Chemopreventive Efficacy of *Punica granatum* and *Silybum marianum* Extracts on Chemically-induced Hepatocellular Carcinoma in Rats

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ABSTRACT----- *Punica granatum (POM) and Silybum marianum (MT) receiving attention as potential potent anti-oxidant and anti-mutant agents. In this context, the present study was designed to highlight their effects either in vitro as well as in vivo model of induced Hepatocellular carcinoma (HCC). Human hepatoma (HepG2 cells) were treated with MT and POM to explore their antitumor activity then in vivo were carried out on thirty-six male albino rats divided into six groups (n=6). Two weeks after induction of HCC, rats were co-treated with either MT or POM ethanolic extract (500 mg/kg, orally) daily for 8 weeks. The results displayed marked reduction in the viability of HepG2 cells with IC_{50} equal to 48.4 and 8.6 µg/mL of POM and MT treatment respectively. Considering, in vivo experiment HCC group displayed significant elevation liver function indices (p<0.05). It also elicited depletion of liver reduced glutathione (GSH), and increased content of liver malondialdehyde (MDA) compared to control group. HCC was proved after a significantly elevated alpha-fetoprotein (AFP) level (p<0.05). All of these measurements were diminished significantly after POM and MT treatments, except the GSH level that was increased significantly. Supplementation of pomegranate and milk thistle extracts had a protective effect against chemically induced HCC.*

Keywords---- Pomegranate, Milk thistle, Chemoprevention, Liver cancer, Rat

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1. INTRODUCTION

The liver takes a fundamental role in order to maintain the performance of many vital functions organisms. Where it represents the chief site for maintenance, and regulating homeostasis of the body [1]. It is mostly prone to chemically induced injury due to its broad metabolic capability and cellular heterogeneity. Excess generation of free radicals can cause oxidative injury to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis [2]. Thus liver diseases pretense a serious challenge to international public health especially liver cancer which represents the sixth most frequently diagnosed cancer worldwide [3]. The vast majority of primary liver cancers, 75%-90%, are hepatocellular carcinomas (HCC), which are malignant tumors of liver parenchymal cells [4]. Sayed-Ahmed et al. [5] reported that Hepatocellular carcinoma represents about 80–90% of all liver cancer and is the fourth most dominant cause of cancer mortality.

Medical plants have always been a good source of new therapeutic properties for human health problems. Recently, a wide range of these plants have been tested for antioxidant and anti-cancer properties [6]. *Punica granatum L.* (Punicaceae), generally called pomegranate, recently termed as nature's power fruit, is a plant used in folk medicine for the management of various diseases [7] . Negi et al. [8] conveyed that pomegranate peel extracts displayed in vitro anti-oxidant and anti-mutant actions due to their content of polyphenols flavonoids and flavones and catechins.

Seeds of milk thistle have been shown to treat liver and gallbladder disorders, comprising hepatitis, fibrosis and jaundice and to conserve the liver against poisoning from chemicals, environmental toxins, poisonous animal's bites and alcohols [9,10]. Due to hepatoprotective effect and antioxidant properties of pomegranate and milk thistle, it is being used

in the current study to determine whether these plant extracts presenting selective growth inhibition of hepatic tumor cell line followed up with *in vivo* testing using chemically induced hepatocellular carcinoma animal model male albino rats for testing their antitumor activity.

2. MATERIALS AND METHODS

Animals

The experiments were carried out with male albino rat, weighing 130-170 g. rats were obtained from National Research Center, Giza, Egypt. The animals were Kept in hygienic condition under natural light and dark program. Placed in well aerated polystyrene cages at room temperature (24±2°C) and were fed on standard laboratory diet. Food and water were given *ad libitum.*

Preparation of Ethanolic extract of pomegranate peel (POM) and milk thistle seed (MT)

The pomegranate peel was manually removed, shade-dried and powdered. Three kilograms of shade dried pomegranate peel and milk thistle, coarsely powdered, charged into aspirator bottles and allowed to soak in absolute ethanol (100%) for 72 hours at room temperature. The extracts were filtered, concentrated using rotary vacuum to get the solid mass. The residue yielded were 16.7 % and 1.16% for pomegranate peel and milk thistle respectively. The concentrate was dissolved in distilled water for oral administration.

In vitro **assay for cytotoxic activity**

Liver cell line Hep G2 obtained from liver biopsy of male Caucasian aged 15 years, received from the American Type Culture Collection, was used. Cells were seeded in 96- well plate. Approximately $1x 10^4$ cells per well in 100µl were cultured and kept under standard conditions (37 °C and 5 % CO_2) in Ham's F-12K medium with L-Glutamine, phenol red and sodium pyurvate supplemented with 10% Fetal Bovine Serum (FBS) and penicillin and streptomycin as antibiotics (Bio Whittaker® Lonza, Belgium) for a period 48 h. Ethanol extract of POM and MT were prepared in 10% DMSO with different concentrations (50, 25, 12.5, 6.25, 3.125 and 1.56 µg/ml), cytotoxic effect of 10% DMSO was tested by inoculating its dilutions on Hep G2 cell line. The anticancer drug doxorubicin hydrochloride was used at the final concentrations (50, 25, 12.5, 6.25, 3.125 and 1.56 µg/ml) respectively. Plates were kept in 5% humidified CO2 atmosphere for next 48 h. After that, cells were trypsinized in 1 mL of trypsin-EDTA solution and counted for number of viable and nonviable cells by staining with trypan blue dye. IC_{50} value of the drug was defined as the concentration of the drug that killed 50% of cells in comparison with the untreated cultures [11,12].

Experimental design for *in vivo* **anti-carcinogenic activity**

A total of 36 adult apparent healthy male albino rats was alienated into six groups of 6 animals each. Rats of Group 1 (control group) were given a single intraperitoneal (i.p.) injection of normal saline. Group 2 (HCC group), the animals treated with single dose of Diethylnitrosamine (DEN (200 mg/kg, I.P.) then intraperitoneal injection of 50% CCl4 were performed 3 times per week for 2 weeks to initiate carcinogenesis according to Ahmed and Tag [13]. Followed by administration of AAF for 2 weeks with 3mg/kg b.wt (3 times per week) as a promoter for hepatocellular carcinoma [14]. Group 3 and 4 were administered with POM and MT daily (500 mg/kg BW). Doses of POM and MT were selected on the basis of doses used by earlier studies [15,16] respectively. Plant extract treatments started 2 weeks after AAF administration and continued for 8week. Animals in Group 5 (POM-supplemented-HCC group) and Group 6 (MT-supplemented-HCC group) treated with DEN, CCL4 and AAF as in group 2 then 2 weeks later the animals treated with POM and MT extract for 8 weeks (500 mg/kg BW, daily). At necropsy, blood was collected from dorsal aorta under anesthesia. After centrifugation, serum was harvested and kept at −20°C until analysis. Liver were dissected free of mesentery, and absolute weight of liver tissue was recorded and normalized with BW (relative weight of liver, i.e., weight of liver per 100 g of BW) of animals. A portion of the liver washed in cold isotonic saline for preparing liver homogenate which were used for determination of hepatic GSH and MDA content.

Determination of biochemical parameters

The activities of Alanine transaminase (ALT) and aspertate aminotransferase (AST) were determined in serum using commercial kit based on 2,4-dinitrophenylhydrazine method [17], Alkaline phosphatase (ALP) based on AACC method described by Point Scientific, INC kit [18]. Gamma glutamyl transferase (γ-GT) was measured using commercial kit (Vitro Scient, Egypt) according to Goldbarg et al. [19]. Determination of total activity of Lactate dehydrogenase (LDH) was by the method of Decker and Lohmann-Matthes [20]. Total Protein (TP) and Albumin Concentrations (ALB) were determined according to methods of Cheesbrough [21]. Bilirubin based on Jendrassic reaction were assayed using reagent kits [22]. Besides, serum alpha-fetoprotein (AFP) concentrations were determined using quantitative sandwich immunoassay kit specific for rat (Kamiya Biomedical Company, Washington, Cat. No. KT-59172).

For estimations of liver Glutathione reduced (GSH) and Liver Lipid peroxide (LPX), hepatic tissue was homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 0.15 M KCl to obtain 25% homogenate (W/V). GSH was estimated by the method of Moron et al [23]. GSH is a non-protein sulphydryl compound. 5,5′-dithiobis (2-nitrobenzoic acid). DTNB is a disulphide compound, which is readily reduced by sulphydryl compound, forming density of this compound was measured at 412 nm. lipid peroxidation was assayed by method of Trush et al. [24]. LPX was measured by formation of Thiobarbituric acid (TBA) reactive metabolites of lipid, such as malondialdehyde (MDA). MDA reacts with TBA to form a pink coloured complex absorbs maximally at 353 nm.

Histopathological studies

For histopathological study, after dissection of animals; liver was isolated washed by normal saline then fixed 10% formalin saline. liver tissue was treated with ascending series of ethanol for dehydration and cleared in xylol for embedding in paraffin wax. Sections (5 μm) of livers stained with hemotoxylin and eosin, were observed microscopically for histopathological studies [25].

Statistical analysis

Data were expressed as the mean ± S.E. Means were compared by one- way analysis of variance (ANOVA) followed by Duncan test, which was used to identify differences between groups. Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

The results of cytotoxicity evaluation for Pomegranate and milk thistle extracts at concentration (50, 25,12.5, 6.25, 3.125 and 1.56 µg/ml) and exposure time 48 hours are summarized in Figure 1. This data displayed that the ethanolic extract of POM and MT showed marked cytotoxicity activity against human cell line of well differentiated hepatocellular. The ethanolic extract of milk thistle showed the highest cytotoxic activity against liver cancer cell line (IC_{50} =8.6 µg/ml) and the ethanolic extract of *pomegranate* was second in effectiveness against cancer cells (IC_{50} =48.4 µg /ml).

Regarding in vivo experiment, a significant reduction in hepatosomatic index (P<0.05) was seen in HCC Group either treated with POM or MT extracts as compared with DEN+CCL4+AAF-intoxicated group (Figure. 2). In the present investigation, hepatoprotective actions of pomegranate and milk thistle extracts at a dose of 500 mg/kg b.wt on chemically induced HCC rats were illustrated in table 1. HCC group showed significant increase in activity of Alanine transaminase (ALT) (+551.5%), aspartate aminotransferase (AST) (+362.5%), and LDH (+630.6%) as well as Bilirubin displayed significant elevation by 9 folds as compared with corresponding control group. Statistically significant decreases in plasma total protein, plasma albumin were identified with percent of change -36.6% and -38.95% respectively versus control. The two tested extracts; POM and MT at a dose 500 mg/kg b.wt. showed non-significant changes as compared to normal group. The current results revealed that administration POM and MT extracts to HCC rats leads to high significant decrease in ALT, AST and LDH activities as well as Bilirubin level as compared with HCC group. POM and MT reduced ALT by 46.48%, 31.96% respectively. Also, SGOT activity significantly decreased by 40.9%, and 29.42%, respectively, as compared with HCC group. The levels of serum bilirubin indicated significant statistical decrease in HCC group treated with POM and MT compared to the HCC group (P < 0.05). There was also a significant statistical difference (P < 0.05) with respect to the activity of serum LDH between the HCC group and HCC rats treated with POM and MT with a percent of change -37.69% for POM and -42.55% for MT as compared with HCC group.

Serum AFP level of HCC were about four times more than that of the normal control group. There was a significant decrease ($P \le 0.05$) of serum AFP level in MT, and POM extracts of HCC rat with percent of change 62.09 % and 68.0% respectively as compared with HCC group (Figure. 3).

The effects of DEN, CCl4 and AAF and plant extract supplementation on the oxidative stress biomarkers in liver tissues are shown in (Figure 4 and 5). Chemically induced HCC rats displayed significant 254.03% increase in LPX and a significant 77.63% decrease in GSH as compared to the control group. Treatment with POM and MT resulted in a significant reversal induced increase in LPX of HCC. Moreover, POM and MT supplementation Significant increase in the depletion hepatic GSH level of HCC group.

Histological examination of hepatic tissue in normal control animals revealed normal hepatocytes architecture with few spaced sinusoids arranged in-between the hepatic cords, while no noticeable changes were detected in livers of POM and MT-treated animals (data not shown). In contrast, chemically induced hepatocellular carcimoma rat's liver sections examination showed loss of normal architecture with oval shaped neoplastic hepatocytes rod cell/sarcomatoid" (Figure 6D and 6G). some degenerated Hepatocytes were had karyolysed nuclei (Figure 6E). Besides, lymphatic infiltrate associated with hepatocyte necrosis were also reported (Figure 6F). Liver sections of POM-HCC group (Figure 6 H-I)

showed that these changes still present as neoplastically transformed cells but generally less than DEN group rats indicated certain improvement as well as MT-HCC group (Figure 6 J-K) which exhibited to some extent normal liver architectures.

4. DISCUSSION

Chemoprevention is a well-known definition in cancer therapy which mean; the use of naturally occurring and/ or synthetic compounds in which incidence of cancer will be entirely prevented, slowed or reversed [26,27]. With the increasing acceptance of chemical diversity of natural products; Bannazadeh et al. [28] stated that drug development will be improved through discovery novel natural products. Medicinal plants play a key role in human health care. Nullification the side effects of allopathic drugs has driven the medical world to take sanctuary in the plant kingdom for the treatment of various diseases [29]. The current study has been planned to study whether Pomegranate and milk thistle supplementation could prevent the development of hepatic carcinogenesis both in vitro and in vivo using chemical induction for hepatic cancer.

Inhibition of cancer growth has been a continuous effort in cancer treatment. A decline in cell growth and promotion of cell death are two major means to inhibit cancerous cells [30]. In this study, we demonstrated that POM and MT extract could cause significant growth inhibition in Hep G2 human liver cancer cell line, in dose dependent manner with IC 50 at about 48.4 and 8.6 µg /ml, respectively. In addition, comparing the effects of POM and MT extract with those of the standard anticancer drug doxorubicin hydrochloride, revealed that the effects of MT extract displayed the same pattern as that of doxorubicin hydrochloride on the cells. Cell death, due to necrosis and apoptosis, was involved using Many medicinal plants as anticancer agent, such as turmeric and green tea [31,32] to cause induction of apoptosis and cell cycle arrest in many types of cancer cells without affecting normal cells. phenolic compounds terpenoids and alkaloids present in pomegranate [34] and milk thistle have been found to stimulate apoptosis in cancer cells [33-35]. Thereby eradicate cancer cells without harming normal body cells.

One of the most widely used animal models for the screening of hepatoprotective drugs against hepatocellular carcinoma is the chemical induction by DEN and AAF [36-38]. The present study validated that HCC group received DEN , CCL4 and AAF displayed increased serum indices of liver function enzymes as well as total Bilirubin and caused emphatic histopathological injuries in liver tissues. These results were in accord with the results obtained from earlier studies Hayashi et al.[40] ; Taha et al.[41] and Adesanoye et al. [42] who recorded an elevation in liver enzymes after administration of DEN , CCL4 and AAF. The elevation in serum liver function indices observed in the HCC group could be due to loss of structural integrity of hepatic tissue; liver enzymes are cytoplasmic compartment of hepatocytes; which are liberated to the blood after hepatic degeneration [39].

Administration of POM and MT after chemical induction of hepatocellular caused significant decrease in hepatic indices as compared with HCC group. This finding indicates that probably POM and MT preserve the architecture of hepatic cells and prohibited additional hurt to the liver parenchyma by virtue of which the leakage of these enzymes into the circulation was decreased. These results are in agreement with Toklu et al [43] who studied the effect of chronic administration of pomegranate on liver fibrosis induced by bile duct legation in rats and found that serum ALT and AST were significantly decreased by POM treatment. These indicate that pomegranate peel extract preserved the structural integrity of the hepatocellular membrane and liver cell architecture which is confirmed by histopathological studies [44].

Regarding effect of MT (*Silybium marianum*) as chemopreventive agent as the present result displayed improvement in hepatic indices; which is in agreement with Pradhan and Girish [45] who proclaimed that silymarin's hepatoprotective effects are achieved through several mechanisms comprising scavenging of reactive oxygen species, antioxidation and inhibition of lipid peroxidation. Moreover, Post-White et al. [46] reported that milk thistle promote liver detoxification via reticence of Phase I detoxification, improve xenobiotic biotransformation and maintenance of glutathione level in tissue [47].

The present study shows a significant rise in the levels of albumin, total protein whereas the concentration of direct bilirubin in serum was significantly decreased following the treatment of the HCC rats with either POM or MT extracts as compared with HCC group which exhibited depletion in both total protein and albumin; besides elevation in level of bilirubin. This result is in concordance with the study of Chtourou et al. [48] who studied the effect of silymarin from milk thistle on hepatic damage induced by manganese intoxication in albino rats. The study of Kaur et al. [49] on the assessment of the hepatic effects of pomegranate also agrees with the present findings. This may be due the potent antioxidant found in the tested extracts, mainly phenols, flavonoids and flavones.

Monitoring the level of LDH as well as alpha-fetoprotein (AFP) in serum was selected as a marker for monitoring and prognosis of cancer. A significant increase in the level of both LDH (approximately 6 -fold) and AFP were detected in the chemically induced hepatocellular carcinoma group. This observation depicted cellular injury that may attributed to the loss of membrane integrity; besides cell proliferation due to DEN, CCl4 and AAF treatments. Our result was consistent

with Kujawska et al. [50] who concluded that DEN is a hepatotoxin and promoter in hepatocarcinogenesis which caused increase in serum LDH level in rats. Regarding AFP as a potent marker for hepatocellular carcinoma Macri [51] stated that Alpha-fetoprotein (AFP) testing is useful in the diagnosis of hepatocellular carcinoma (HCC). A significant decrease was observed in both LDH activity and AFP level in HCC group treated with POM and MT when compared with HCC group.

Regarding the hepatosomatic index of the rats during the in vivo experiment, we observed that the rats of the cancer group (HCC group) showed significant increase in hepatosomatic indices when compared to normal group rats, whereas HCC animals treated with either POM or MT showed significantly reduced hepatosomatic indices when compared to HCC group rats. these findings may be due to significantly downregulated the elevation of serum AFP levels in rats treated with MT or POM.

The liver malondialdehyde (MDA) concentrations significantly ($P < 0.05$) increased in the untreated DEN/CCl4/AAF rats indicating hepatic lipid peroxidation. There was also significant ($P < 0.05$) reduction in the hepatic tissue glutathione (GSH) concentrations. The present results were in agreement with Sayed-Ahmed et al. [5] who reported that model of chemically induced hepatocellular carcinoma using DEN; characterized by generation of reactive oxygen species and depletion antioxidant enzymes in liver tissues. Michiels et al. [52] had been described that tumor promotion resulted from generation of reactive oxygen species which destroy critical macromolecules including lipids and DNA. Moreover, Husain Kha. [53] stated that CCl4 is well known to generate free radicals, disturbing the antioxidant status and ultimately leading to oxidative stress and carcinogenesis. Malondialdehyde (MDA) reported as a potent toxic product which plays an important role in carcinogenesis [54]. The present work showed that HCC group treated with POM and MT displays significant reduction in MDA in correspondence with elevation of hepatic GSH. This results could be explained by Orak et al. [55] and Chalfoun-Mounayar et al. [56] who reported that pomegranate is among the richest fruit in antioxidants of the polyphenolic class which includes tannins and anthocyanins. As investigated by Middleton et al. [57], Polyphenols like catechin or quercetin exhibited antioxidant activities and directly scavenge ROS, which can be very injurious causing lipids, proteins and DNA damage [58,59]. Furthermore, polyphenols and quercetin in particular, can chelate metals like iron involved in free radical formation [60]. Indirectly, polyphenols can inhibit cellular detoxification systems, and enhance antioxidant enzymes [61]. Besides, polyphenols can inhibit enzymes generating ROSas xanthine oxidase and nicotinamide adenine dinucleotidephosphate (NADPH) oxidase [62]. Because of its potent antioxidant activity, pomegranate considers one of the commonly used natural antioxidants. Pomegranate fruit, juice, and peel extracts is a rich source of polyphenols and hence possess potent antioxidant properties [63,64]. The effectiveness and safety of its isolated antioxidants have been tested [65]. Chidambara et al. [66] added that methanolic extract of the peel has shown a higher antioxidant potential than that of seeds and could prevent CCl4-induced hepatotoxicity.

Histopathologic studies also reinforced the findings of biochemical analysis. Histological examination of HCC group shows significant carcenogenesis characterized by Centrilobular hepatocellular hypertrophy with neoplastic cells besides, spindle cell/sarcomatoid" morphology of hepatocytes. There was infiltration of the lymphocytes and necrotic area. However, POM and MT treatment frustrated the severity of hepatic damage which diminished as compared with those in HCC group. Supplementation of Pomegranate and milk thistle ethanolic extract reduced the hyperplasia of hepatocytes and lymphocyte infiltration, which indicated its role as hepatoprotective agents. These results indicate the potent antioxidant property of both extracts.

Regarding the antioxidant activity of milk thistle, it could be noticed that administration of MT enhances liver regeneration more than POM extract. Silymarin supports the liver cells through multifactor action including binding to cell membrane to suppress toxin penetration into the hepatic cells, increasing superoxide dismutase activity, increasing glutathione tissue level [67] , inhibition of lipid peroxidation [68]. In addition, silymarin has been shown to increase hepatocyte protein synthesis which revealed enhanced regeneration of hepatic tissue as concluded with Carrillo et al. [69]. It was formulated by Milijasevic. [70] that silymarin is a potent antioxidant due to its richness with phenolic compound. The author also supported this formulation through its ability to stimulate liver cells regeneration and cell membrane stabilization which had a major role in prevention of hepatotoxic agents from entering hepatocytes

In conclusion, the present findings suggest that POM and MT extracts (500 mg/kg) ameliorates chemically induced hepatocellular carcinoma in rats. The efficient recovery in liver indices, alleviation of oxidative damage as well as enhance tissue regeneration. Besides suppression of alpha-fetoprotein highlights the therapeutic efficacy of POM and MT in alleviating induced hepatocellular carcinoma.

5. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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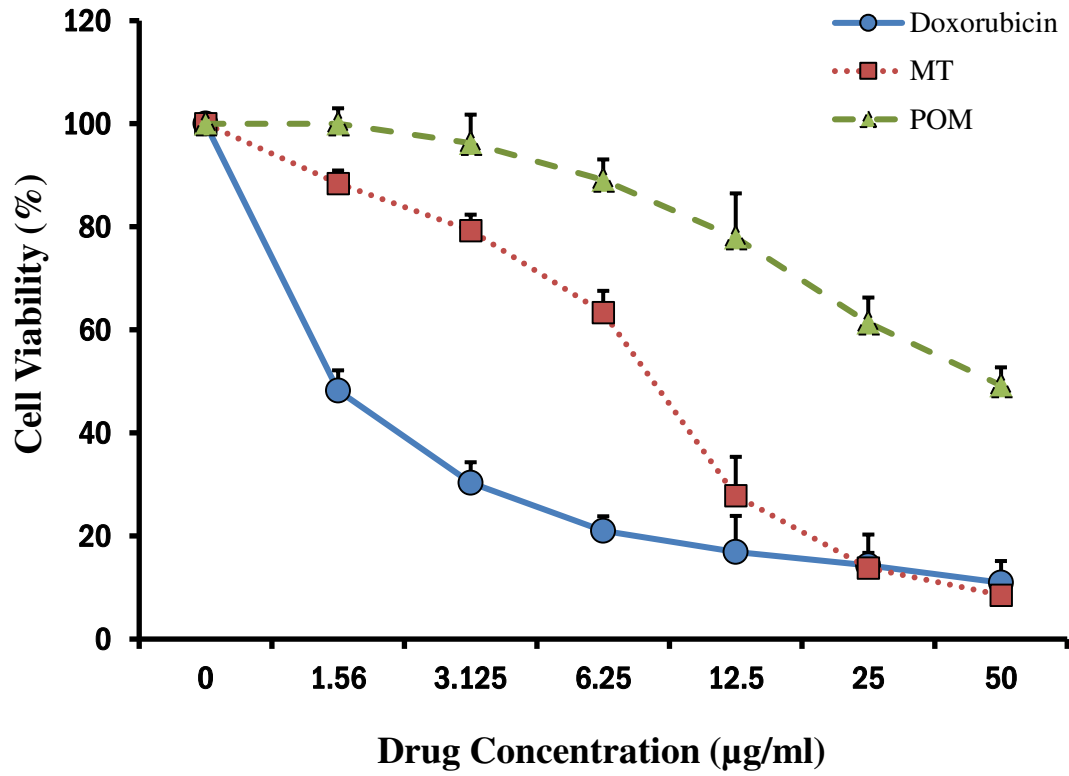
Table 1: Effects of ethanol extract of *Punica granatum* and *Silybum marianum* on various biochemical parameters in rats with chemically induced liver carcinogenesis.

| Groups		SGPT Level (U/l)	SGOT Level (U/l)	Bilirubin (mg/dl)	Total protein g/dl	Albumin g/dl	LDH U/l	AFP ng/mL
Control	Mean ± s.e	22.40± 2.19	34.65±1.42	0.05±0.01	7.06±0.27	4.75±0.33	128.83±18.63	3.36±0.13
MT	Mean ± s.e	27.30±2.47	33.35±1.88	0.06±0.02	7.18±0.25	4.80±0.20	161.15±21.89	3.61±0.48
	% of change vs control	+6.47	-7.85	+20	-10.18	-19.37	+26.52	+7.44
POM	Mean ± s.e	23.85±2.18	31.93±2.36	0.06±0.03	6.35±0.26	3.83±0.11	163.00±9.67	3.23±0.18
	% of change vs control	+6.47	-7.85	+20	-10.18	-19.37	+26.52	-3.87
HCC	Mean ± s.e	145.49±5.28□	160.25±3.57□	0.50±.02□	4.49±0.19□	2.9±0.22□	941.25±38.77□	19.81±4.03□
	% of change vs control	+551.52	+362.48	+900.0	-36.63	-38.95	+630.61	+489.58
HCC+POM	Mean ± s.e	78.11±6.78 [#]	94.70±8.50 [#]	0.28±0.04[#]	4.92±0.27[#]	3.28±0.15[#]	586.45±37.50[#]	7.54±0.51[#]
	% of change vs HCC	-46.48	-40.9	-44.0	+30.13	+13.1	-37.69	-62.0
HCC+MT	Mean ± s.e	99.30±4.06[#]	133.10±8.42[#]	0.37±0.02[#]	5.83±0.53[#]	3.71±0.22[#]	540.75±22.98[#]	6.51±0.28[#]
	% of change vs HCC	-31.96	-29.42	-26.0	+9.82	+27.23	-42.55	-68.09

Values are expressed as mean ± S.E. for six rats in each group. % of changes of group MT, POM and HCC are relative to control group.% of changes of groups HCC+ MT and HCC+POM are relative to HCC group. *$P< 0.05$ denotes value significantly different from controlusing one way ANOVA followed by Duncan. [#] $P<0.05$ experimental groups compared with HCC group. using one way ANOVA followed by Duncan. Other values proved no significance.

HCC = Single dose of DENA, followed by CCL4 then AAF. **POM** = thrice dose of 500 mg/kg b.wt/week of ethanol extract of pomegranate (*Punica granatum*).

**MT**=thrice dose of 500 mg/kg b.wt/week of ethanol extract of Milk thistle (*Silybum marianum*).

Figure 1: Cytotoxicity activity (IC50 µg/ml) of *Punica granatum* (POM) and *Silybum marianum* (MT) on human hepatocellular carcinoma cell line (HEP-G2). Cells were treated with different concentrations of POM and MT extract for 48 hr when the cell viability was determined by the MTT assay. The Cytotoxicity activity was calculated as percentage of inhibition compared with the control.
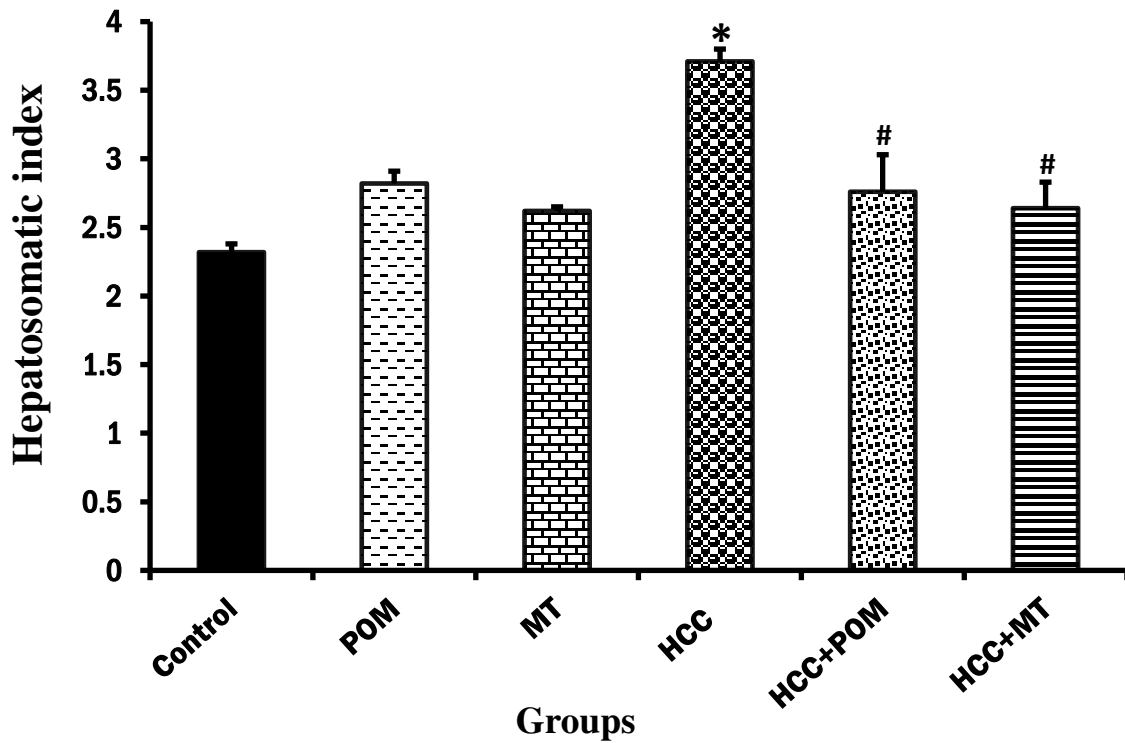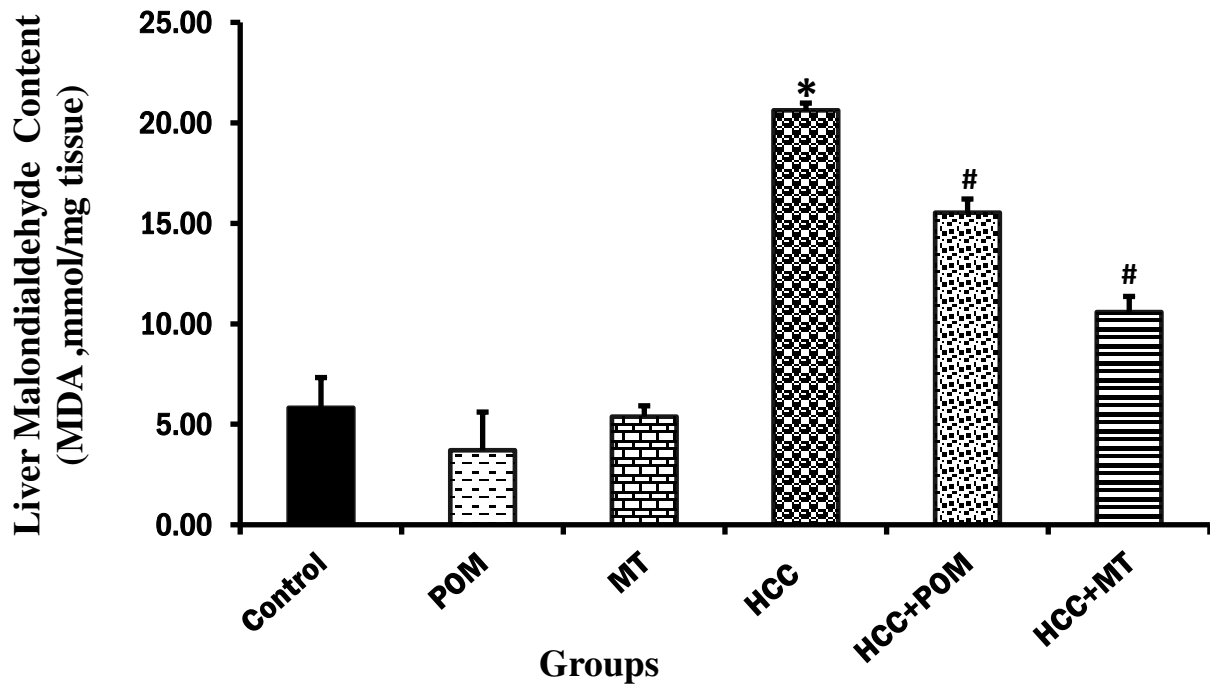
Figure 2: Effects of ethanol extract of *Punica granatum* and *Silybum marianum* on Hepatosomatic index in rats with chemically induced liver carcinogenesis. Values are expressed as mean ± S.E. for six rats in each group. *$P<$ 0.05 denotes value significantly different from controlusing one way ANOVA followed by Duncan. # $P<0.05$ experimental groups compared with HCC group. using one way ANOVA followed by Duncan. Other values proved no significance. **HCC** = Single dose of DENA, followed by CCL4 then AAF. **POM** = thrice dose of 500 mg/kg b.wt/week of ethanol extract of pomegranate (*Punica granatum*). **MT**=thrice dose of 500 mg/kg b.wt/week of ethanol extract of Milk thistle (*Silybum marianum*).
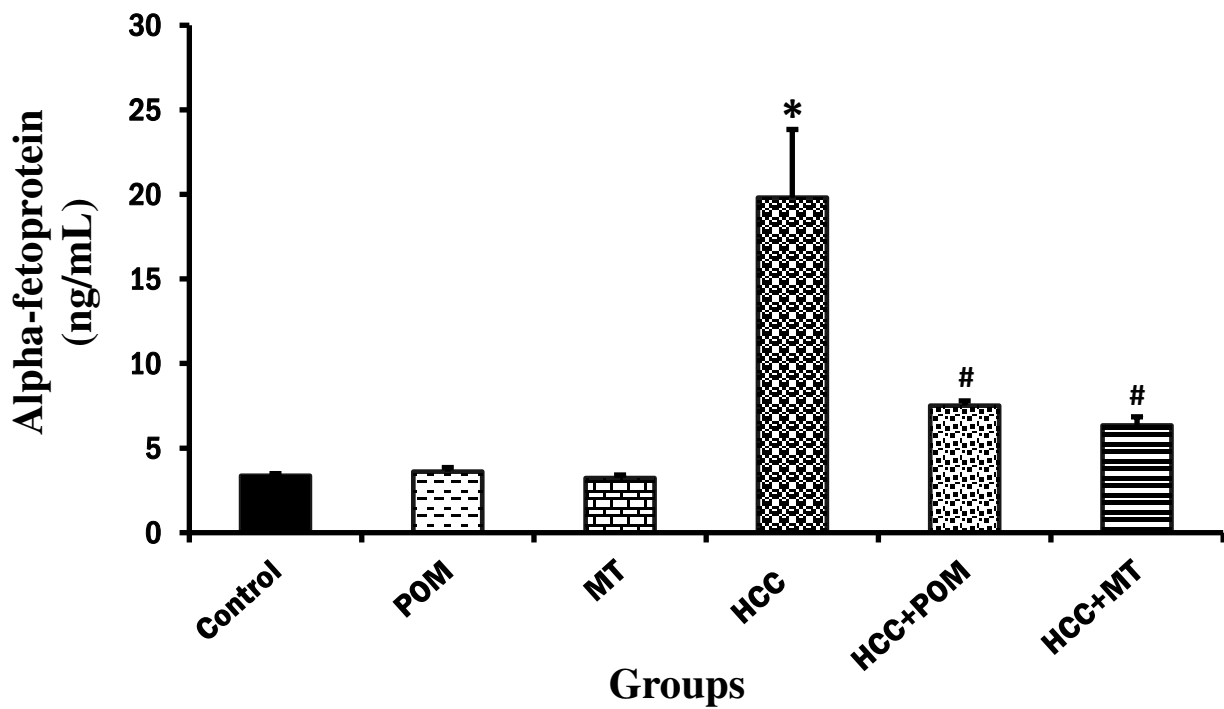
Figure 3: Effects of ethanol extract of *Punica granatum* and *Silybum marianum* on Alpha-fetoprotein in rats with chemically induced liver carcinogenesis. Values are expressed as mean ± S.E. for six rats in each group. *$P<$ 0.05 denotes value significantly different from controlusing one way ANOVA followed by Duncan. # $P<0.05$ experimental groups compared with HCC group. using one way ANOVA followed by Duncan. Other values proved no significance. **HCC** = Single dose of DENA, followed by CCL4 then AAF. **POM** = thrice dose of 500 mg/kg b.wt/week of ethanol extract of pomegranate (*Punica granatum*). **MT**=thrice dose of 500 mg/kg b.wt/week of ethanol extract of Milk thistle (*Silybum marianum*).
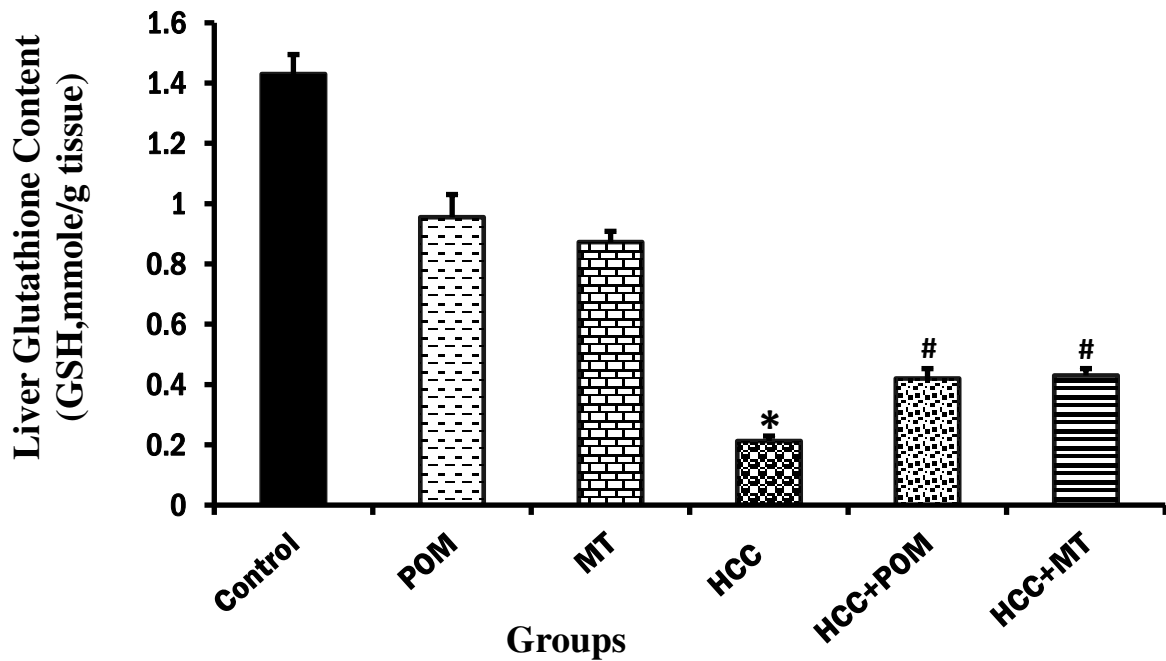
Figure 4: Effects of ethanol extract of *Punica granatum* and *Silybum marianum* on liver glutathione content in rats with chemically induced liver carcinogenesis. Values are expressed as mean ± S.E. for six rats in each group. *$P<$ 0.05 denotes value significantly different from controlusing one way ANOVA followed by Duncan. # $P<0.05$ experimental groups compared with HCC group. using one way ANOVA followed by Duncan. Other values proved no significance. **HCC** = Single dose of DENA, followed by CCL4 then AAF. **POM** = thrice dose of 500 mg/kg b.wt/week of ethanol extract of pomegranate (*Punica granatum*). **MT**=thrice dose of 500 mg/kg b.wt/week of ethanol extract of Milk thistle (*Silybum marianum*).

Figure 5: Effects of ethanol extract of *Punica granatum* and *Silybum marianum* on liver malondialdehyde content in rats with chemically induced liver carcinogenesis. Values are expressed as mean ± S.E. for six rats in each group. *P< 0.05 denotes value significantly different from controlusing one way ANOVA followed by Duncan. #P<0.05 experimental groups compared with HCC group. using one way ANOVA followed by Duncan. Other values proved no significance. **HCC** = Single dose of DENA, followed by CCL4 then AAF. **POM** = thrice dose of 500 mg/kg b.wt/week of ethanol extract of pomegranate (*Punica granatum*). **MT**=thrice dose of 500 mg/kg b.wt/week of ethanol extract of Milk thistle (*Silybum marianum*).
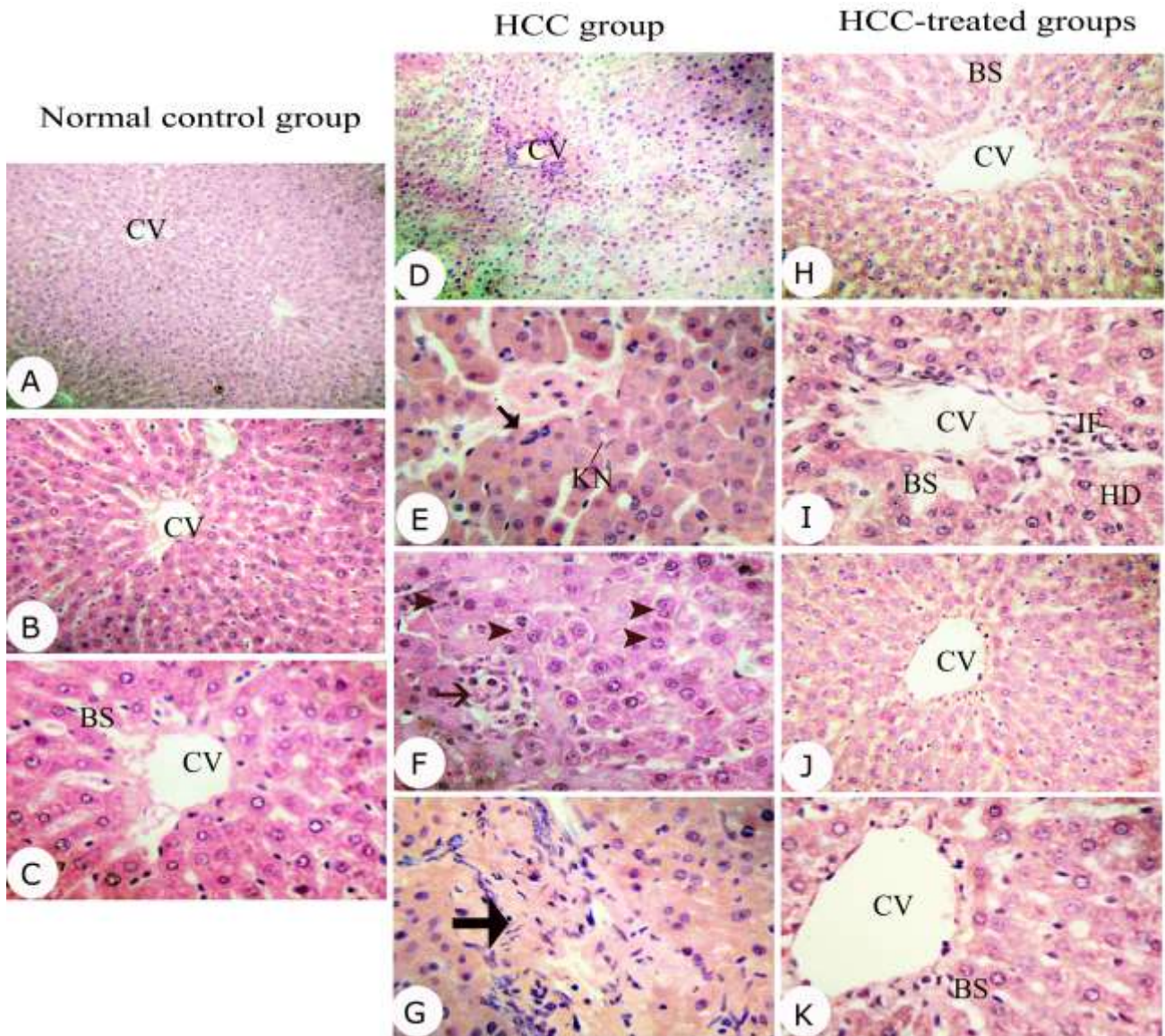
Figure 6: Hematoxilin-eosin stained sections of normal control liver **(A-C)** show normal architecture and granulated cytoplasm with uniform nuclei; Central vein: CV; and Blood sinusoids: BS **(A)**X 100, **(B)** X200, **(C)** X400 . Pronounced histopathological abnormalities seen in HCC group **(D-G). (D)** Centrilobular (CV) hepatocellular hypertrophy with neoplastic cells are irregular with hyperchromatic nuclei was observed (Hepatocellular adenoma) X100. **(E)** hepatocellular hypertrophy with Cytoplasmic alteration, degenerated cells has karyolysed nuclei (KN) X400 **(F)** neutrophil infiltrate associated with hepatocyte necrosis (arrow) and diploid nuclei (arrow head) X400. **(G)** spindle cell/sarcomatoid" morphology of hepatocytes (arrow) X400. These changes were reduced in livers of POM-HCC group rats **(H and I)** with sinusoid dilatation (BS), mild lymphatic infiltration (IF) and hydropic degeneration (HD) (X200 and X400, respectively). **(J-K)** MT-HCC showed normal liver architectures (J: X200, K: X400)