4	[14]
Syzygium cumini	Antioxidant, anthocyanin Flavonoid	Nitric oxide, Fe, CCl_4 Fe, methylmercury	[15,16]
Moringa oleifera *Hippophae rhamnoides*	Phytochemicals	Cr	[17,18]
Plants, fruits, vegetable	Vitamins E/C	Heavy metals Pb	[19,20]
Mangifera indica Red cabbage	Phytochemicals	Fluoride Heavy metals	[21,22]
Plants *Salvia plebeia*	L-carnitine Flavonoid	CCl_4	[23,24]
Plants	Ascorbic acid, tocopherol	Arsenic	[25]
Plants derived drug	Deferiprone	Fe, Al	[26]

damage [50] and chamomile (*Matricaria recutita*) as antioxidant and anticancer activities [51]. The enzymatic changes and gene expressions are almost same in humans and rodents [52] so mice mammalian model was selected for this study, and the cheapest economical fruit Morus and Jamun were selected to probe the poisonous effects of heavy metals and shield against andro-hepatic anomalies at micrometric level.

2. MATERIALS AND METHODS

The study was conducted on 25-30g/3-4months old 50 male albino laboratory mice (*Mus musculus*), kept at 26±4 C˚/45% relative humidity with 12-hr L/D cycles throughout experimental duration.

2.1 Preparation of Solution and Fruit Extracts

Dose for various groups were prepared from pure Potassium dichromate (K_2 Cr_2 O_7), stock solution of 1000ppm and diluted up-to 50ppm required solutions. Ripe black berry of *Morus nigra* and *Syzygium cumini* were washed, and 100g of their pulp was blended in an electric juicer in 100ml cooled boiled drinking water for 5minutes and finally centrifuged at 500rpm for 10minutes. The supernatant was immediately placed in sterilized 5ml capacity ice-cube dishes, store in sterilized plastic bags at -30^{o}C and a fresh thawed cube was used for each experiment at room temperature.

2.2 Experimental Groups

Animals were randomly divided into 5 groups (n=10) as: Control group (C); provided boiled cooled mineral water for 15days, Cr-group (Cr); provided 50ppm Cr-solution for first 10days at *ad libitum* followed by simple mineral water for next 5days, Cr +Mulberry group (Cr-M); as Cr group but the last 5days they were given MFE, similarly Cr +Jamun group (Cr-J) given JFE and Cr +Mulberry+Jamun group (Cr-MJ) were given equally mixed MFE and JFE through gavage as post-treatment 0.25ml/12hrs for next 5 days.

2.3 Histological and Micrometric Analysis

The animals were euthanized by cervical dislocation and dissected out on day 16th for histological hematoxylin and eosin (HE) staining, testicular smear formation and micrometric analysis following [74] protocol. Photograph at 100×, 400× and 1000× with 7.2 MP digital camera (Sony) were processed in CorelDRAW11 for micrometry. The results were expressed as mean ± SD and the data was analyzed through ANOVA (two factors, without replication) and Duncan's Multiple Range Test (post hoc analysis).

3. RESULTS

In control group testes of animals specify the clear boundaries of seminiferous tubules (ST) with integral basement membranes, spermatogonia (Sp.g) with multiple whirls of spermatocytes (Sp.c) and secondary spermatocytes (Se.c). The sperms with parrot beak headed (PBH) and club headed (CH) along with prominent elongated tail (Sp.T) were clearly visible. All whirls are equally and systematically are arranged and there was no sign of anomalies in all animals of control group (Fig. 1).

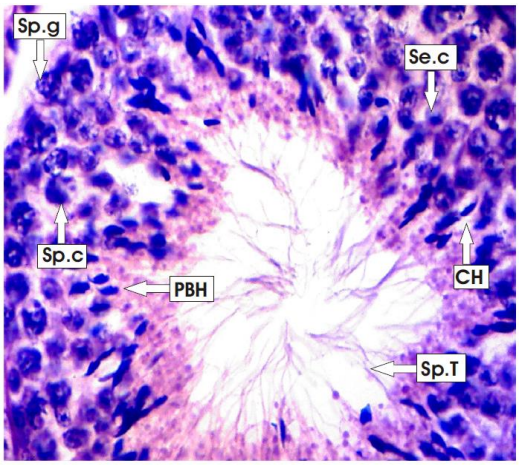

Fig. 1. Mice Seminiferous Tubule, control group

Sp.g; spermatogonium, Se.c; sertoli cell, Sp.c; spermatocyte, PBH; parrot beak headed spermatozoa, CH; club headed spermatozoa, Sp.T; Spermatozoa Tail

The seminiferous tubules (ST) in animals without Cr treatment were symmetrical equally distributed rounded with prominent Leydig's cells (Fig. 2-a), spermeogenesis (Fig. 2-b) and spermatozoa with elongated tails directed towards the lumen (Fig. 2-c). The spermatogonium with prominent dark stain nuclei without vacuolation and cracks (Fig. 2-d). In Cr treated group there was breakdown of basement membrane, destruction of smooth muscles, Leydig's cell (Fig. 2-e) and spermatid without spermeogenesis (Fig. 2-f). The tailless sperm (Fig. 2-g) and debris filled in ST lumen appear like vortex (Fig. 2-h). The ST with halted spermatogenesis, with irregular boundary and basement membrane loses inter-tubular junction (Fig. 2-i). The dead spermatogonia and Leydig's cells were disorganized (Fig. 2-j, l) and whirls of primary and secondary spermatocytes lost their regular symmetry (Fig. 2-k).

Morus as post-treatment ameliorate the basement membrane of ST as shown in Fig. 2-CrM-m. There are prominent Sertoli cells (Fig. 2-CrM-n) after treatment indicate clear systematic spermatogenesis (Fig. 2-CrM-o). The regular whirls of Sp.g, Se.c, and Sp.c, with healthy PBH, CH and Sp.T (Fig. 2-CrM-p in Morus post-treatment specify prominent recovery of testicular anomalies.

Similarly Jamun improve the Leydig's cell, basement membrane along with smooth muscles around vacuolated regions (Fig. 2-CrJ-r). There is reorganization of spermeogenesis (Fig 2-CrJ-s) and improvement in primary and secondary spermatocytes whirls (Fig. 2-CrJ-t,u) after Jamun treatment. The Morus and Jamun co-treatment synchronized their effects by reducing vacuolated cells and removing debris. The re-organizing cellular texture and symmetrical whirls of ST (Fig. 2-CrJM-w,x,y,z) also intimate the ameliorative aptitude.

The testicular and seminal vesicle smear of normal animals as control group with PBH along with long tail; without any kink (Fig. 3-1, 5). In Cr treatment sperm show kink in their tails and weak connection b/w sperm head and tail (Fig. 3-2,7); sperm heads are frequently distributed specify halted spermatogesis and spermeogenesis (Figs. 3-8,9), while slides (Figs.3-3,4,6) indicate Morus and Jamun amelioration.

3.1 Micrometric Histological Study of Mice Testes

3.1.1 Sperm micrometric study

The mean CSA of head, length of tail and diameter of mid piece was measured from testicular smear as;

3.1.2 Micrometric histological study of mice liver

The cross sectional of liver indicate destruction of hepatocytes in Cr treated group (Fig. 4). There is significant ($p \leq 0.001$) elevation of hepatocytes nuclei size, central vein CSA and mean width (µm) of Sinusoidal Spaces (S.S) after Cr exposure. The mean number of hepatocytes and relative area occupied by hepatocytes per unit area were significantly ($p \leq .0001$) reduced in Cr treatment as compared to control.

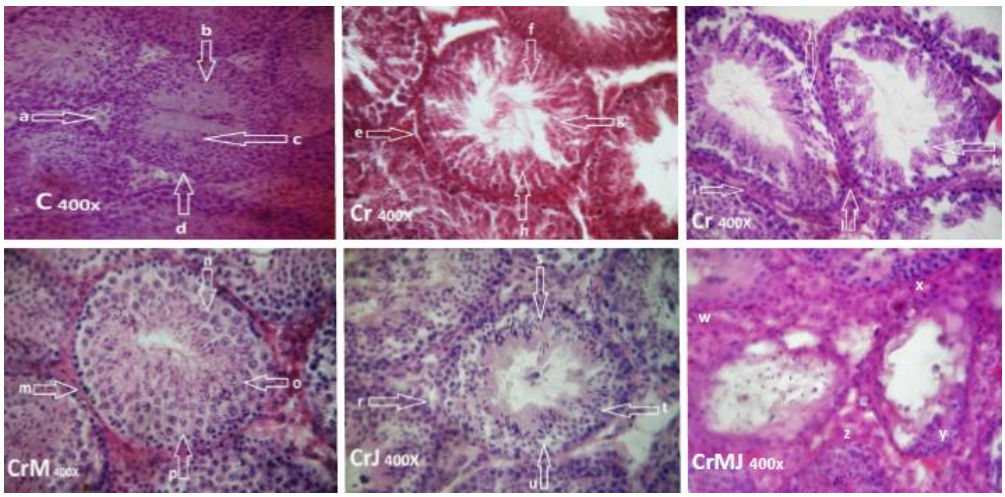

Fig. 2. Histological sections of mice testis in control (C), Cr treated (Cr), Morus (CrM), Jamun (CrJ) and combine co-postreatment of Morus and Jamun (CrMJ) groups

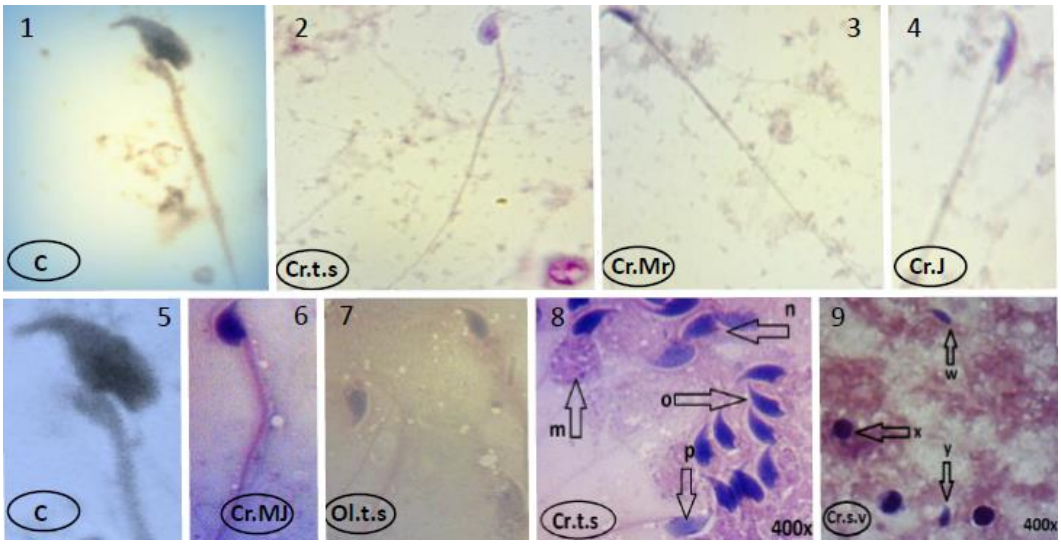

Fig. 3. Testicular smear with sperm morphology in control, Cr-treated and fruit extracts groups
C; control, Cr; chromium, t.s; testicular smear, Mr; Morus, J; Jamun, ol; oil emulsion, s.v; seminal vesicle fluid

Table. 2. Sperm micrometry, ameliorative effects of fruit extracts on Cr induced anomalies

Parameters	Groups				
	C	Cr	Cr-M	C-J	Cr-MJ
PBH CSA(μ^2) of head ***	† 13.91±0.64^{a}	7.86±0.65^{b}	15.39±0.74^{a}	12.89±1.25^{a}	15.78±1.59^{a}
PBH tail length (μ)*	85.57±12.11^{a}	75.92±12.05^{a}	102.10±5.45^{b}	85.99±3.25^{a}	86.84±1.70^{a}
PBH Middle piece. DM (μ)**	0.08±0.06^{a}	0.58±0.09^{b}	0.75±0.05^{c}	0.95±0.14^{a}	1.05±0.05^{d}
CH CSA(μ^2) of head*	11.37±0.98^{a}	9.16±1.85^{a}	14.05±4.05^{a}	15.7±7.04^{a}	11.19±0.73^{a}
CH tail length(μ)*	81.07±5.55^{a}	56.27±14.43^{b}	86.28±16.89^{a}	87.46±5.55^{a}	67.51±10.90^{ab}
CH Middle piece. DM (μ)*	0.96±0.12^{a}	0.39±0.15^{c}	0.78±0.61^{ac}	0.67±0.08^{bc}	1.25±0.35^{d}

*C: control. Cr: chromium treated, Cr-M: chromium+MFE, Cr-J: chromium+JFE, Cr-MJ: chromium+MFE+JFE. CSA: mean cross- sectional area of (PBH) sperm, DM: diameter (thickness) of parrot beak headed sperm and club headed (CH) sperm mid piece at 400X. * : p ≤ 0.05-0.01, * * : p ≤ 0.001 * * * : p ≤ .0001 {Statistical analysis (ANOVA: two factors without replication)} , † group means ±SEM, μ = μm, $^{a\,b\,c}$: Anyone two groups not sharing a lower case letters differ significantly from each other (Duncan's Multiple Range comparison- post hoc analysis), n=10*

4. DISCUSSION

Cr as environmental toxicant produce ROS and induce andro-hepatic anomalies evident by presence of necrosis and steatosis in testes and liver due to β-oxidation which divert metabolic pathway towards lipogenesis [53]. That process effect hepatocytes and kuffer cells to induce cirrhosis, dehydration and fibrosis. The lipid peroxidation interrupt steroid level and elevate testosterone. The ketonic bodies from fatty acids also drop pH which induce atrophy and biochemical changes (Figs. 2, 4) as Cd induce atrophy, testicular architecture disorganization and germinal epithelium disruption [54]. These process increases the acidity which ruptured the endothelial lining of testes indicate the sloughing and degeneration by the exposure of Cr as debris and dislodge spermatid in the lumen of ST, which altered their diameter which exactly co-related with [55] study about Cd toxicity. The primary hypertrophy with Cr exposures seems to cause accumulation of undifferentiated cells due to the lack of meiosis and spermatogenesis to produce spermatogenic arrest [56]. The necrosis of sertoli cells leading to the dislodgment of dead spermatic cells in ST due to the deficiency of nutrition, evident by significant elevation of CSA of ST may cause testicular hypoplasia and destruction of Leydig's cell; may cause infertility

[57]. ROS by Cr exposure damage mitochondrial membrane permeability with deficiency /insufficiency of sugar metabolism which causes the depletion of germinal epithelium in the ST and destruction of ST basement membrane (Fig. 2). Sertoli cells are interconnected by tight junctions; for blood-testis barrier, which temporarily permits the passage of spermatogenic cells and the loss of interstitial cells affects the process of spermatogenesis and spermiogenesis. Cr exposure detach the sperm heads and significant elevation of tail-less spermatozoa specify androgen receptor deformities during terminal spermatids differentiation (Fig. 3, Tab; 2). The rodent sperms have one or more apical regular symmetrical hooks [58]. After Cr exposure the spermatozoa show the prominent anomalies like sperm head with irregular and wavy intermediate appearance (Fig. 3). Micrometric analysis (Tab; 2) of spermatozoa specified the alterations in CSA of sperm head, thickness of mid-piece, and length of the tail, that can be extrapolated with Cr related DNA damage and loss of polymerization of the micro tubular array into microtubules of the sperm tail [59]. The anabolic androgenic steroids can be ameliorated by drugs and the pharmacological products of plants like anthocyanin, β-sitosterol and α-Tocopherol (vitamin E). Phytochemicals are the most important lipid-soluble antioxidants normalize chain reactions of lipid peroxidation to scavenge the free radicals [60]. The phytochemicals are best natural sugar and cholesterol regulators as compared to synthetic drugs [61] in the same token synchronized effect of Morus-Jamun ameliorate and improve the histological and micrometric cellular organization of testis and liver.

Apart from hepato-lipogenicity, atrophy, dehydration, fibrosis and hepatocytic necrosis reported in this study oxidative stress contributed by Cr exposure. The defense against free radicals is associated with activities of SGOT, SGPT and bio-chelation of heavy metals from body. The environmental chemical exposure in the induction and progression of various diseases is significant [62]. The hyperlipidemia may indicate anomalies of anterior pituitary, liver-biliary dysfunction, and lipoprotein lipase cofactor deficiency corticosteroids [63]. LDL cholesterol and excess lipid with rise in HDL and VLDL damage liver and cause hepatic steatosis and displace the nucleus towards periphery. Histological studies of liver at micrometric level indicate the alteration central vein CSA in Cr treated group, due to accumulation of debris by

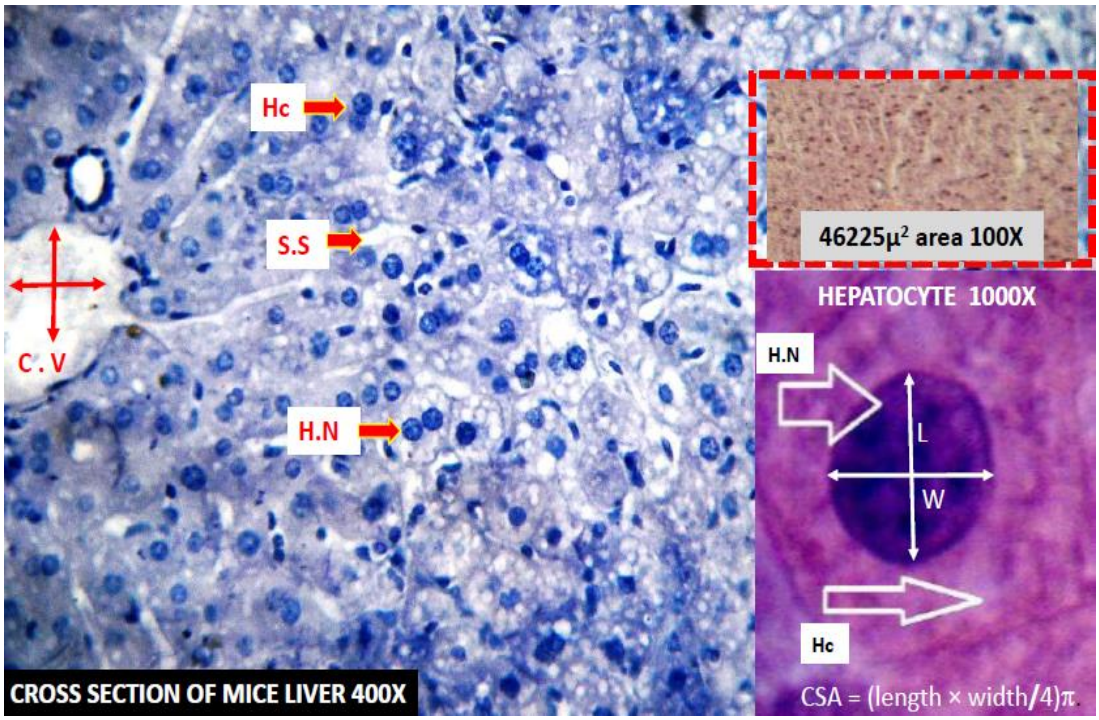

Fig. 4. Histology of Liver

The cross sectional of liver indicate hepatocytes, their nuclei and central vein CSA, Mean width (µm) of Sinusoidal Spaces (S.S) at (400×), Mean number of hepatocytes (N. Hepat) in 46225µ² area (100×), Mean relative area occupied by hepatocytes (R.A.Hepat) in 46225µ² area (100×)

Table 3. Micrometry of mice liver, ameliorative effects of fruit extracts on Cr induced anomalies

PARAMETERS	GROUPS				
	C	Cr	Cr-M	Cr-J	Cr-MJ
CSA (μ^2) Hc***	† 237.95±12.56^{a}	187.39±19.19^{b}	143.14±5.58^{c}	171.95±8.59^{b}	228.76±10.78^{a}
CSA (μ^2) H.N***	32.59±1.29^{a}	43.98±1.17 ^{b}	29.18±1.25^{c}	39.88±1.87^{d}	32.89±0.75^{a}
CSA (μ^2)	2849.14	4213.55	2090.25	2250.24	6748.31
C.V ***	±255.09^{a}	±773.89^{b}	±329.59^{a}	±201.58^{a}	±1466.29 ^{c}
Width(µm)S.S***	6.17±0.63^{a}	7.25±0.83^{b}	5.18±0.66^{c}	7.09±0.67^{b}	5.05±0.26^{c}
N. Hc /area ***	112.06±6.07^{a}	80.64±3.47^{b}	56.75±5.49^{c}	95.13±4.45^{d}	85.89±0.09^{b}
R.A.Hc /area***	26504.77 ±1457.93^{a}	12995.14 ±555.12^{b}	7593.72 ±722.14^{c}	16380.55 ±699.12^{d}	19639.12 ±762.14^{e}

C: control. Cr: chromium treated, Cr-M: chromium+MFE, Cr-J: chromium+JFE, Cr-MJ: chromium+MFE+JFE. Mean cross sectional area (μ^2) of hepatocytes (Hc) at (400×), Mean CSA (μ^2) of hepatocytic nucleus (H.N) at (400×), Mean CSA (μ^2) of central vein (C.V) at (40×), Mean width (µm) of Sinusoidal Spaces (S.S) at (400×), Mean number of hepatocytes(N. Hepat) in 46225μ^2 area (100×), Mean relative area occupied by hepatocytes (R.A.Hepat) in 46225μ^2 area (100×). n=10. Statistical analysis (ANOVA: two factors without replication).: p ≤ 0.05-0.01; **: p ≤ 0.001; ***: p ≤ .0001, † group means ±SEM, $^{a\,b\,c}$: Anyone two groups not sharing a lower case letters differ significantly from each other (Duncan's Multiple Range comparison- post hoc analysis)*

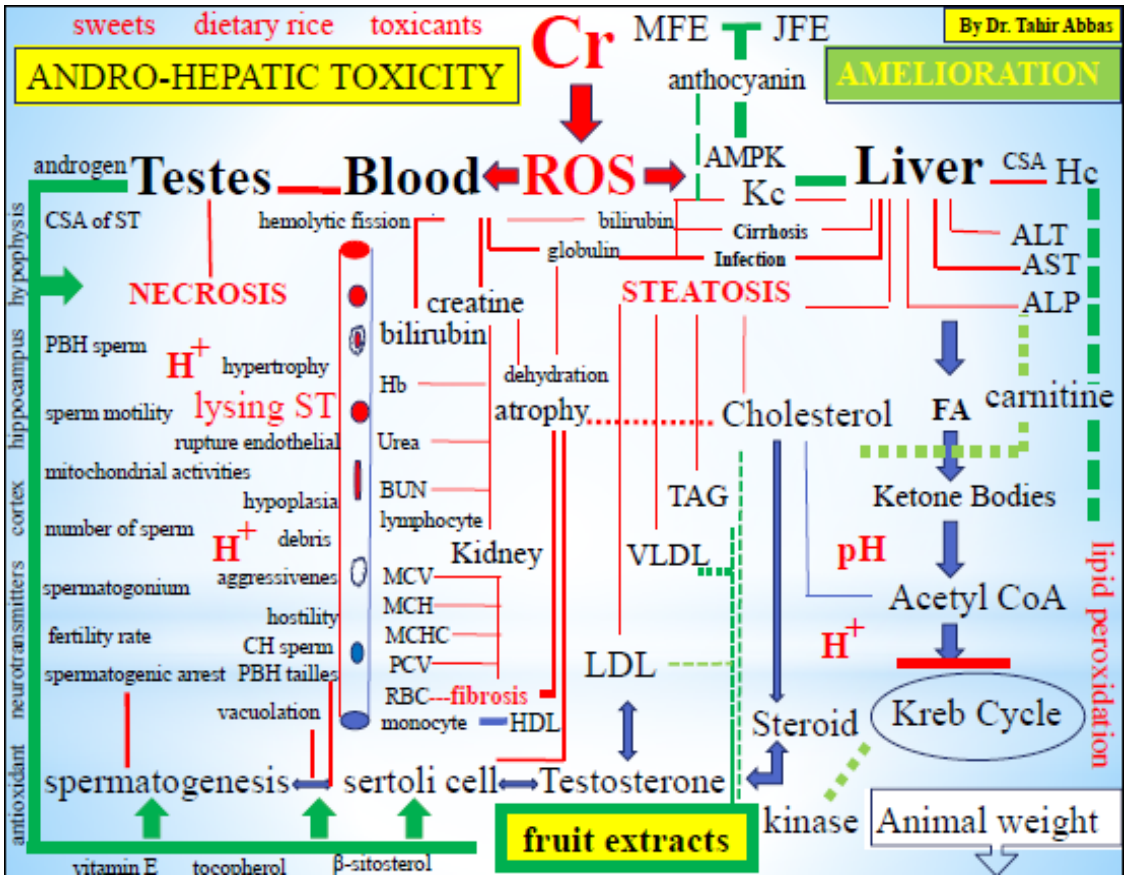

Fig. 5. Comprehensive summary of Ameliorative role of phytochemicals against Cr induced Andro-hepatic anomalies in mice

necrosis. The significant reduction in the CSA of central veins (CV) and number of hepatocytes while the elevation of hepatocytes nuclei size and sinusoidal spaces (SS) clearly intimate liver necrosis [64, 74]. Such changes also specify hypothyroidism, excess glucocorticoid and cortisol due to heavy metals exposure [65]. MFE and JFE like citrus fruits flavonoids protect the membrane integrity during RBC hemolysis [66].

In humans the stomach acts as a site for anthocyanin absorption from food, cyanidin-3-glucoside and cyanidin-3, 5-diglucoside, penetrate into human's blood through liver cells [67]. The anthocyanin-rich extract decreases the lipid peroxidation; to normalize cholesterol by up regulating the peroxisome proliferator-activated receptors and influence the testosterone metabolism to ameliorate hyperglycemia and reproductive anomalies [68].

The ameliorative effect of melatonin also affects the spermatogenic proliferation in ST and metalloid exposure like vitamin-E reduces the oxidative damage induced by Pb [69]. Protective activities of plants (deferiprone) act as an aluminum chelator and nullify the Al-induced toxicity [26]. Chemical compounds isolated from *Morus nigra* like anthocyanin, betulinic acid, flavonoid, β-sitosterol and germanicol, and from *Syzygium cumini* [68] are oleanolic acid, isoquercetin, quercetin, myricetin, tannins, delphinidin, petunidin, malvidin-diglucosides, friedelin, ellagic acid, gallotannin, ellagitannin betulinic acid, kaempferol, gallic acid, myricetine, pinocarveol, cineole, gerany-l-acetone, α-cadinol, eucarvone, muurolol and α-myrtenal have medicinal abilities and can be used in drugs [70].

The heavy metals induced testicular and hepatic histoarchitecture grievances which can be attenuated and chelated by using specific plants extracts as supplements [71]. Traditional medicinal plants are used in amelioration of diseases because plants components like antioxidants [68] have ability to chelate the heavy metals and organs can be recovered (Fig. 5) after chelation. Antioxidant have significant role against Cr induced oxidative stress by efficient chelation and radical scavenging ability during

apoptosis. Natural fruit extracts are without any side effect and never alter the normal cecal microbial composition and intestinal microbiota; essential for carnitine palmitoyl transferase-1 pathway during amelioration [68,72]. The traditional herbal medicines are preferred than modern synthetic drugs and chimeric antigen receptor T-cell therapy against cancer [73,74] along with phytochemicals supplementations will ensure amelioration without side effects in future should be encouraged and sponsored.

5. CONCLUSION

There is no literature available for the co-treatment of Morus and Jamun, but these findings open the door and given results authorized findings about amelioration against heavy metals. The modern allopathic medicines have limited therapeutic options due to their huge serious side effects while the local herbal drugs must be used as an alternative way to cure diseases. Heavy metals like chromium is frequently used in cultural sweets and rice coloring but the hexavalent form should be banned after scientific authentication while Morus (Toot) and Jamun (Jambul) bio-products should be sponsored for clinical recommendation. There is need of another study to find out Morus and Jamun phytochemicals composition for cheapest medicinal formulation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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