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Biochemistry

SYNERGISTIC HEPATOAMELIORATIVE POTENTIAL OF SILYMARIN AND ETHANOLIC EXTRACT OF *JASONIA MONTANA* VERSUS BISPHENOL INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT The current work is designed to explore the hepatoameliorative impacts of silymarin and ethanolic extract of *jasonia montana* versus bisphenol A (BPA) induced hepatotoxicity in a time dependent manner. Rats were allocated into five groups as follows; Group i: rats orally received Propylene glycol (1ml/kg B.W/day), served as control. Groups ii: rats orally received Propylene glycol for 2 weeks, then received BPA (50 mg/kg) in Propylene glycol. Groups iii-v: rats orally received (Silymarin, *Jasonia Montana*, both herbs) (200 mg/kg bw,150 mg/kg B.W/day, both doses) respectively for 2 weeks, then administered BPA in combination with herbs for 6 weeks. BPA induced hepatotoxicity as confirmed by increased liver enzyme leakage, hepatic 4-HNE, reduced TAC activity, IGF-1 and GSH-PX, GST gene expression. Further, BPA evoked apoptotic effect as expressed by high hepatic caspase-3 activity, TNF-α level with lower BCL-2 level. As well as fibrotic effect as indicated by increased hepatic TGF-B1 and confirmed by histopathological investigation. On contrary, oral Silymarin and ethanolic extract of *jasonia montana* supplementation attenuated bisphenol-A evoked alterations in the above-mentioned markers all the experimental periods specially after 8 weeks, meanwhile both herps don't affect GSH-PX, GST gene expression. Taken together, the present results summarize that *J. Montana* ethanolic extract supplementation showed hepatoprotctive effects versus bisphenol- induced hepatic toxicity, while silymarin can be served as a novel hepatoprotective medication and combination of both herps was better than each alone due to their synergistic antioxidant properties.

KEYWORDS : bisphenol A, liver, oxidative stress, apoptosis, fibrosis, silymarin, *j. montana.*

Introduction

Modern food styles and exposure to pollutants besides many other factors are well known to responsible for liver damage and Production of reactive oxygen species ***(Elberry et al., 2010***). Hepatic damage occurs due to various xenobiotics such as Bisphenol A- induced oxidative stress and disrupted liver functions (***Bindhumol et al., 2003; Moon et al., 2012; Hamza and Al-Harbi, 2015***).

Bisphenol A (BPA; 2,2Bis (4-hydroxyphenyl) propane,4,4'-isopropylidenedi-phenol)) has two functional groups of phenol and belong to organic compound. BPA is defined as xeno-estrogenic compound that is used in manufacture of Polycarbonate plastics such as drink, food and baby bottles ***(Ben-Jonathan and Steinmetz, 1998; Liu et al, 2013; Karnam et al., 2016).*** BPA has been recorded as safe in polymeric state, while loosed its stability in basic, acidic medium and by UV light exposure that converting BPA to monomeric state. Thus, BPA has been released into drink, food or environment (***Talsness et al., 2009***). It was reported that, BPA act as endocrine disruptor ***(Kharrazian, 2014).*** BPA was known to dysregulate inflammatory cytokine (***Wetherill et al., 2007; Ben-Jonathan et al., 2009)***. Several researches have been reported the hepatoxic effect of BPA at acute, short term and sub-chronic levels (***Tyl et al., 2002; Tyl, 2008***). Many experiments highlight the toxic effects of BPA at a dose of (50 mg/kg bw) and at higher doses on liver, kidney and body weight ***(Tyl, 2008***; ***Stump et al., 2010)***. As well as, BPA LD50 is 3.25 g/kg per os in rat (***Chapin et al., 2008)***. Daily accepted dose of BPA is 50 g/kg that was approved by American environmental protection (***Rubin et al., 2001***). BPA induces ROS production and dysfunction of the mitochondria leading to hepatic apoptosis (***Asahi et al., 2010; Moon et al., 2012; Xia et al., 2014***). Such reactive oxygen species involved in hepatic membranes damage and subsequently leakage of liver enzymes. So, controlling of oxidative damage is extensively considered**.**

Recently, many studies focused on polyphenols- enriched plant and its role in alleviating oxidative stress and many diseases such as non-alcoholic fatty liver disease (***Rodriguez-Ramiro et al., 2016***) obesity (***Ohta et al., 2006***), diabetes (***Klein et al., 2007***), colon cancer (**Peng et al., 2006**). Silymarin is well known enriched polyphenol hepatoprotective plant isolated from milk thistle (Silybum marianum) and contained a mixture of flavonoids such as silybin, silychristin, silybinin, and silydianin that well known for their radical scavenging activities and thus plays a role in prevention of oxidative damage caused by reactive oxygen species (***Flora et al., 1996***; ***Hanafy et al., 2016).*** Seeds used in treatment of hepatitis, cirrhosis and jaundice (***Kren and Walterova, 2005***). Silymarin is a well-known natural antioxidant product and it had been used in many oxidative stress models in rats ***(Amin and Arbid, 2015***; ***Kim et al., 2016).*** It is well established as anti- inflammatory, antioxidant, hepatoprotective versus ethanol toxicity (***Das and Mukherjee, 2012***)**,** anti-fibrotic ***(El-Lakkany et al., 2012)***, anti-fatty liver **(*Grattagliano et al., 2013*)**, anti-hepatic steatosis (***Ni and Wang, 2016***), anti- renal damage (***Amien et al., 2015),*** antipyretic, anti-nociceptive, anti-hyperlipidemic **(*Amin and Arbid, 2015***).

Jasonia Montana (Haneida) is another enriched polyphenol plant, a member of the Asteraceae occurred in the *Sinai Peninsula*. About 13 flavonoid aglycones were extracted from *Jasonia Montana* aerial parts such as 9 methyl ethers of quercetagetin, 1 methyl ether of 6-hydroxykaempferol, and 3 methyl ethers of quercetin. *J. Montana* is characterized by aromatic odor and recorded in digestive diseases, stomachache and chest diseases treatment (***Tackholm, 1974; Ahmed et al., 1989; Scartezzini and Speroni, 2002***). The various extracts of the plant were screened for hypoglycemic, anti-diabetic **(*Hussein and Farghaly, 2010),*** antioxidant and protect against lipid Peroxidation in liver and kidney ***(Hussein and Farghaly, 2010)***. It well known as protector against Alzheimer's disease (***Ahmed et al., 2013).*** As well as anti-bacterial and antifungal activity ***(Hegazy et al., 2014).*** Taking altogether, the current work is designed to assess the hepatoameliorative activities of Silymarin and *J. Montana* versus bisphenol induced hepatotoxicity in a time dependent manner.

Materials and Methods

1-Rats and housing condition

A total number of 90 healthy male Spraque Dawely rats, weighing 130-180 gm were obtained from Lab. Animal House, faculty of Vet. Medicines, Suez Canal University. Rats were allowed to acclimatize to experimental conditions for 2 weeks. Rats were housed in separated metal cages under controlled environmental conditions (20-24°C) and a normal dark light cycle. Rats had free access to conventional eating regimen and ingesting water ad libitum. Experimental design of the current work was conducted according to that authorized by institutional research ethics committee of faculty of Vet. Medicine, Suez Canal University.

Induction of hepatotoxicity

Bisphenol A (BPA; 2,2Bis (4-hydroxyphenyl) propane,4,4'-isopropylidenedi-phenol) was obtained from Sigma- Aldrich**,** USA. BPA was used to induce hepatotoxicity at a dose of 50mg/kg b.w. (***Hassan et al., 2012***). BPA was formulated by using propylene glycol as a vehicle at a dose of 1ml/kg B.W. ***(Gilibili et al., 2014)*.**

Medicinal plants

Silymarin

Mepasil is a tablet preparation of silymarin standardized extract containing 160 mg which equivalent to 200 mg Silybium marianum (plant extract from ripe seeds of milk thistle plant) was purchased from Arab Company for Pharmaceuticals & Medicinal Plants (MEPACO - MEDIFOOD), Enshas El Raml, Sharkeya, Egypt.

Jasonia Montana

Fresh aerial components of *J. Montana* have been gathered from the Saint Katherine; Sinai, Egypt. Air-dried aerial parts of the plant (2 kg) were crushed to coarse powder and extracted exhaustively in a Soxhlet apparatus with 95% ethanol. The extract was concentrated in hot air oven to yield viscous mass. The ethanolic extract was kept in airtight containers in a deep freeze and maintained at 4 °c till the time of usage.

Phytochemical screening to ethanolic extraction of *J.Montana*

A phytochemical analysis of aerial components of *J. Montana* was revealed the presence of alkaloids, cardiac glycosides, flavonoids, tannins, anthraquinones, saponins, volatile oil, coumarins and triterpenes ***(Hussein and Abdel-Gawad, 2010).***

Experimental Regimens

Rats were randomly allocated in to five groups, 18 rats in each group;
Group i (control group): rats received Propylene glycol (1ml/kg B.W) once daily and continued till end of the experiment.

Groups ii (BPA group): rats orally received Propylene glycol (1ml/kg B.W) once daily for 2 weeks, then received BPA (50 mg/kg) per os till end of the experiment.

Groups iii (BPA+ Silymarin): rats orally received silymarin in propylene glycol as a vehicle (200 mg/kg b.w**) (*Sabiu et al., 2015*)** for 2 weeks, then administered BPA in combination with Silymarin for 6 weeks.

Groups iv (BPA+ *J. Montana*): rats orally received ethanolic extract of *J. Montana* (150 mg/kg b.w) suspended in propylene glycol **(*Hussein and Abdel-Gawad, 2010*)** for 2 weeks, then administered BPA in combination with ethanolic extract of *J. Montana* for 6 weeks.

Groups v (BPA+ Silymarin+ *J. Montana*): rats orally received silymarin (200 mg/kg b.w**)** and ethanolic extract of *J. Montana* (150 mg/kg b.w) for 2 weeks, then administered BPA in combination with Silymarin and ethanolic extract of *J. Montana* for 6 weeks.

Handling of blood and tissue samples

A total number of 30 rats from five groups (6 rats from each group) were decapitated and blood samples have been accrued at 3 different time after 4,6 and 8 weeks from the onset of the experiment after overnight fasting from the medial canthus of eye by using micro-hematocrit tubes. Blood was taken in to a clean and dry screw capped centrifuge tubes and left to clot at room temperature, then centrifuged at 3000 r.p.m. for 15 min to separate clear serum samples and stored at -20° C for determination of liver function enzymes. Rats were sacrificed by cervical decapitation. Liver samples were dissected and weighted then cut into 3 parts. First part was rapidly frozen at -80° C and homogenized in phosphate buffered saline (PH=7.4) using Teflon homogenizer (Glass Col homogenizer system, Vernon hills, USA). Then centrifuged at 3000xg for 15 minutes at 4° C. The supernatant was collected in dry tubes until use. Second part was fixed in 10% phosphate buffered formalin for (18-24hr) at room temperature and then embedded in to paraffin, then sectioned and stained by H& E for histopathological examination. Third part was snap frozen by liquid nitrogen, kept at -80°C for GSH-PX& GST gene expression analysis.

Determination of weight indices. Changes in body weight of rats were noted by recording the difference between weight at the starting time of the experiment (baseline) and weight before samples collection for each rat individually after 4,6 and 8 weeks.

Liver somatic index is calculated as follow: liver weight/ body weight x 100 (***Novaes et al., 2012)***.

Estimation of liver function enzymes. ALT& AST were estimated using kits of Spectrum, Cairo, Egypt. GGT was estimated using kits of Meril Diagnostics Pvt. Ltd, Gujarat, India.

Determination of hepatic oxidative and inflammatory and apoptosis markers. Total antioxidant capacity (TAC) was measured using ABTS Antioxidant Assay Kit, Zenzbio, Cat# AOX-1. 4-Hydroxynonenal (4-HNE) was estimated using rat 4-Hydroxynonenal ELISA Kit, MyBioSource, Cat. # MBS006080. Tumor necrosis factor-alpha (TNF-α) was Determined by the aid of Rat TNF-alpha ELISA Kit, RayBio. Transforming Growth Factor Beta 1 (TGF-β1) was estimated by aid of ELISA using TGF-B1 ELISA, Kamiya Biomedical Company, USA. Cat. # KT-30309. Caspase-3 was estimated using colorimetric caspase 3 activity assay kit (Sigma-Aldrich, USA). B-cell CLL/lymphoma 2 (BCL2) was measured using rat B-cell CLL/lymphoma 2 ELISA Kit, CUSABIO, Cat. # CSB-E08854r.

Assessment of hepatic Insulin like growth factor-1. IGF-1 was assessed using Mouse/Rat IGF-I Immunoassay, Quantikine, Cat. # MG100, SMG100, PMG100.

Analysis of Glutathione peroxidase (GSH-Px) & glutathione-*s*-transferase (GST) gene expression level by real time- PCR

Liver total RNAs were extracted using total RNA purification kit (Jena Bioscience kit) following the manufacture protocol. RNA concentration was assessed by Nano-Drop (ND1000) spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA). cDNA was produced in presence of SCRIPT cDNA synthesis kit (Jena Bioscience kit). Quantification of GSH-PX& GST gene expression were applied using q PCR GreenMaster Kit (Jena Bioscience) and Sybr green assay. Primers were produced from (Bio Basic Canada Inc.) were showed in Table 1 (**Hassan et al, 2012**). Glyceraldehyde 6-phosphate dehydrogenase (GAPDH) was used as a house keeping gene for normalization ***(Gao et al., 2011)***. PCR was applied as follow: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative quantification method was performed to calculate gene expression (***Livak and Schmittgen, 2001)*** by using Real Time PCR (software Applied Biosystem 7500).

Table 1 – Sequences of Primers for GAPDH, GSH-Px and GST

Gene	Forward primer	Reverse primer
GAPDH	5?-TTCAACGGCACAGTCAAG-3?	5?- CACACCCATCACAAACAT-3?
GSHPx	5?-GGGCAAAGAAGATTCCAGGTT-3?	5?-AGAGCGGGTGAGCCTTCT-3?
GST	5?-GCCTTCTACCCGAAGACACCTT-3?	5?-GTCAGCCTGTTCCCTACA-3?

Statistical analysis

Data are given as mean± SE and were analyzed using Two-way Analysis of Variance (ANOVA) for normally distributed samples according to ***(Snedecor and Cochran, 1989)***. Means separation and pairwise comparisons were done by Duncan's Multiple Range test according to ***(Duncan, 1955).*** Regarding Statistical analyses of GST and GSH-Px relative gene expression data which were not normally distributed, it was analyzed by non-parametric ANOVA test (Kruskal-Wallis Test). Results are considered significant at probability level of 0.05 for each ($P \leq 0.05$). Statistical analyses were conducted by SPSS version 20.

RESULTS

Effect of Silymarin and ethanolic extract of *jasonia Montana* weight changes and liver somatic index in bisphenol A treated rats.

Oral exposure to bisphenol A reduced body weight after 6& 8 weeks (table 2), while an increase in liver somatic index after 8 weeks (table 3) in BPA- treated rats was recorded comparing to control. On the other hand, these alterations were returned to normal levels in BPA+ *J. Montana*, BPA+ silymarin, BPA+ silymarin+ *J. Montana* after 6& 8 weeks.

Effect of Silymarin and ethanolic extract of *jasonia Montana* on serum liver function enzymes activity in bisphenol A treated rats

BPA induced elevation in the activity of AST& GGT activity than control all over the experimental periods (table 4& 6) and ALT after 8 weeks (table 5). Higher enzymes leakage was observed after 8 weeks. *J. Montana* and silymarin supplementation restored enzymes activity to normal values.

Effect of Silymarin and ethanolic extract of *jasonia Montana* on hepatic oxidative stress, inflammatory, apoptosis markers and IGF-1 in bisphenol A treated rats

Oral exposure to bisphenol A induced hepatic toxicity as expressed by reduction in TAC activity (table 7), BCL-2, IGF-1 levels (table 12, 13) respectively along with elevation in 4-HNE, TNF-α, TGF-β1 levels (table 8, 9, 10) respectively & caspase-3 activity (table 11) all over the experimental periods. Higher damage was evoked after 8 weeks. On contrary, an amelioration in hepatic oxidative stress, apoptosis, fibrosis cascade was established in BPA+ *J. Montana*, then BPA+ silymarin, then BPA+ silymarin+ *J. Montana* - treated groups respectively all the experiment specially after 6& 8 weeks.

Effect of Silymarin and ethanolic extract of *jasonia Montana* on GSH-PX gene expression in bisphenol A treated rats.

Figure 1 summerize that, GSH-Px gene expression is down regulated in BPA, BPA+ Silymarin, BPA+ J. Montana& BPA+ Silymarin+ J. Montana- treated groups comparing to control. No significant difference between the periods was observed. Although GSH-Px gene expression was higher in BPA+ *J. Montana* treated group after 8 weeks and in BPA+ Silymarin+ *J. Montana* after 6,8 weeks comparing to BPA& BPA+ Silymarin -treated groups, there is no significance difference.

Effect of Silymarin and ethanolic extract of *jasonia Montana* on GST gene expression in Bisphenol A treated rats.

Figure 2 evoke that, GST gene expression is down regulated in BPA, BPA+ Silymarin, BPA+ *J. Montana*, BPA+ Silymarin+ *J. Montana*-treated groups comparing to control. No significant difference between the periods was observed. Although GST gene expression was higher in BPA+ Silymarin+ *J. Montana* after 4 weeks comparing to BPA, BPA+ *J. Montana*& BPA+ Silymarin -treated groups, there is no significance difference.

Histopathological investigations in hepatic tissue in experimental groups

Liver of control group after 4,6 and 8 weeks showed normal polyhedral hepatic cells which arranged in cords that radically arranged around the central veins (Figure 3a, 4 a, 5a). On the other hand, liver in BPA treated group exhibited mild dilated and congested central vein, some of hepatic cells showed hydropic degeneration. Some hepatic cells are apoptotic with pyknotic nuclei with focal leukocytic infiltrations after 4 weeks (Figure 3b). After 6 weeks, hepatic areas showed mild infiltration of lymphocytes and mild to moderate proliferation of fibroblasts (Figure 4b). Severe intrahepatic damage, apoptosis and fibroblasts proliferation and in portal area were observed after 8 weeks (Figure 5 b). Liver in BPA+ *J. Montana* treated group evoked mild dilated and congested blood vessels. Hepatic cells showed mild hydropic degeneration with mild lymphocytic infiltration and proliferations of fibroblast with some apoptotic hepatocytes after 4weeks (Figure 3c). After 6&8 weeks, liver in BPA+ *J. Montana* treated group evoked mild dilated and congested blood vessels. Hepatocyte showed moderate hydropic degeneration, lymphocytic infiltration and proliferations of fibroblast with some apoptotic hepatocytes (Figure 4 c& 5c). Liver in BPA+ silymarin showed mild dilated and congested central vein and sinusoid. Hepatocytes showed mild hydropic degeneration, lymphocytic infiltration and proliferation of fibroblast and some apoptosis after 4&6weeks (Figure 3d& 4d). After 8 weeks, liver pointed mild dilated and congested central vein. Hepatic cells showed moderate hydropic degeneration with some hepatocytes apoptosis and mild lymphocytic infiltration and proliferation of fibroblast (Figure 5 d). While liver in BPA+ *J. Montana* + Silymarin- treated group evoked mild dilated and congested blood vessels with mild apoptotic cells, lymphocytic infiltration and fibroblastic proliferation after 4, 6& 8 weeks (Figure 3e, 4 e& 5e). Therefore, this group showed the most ameliorative effect indicating synergistic effect of both herps.

Table (2): Effect of Silymarin and ethanolic extract of *jasonia Montana* on body weight change (gm) in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$105.4^{bc} \pm 10.29$	$123^{ab} \pm 6.99$	$135^{a} \pm 7.06$
BPA	$103.2^{bc} \pm 8.16$	$93^{c} \pm 7.16$	$60.6^{d} \pm 3.82$
BPA + Sil.	$104b^{c} \pm 5.09$	$123^{ab} \pm 3.74$	$120^{abc} \pm 7.4$
BPA+ J.M.	$92.4^{c} \pm 8.28$	$125^{ab} \pm 5.69$	$114^{abc} \pm 4.29$
(BPA+Sil.+J.M.)	$93^{c} \pm 9.01$	$114^{abc} \pm 1.87$	$135^{a} \pm 7.89$

Results are expressed as mean ± SEM. Means with different superscripts are significantly different at ($P \leq 0.05$). BPA: bisphenol A, Sil.: silymarin, J.M.: *Jasonia Montana*.

Table (3): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Liver Somatic index (gm) in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$3.09^{bcd} \pm 0.15$	$3.13^{bcd} \pm 0.12$	$3.09^{bcd} \pm 0.09$
BPA	$3.93^{bc} \pm 0.1$	$4.04^{b} \pm 0.24$	$5.92^{a} \pm 0.45$
BPA + Sil.	$3.46^{bc} \pm 0.27$	$3.09^{bcd} \pm 0.12$	$2.99^{cd} \pm 0.09$
BPA+ *J.M.*	$3.73^{bc} \pm 0.1$	$3.34^{bcd} \pm 0.14$	$3.11^{bcd} \pm 0.17$
(BPA+Sil.+J.M.)	$3.62^{bc} \pm 0.06$	$3.42^{bc} \pm 0.12$	$2.42^{d} \pm 0.09$

Table (4): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Aspartate Amino Transferase (AST)(U/L) activity in Bisphenol treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$111.99^{de} \pm 2.59$	$113.54^{de} \pm 3.79$	$105.21^{de} \pm 4.66$
BPA	$134.16^{bc} \pm 4.39$	$142.39^{ab} \pm 10.51$	$157.09^{a} \pm 10.99$
BPA + Sil.	$114.63^{d} \pm 8.13$	$112.25^{de} \pm 4.26$	$108.49^{de} \pm 3.49$
BPA+ *J.M.*	$121.36^{cd} \pm 3.65$	$114.16^{d} \pm 4.91$	$103.25^{de} \pm 3.64$
(BPA+Sil.+J.M.)	$103.48^{de} \pm 9.36$	$103.71^{de} \pm 3.48$	$92.55^{e} \pm 4.82$

Table (5): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Alanine Amino Transferase (ALT)(U/L) activity in Bisphenol treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$78.05^{bcd} \pm 5.87$	$73.07^{bcd} \pm 7.17$	$75.41^{bcd} \pm 4.34$
BPA	$94.49^{bc} \pm 5.17$	$98.54^{b} \pm 5.42$	$180.8^{a} \pm 6.77$
BPA + Sil.	$87.51^{bcd} \pm 1.95$	$69.15^{bcd} \pm 6.26$	$62.14^{cd} \pm 3.26$
BPA+ *J.M.*	$85.98^{bcd} \pm 4.35$	$86.59^{bcd} \pm 3.96$	$68.77^{bcd} \pm 5.09$
(BPA+Sil.+J.M.)	$82.09^{bcd} \pm 1.86$	$66.22^{bcd} \pm 5.21$	$55.52^{d} \pm 5.39$

Table (6): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Gamma Glutamyl Transferase (GGT) (U/L) activity in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$6.39^{e} \pm 0.3$	$7.45^{cde} \pm 0.37$	$7.2^{cde} \pm 0.46$
BPA	$14.24^{b} \pm 1.19$	$16.25^{b} \pm 0.59$	$21.69^{a} \pm 0.94$
BPA + Sil.	$8.23^{cde} \pm 0.45$	$7.08^{cde} \pm 0.29$	$5.88^{e} \pm 0.4$
BPA+ *J.M.*	$9.58^{cd} \pm 0.9$	$9.98^{c} \pm 0.25$	$6.49^{de} \pm 0.42$
(BPA+Sil.+J.M.)	$6.76^{de} \pm 0.63$	$6.86^{de} \pm 0.32$	$5.25^{e} \pm 0.22$

Table (7): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Total Antioxidant Capacity (TAC) (µm/gm tissue) activity in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$44.77^{b} \pm 0.24$	$46.73^{a} \pm 0.44$	$47^{a} \pm 0.74$
BPA	$9.57^{i} \pm 0.19$	$8.73^{ij} \pm 0.27$	$7.7^{j} \pm 0.21$
BPA + Sil.	$28.3^{f} \pm 0.21$	$29.53^{ef} \pm 0.38$	$30.73^{e} \pm 0.35$
BPA+ *J.M.*	$23.8^{h} \pm 0.21$	$24.63^{h} \pm 0.22$	$26.4^{g} \pm 0.4$
(BPA+Sil.+J.M.)	$33.83^{d} \pm 0.23$	$35.67^{c} \pm 0.26$	$37.3^{c} \pm 0.35$

Table (8): Effect of Silymarin and ethanolic extract of *jasonia Montana* on 4-Hydroxynonenal (4-HNE) (µmol/g tissue) level in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$11.8^{j} \pm 0.21$	$11.4^{j} \pm 0.06$	$10.17^{j} \pm 0.51$
BPA	$32^{b} \pm 0.7$	$33.93^{a} \pm 0.15$	$35.33^{a} \pm 0.43$
BPA + Sil.	$20.07^{ef} \pm 0.24$	$18.87^{fg} \pm 0.33$	$18.23^{fgh} \pm 0.2$
BPA+ *J.M.*	$23.7^{c} \pm 0.33$	$22.17^{cd} \pm 0.18$	$21.1^{de} \pm 0.32$
(BPA+Sil.+J.M.)	$17.8^{gh} \pm 0.42$	$16.43^{hi} \pm 0.29$	$15.1^{i} \pm 0.46$

Table (9): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Tumor necrosis factor-alpha (TNF-α) (pg/gm tissue) level in Bisphenol A treated rats.

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$12.43^{g} \pm 0.29$	$11.33^{gh} \pm 0.26$	$10.2^{h} \pm 0.23$
BPA	$43.8^{b} \pm 0.32$	$45.77^{ab} \pm 0.3$	$46.6^{a} \pm 0.68$
BPA + Sil.	$22.7^{d} \pm 0.31$	$21.8^{d} \pm 0.26$	$20.97^{d} \pm 0.49$
BPA+ *J.M.*	$26.57^{c} \pm 0.18$	$25.63^{c} \pm 0.32$	$22.9^{d} \pm 1.32$
(BPA+Sil.+J.M.)	$18.8^{e} \pm 0.21$	$17.37^{ef} \pm 0.24$	$16.2^{f} \pm 0.21$

Table (10): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Transforming Growth Factor Beta 1 (TGF-β1) (pg/gm tissue) level in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$9.83^{g} \pm 0.23$	$8.47^{g} \pm 0.22$	$7.4^{g} \pm 0.29$
BPA	$29.13^{a} \pm 0.92$	$30^{a} \pm 0.63$	$30.6^{a} \pm 1.51$
BPA + Sil.	$17.17^{cd} \pm 0.93$	$16.07^{de} \pm 0.35$	$15.07^{de} \pm 0.15$
BPA+ *J.M.*	$20.03^{b} \pm 0.43$	$19.47^{bc} \pm 0.35$	$17.27^{cd} \pm 0.69$
(BPA+Sil.+J.M.)	$14.4^{ef} \pm 0.26$	$15.27^{de} \pm 1.94$	$12.43^{f} \pm 0.24$

Table (11): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Caspase-3 (ng/gm tissue) activity in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$14.2^{h} \pm 0.17$	$15.47^{gh} \pm 0.15$	$15.93^{gh} \pm 0.15$
BPA	$45.87^{c} \pm 0.18$	$49.37^{b} \pm 0.47$	$52.3^{a} \pm 0.21$
BPA + Sil.	$22.4^{f} \pm 0.17$	$24.2^{e} \pm 0.17$	$25.3^{e} \pm 0.12$
BPA+ *J.M.*	$32.37^{d} \pm 0.29$	$33.37^{d} \pm 0.21$	$34.1^{d} \pm 0.12$
(BPA+Sil.+J.M.)	$16.8^{g} \pm 0.06$	$16.6^{g} \pm 0.06$	$16.47^{g} \pm 0.09$

Table (12): Effect of Silymarin and ethanolic extract of *jasonia Montana* on B-cell CLL/lymphoma 2 (BCL2) (ng/gm tissue) level in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$36.23^{b} \pm 0.2$	$37.17^{ab} \pm 0.18$	$38.27^{a} \pm 0.19$
BPA	$7.13^{i} \pm 0.18$	$6.63^{ij} \pm 0.24$	$5.77^{j} \pm 0.24$
BPA + Sil.	$18.4^{f} \pm 0.29$	$19.5^{f} \pm 0.44$	$20.87^{e} \pm 0.2$
BPA+ *J.M.*	$14.43^{h} \pm 0.29$	$15.47^{gh} \pm 0.4$	$15.9^{g} \pm 0.35$
(BPA+Sil.+J.M.)	$23.57^{d} \pm 0.2$	$24.93^{c} \pm 0.43$	$25.63^{c} \pm 0.32$

Table (13): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Insulin-like Growth Factor I (Pg/ gm tissue) level in Bisphenol A treated rats

Groups \ Duration	4 weeks	6 weeks	8 weeks
Control	$45.83^{c} \pm 0.38$	$47.77^{b} \pm 0.24$	$49.73^{a} \pm 0.23$
BPA	$13.27^{j} \pm 0.3$	$12.5^{j} \pm 0.21$	$11.6^{j} \pm 0.4$
BPA + Sil.	$26.07^{g} \pm 0.22$	$27.47^{g} \pm 0.35$	$29.2^{f} \pm 0.51$
BPA+ *J.M.*	$21.8^{i} \pm 0.26$	$22.83^{hi} \pm 0.18$	$23.8^{h} \pm 0.67$
(BPA+Sil.+J.M.)	$33.77^{e} \pm 0.2$	$34.4^{e} \pm 0.36$	$36.13^{d} \pm 0.67$

Figure (1): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Glutathione peroxidase gene expression in Bisphenol A treated rats. Fold-change for GSH-PX was normalized to GAPDH and calculated using delta-delta CT method [=2 (−ΔΔCT)] compared to control group. Data are analyzed by non-parametric ANOVA test (Kruskal-Wallis Test) at P < 0.05. *P compared to control

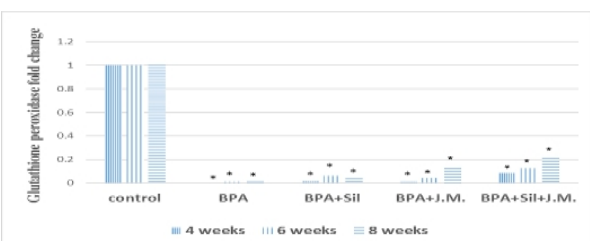

Figure (2): Effect of Silymarin and ethanolic extract of *jasonia Montana* on Glutathione-S- transeferase gene expression in Bisphenol A treated rats. Fold-change for GST was normalized to GAPDH and calculated using delta-delta CT method [=2 (−ΔΔCT)] compared to control group. Data are analyzed by non-parametric ANOVA test (Kruskal-Wallis Test) at P < 0.05. *P compared to control.

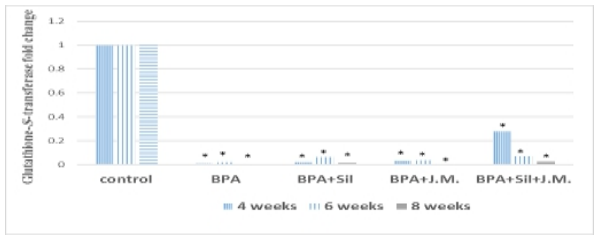

Figure (3): Photomicrographs of liver sections from rats treated with Silymarin and ethanolic extract of *jasonia Montana* versus Bisphenol A induced hepatic toxicity after 4 weeks

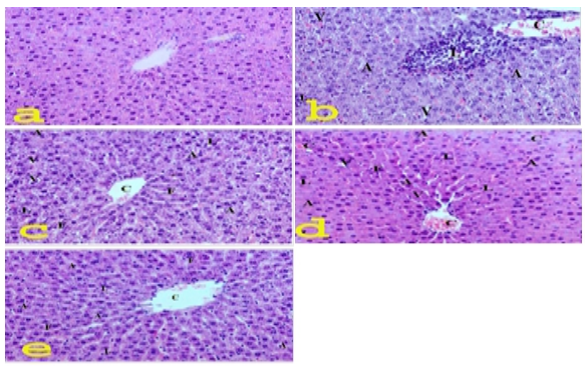

Figure 3: (a) liver of control group showed normal polyhedral hepatic cells which arranged in cords that radically arranged around the central veins. (b) liver of BPA treated group showed multifocal lymphocytic infiltration(L), mild apoptosis (A), congestion of central vein (C) and vacuolar degeneration (V). (c) liver of BPA+ *Jasonia Montana* treated group showed mild dilated and congested central vein (C), mild hydropic degeneration (V), lymphocytic infiltration (L), proliferation of fibroblasts (F), and apoptosis (A). (d) liver of BPA+ Silymarin treated group showed mild dilated and congested central vein and sinusoid (C), mild hydropic degeneration (V) and apoptosis (A), lymphocytic infiltration (L) and proliferation of fibroblast (F). (e) liver of BPA+ Silymarin+ *Jasonia Montana* treated group showed mild dilated and congested blood vessels (C) with mild apoptotic cells (A), lymphocytic infiltration (L) and fibroblastic proliferation (F). H&E X40

Figure (4): Photomicrographs of liver sections from rats treated with Silymarin and ethanolic extract of *jasonia Montana* versus Bisphenol A induced hepatic toxicity after 6 weeks

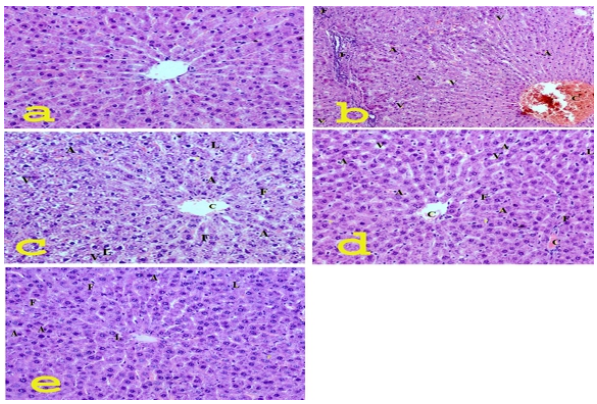

Figure 4: (a) liver of control group showed normal polyhedral hepatic cells which arranged in cords that radically arranged around the central veins. (b) liver of BPA- treated group showed moderate apoptosis (A), congestion of central vein(C) and vacuolar degeneration (V), and proliferation of fibroblasts (F). (c) liver of BPA+ *Jasonia Montana* treated group showed dilated and congested central vein (C), moderate hydropic degeneration (V), lymphocytic infiltration (L), proliferation

of fibroblasts (F), and some apoptosis (A). (d) liver of BPA+ Silymarin treated group showed mild dilated and congested central vein and sinusoid (C), mild hydropic degeneration (V), apoptosis (A), lymphocytic infiltration (L) and proliferation of fibroblast (F). (e) liver of BPA+ Silymarin+ *Jasonia Montana* treated group showed mild dilated and congested blood vessels (C) with mild apoptotic cells (A), lymphocytic infiltration (L) and fibroblastic proliferation (F). H&E X40

Figure 5. Photomicrographs of liver sections from rats treated with Silymarin and ethanolic extract of *jasonia Montana* versus Bisphenol A induced hepatic toxicity after 8 weeks

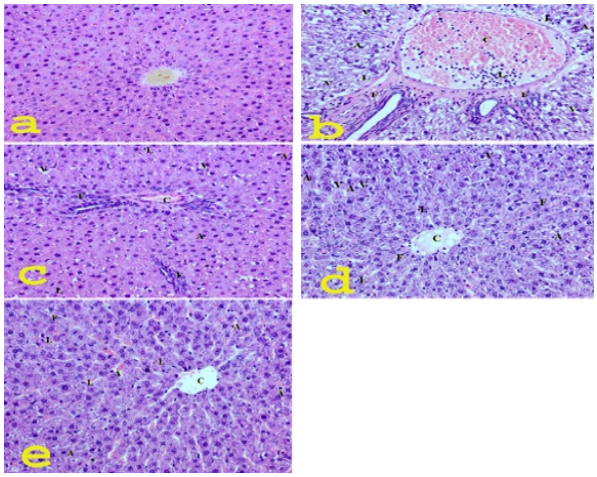

Figure 5: (a) liver of control group showed normal polyhedral hepatic cells which arranged in cords that radically arranged around the central veins. (b) liver of BPA- treated group showed lymphocytic infiltration (L), congestion of central vein (C), apoptosis (A), vacuolar degeneration (V), and sever proliferation of fibroblasts (F). (c) liver of BPA+ *Jasonia Montana* treated group showed moderate hydropic degeneration (V), lymphocytic infiltration (L), proliferation of fibroblasts (F), and some apoptosis (A). (d) liver of BPA+ Silymarin treated group showed mild dilated and congested central vein and sinusoid (C), moderate hydropic degeneration (V) and mild lymphocytic infiltration (L), proliferation of fibroblast (F) and apoptosis (A). (e) liver of BPA+ Silymarin+ *Jasonia Montana* treated group showed mild dilated and congested blood vessels (C) with mild apoptotic cells (A), lymphocytic infiltration (L) and fibroblastic proliferation (F). H&E X40

Discussion

Liver is important organ for xenobiotic metabolism and detoxification involving BPA. Hepatic estrogen receptor found in the liver play a role in BPA metabolism where glucuronidation by UGT2B1 has been occurred, then excreted in bile (***Inoue et al, 2005***). Taking altogether, the current work is designed to assess the hepatoameliorative activities of Silymarin and *J. Montana* versus bisphenol induced hepatotoxicity in a time dependent manner.

Our study established that the hepatic toxic effect of BPA through elevated serum liver function enzymes which may contribute to BPA produced ROS stimulating oxidative stress and plasma membrane damage, by turn increase liver enzyme leakage ***(Yamasaki et al., 2002; Abdel- Wahab, 2014).*** A reduction in body weight and hepatic IGF1 were observed in BPA- treated group that agree with the finding of **(*Buehlmeyer et al., 2007; Hassan et al., 2012*)** who reported a positive correlation between circulating IGF1 and body mass. Reduced IGF1 expression here may be contributed to that IGF1 is generated by the liver that already exposed to BPA- induced ROS production (***Bassanello et al.,2004; Asahi et al., 2010; Moon et al., 2012***). Additionally, IGFI is considered an important growth factor since it stimulates growth of all cell types, enhance cellular protein synthesis and induced antioxidant effect **(Buehlmeyer *et al., 2007*; *Aluru et al., 2010; Otunctemur et al., 2016).*** Concerning the pathway for bisphenol A- induced ROS generation may relate to down regulation of antioxidant enzymes gene expression (GSH-Px& GST) as confirmed here by real time PCR analysis. Glutathione is very vital antioxidant that detoxify H2O2 in presence of GSH-Px& GST enzymes (***Cheeseman and Slater, 1993; Ursini et al., 1995***). Therefore, decline of their gene expression resulted in H2O2 accumulation inducing hepatic injury. As well as, BPA disrupted cytochrome P450 which required for xeno-estrogens biotransformation resulting in increased ROS and hepatic damage (***Wang et al., 2011; Padgett et al., 2013***). GST catalyzes the conjugation of reduced glutathione through SH-group to electrophilic centers of many substrates during detoxification of xenobiotics ***(Douglas, 1987*; *Leaver and George, 1998)***. The hepatic biotransformation of BPA depends on phase I oxidation/reduction involving glutathione and phase II glucuronidation, glutathione, and sulfate conjugation **(*Kurebayashi et al., 2003*)**. Subsequently, hepatic TAC activity decreased in response to elevated ROS production during BPA metabolism which agree with (***Karafakioglu et al., 2010; Mendoza and Fregoso, 2013*)**. Reduced TAC activity resulted in elevated ROS that can induce cell membrane damage producing 4-HNE as lipid peroxidation product which agree with the findings of ***(Mourad and Khadrawy, 2012; Eid et al., 2015*)**. Interestingly, pathways involved in BPA induced hepatic damage may be apoptosis and fibrosis as confirmed in the current study. Elevated hepatic caspase-3 activity, TNF-α level and decreased BCL-2 expression in BPA treated rats that approved with the results of (***Moon et al., 2012)***. ROS play important role in NF_kB activation and TNF-α production (***AverillBates and Pallepati, 2010***). BCL2 is well known as anti-apoptotic marker that regulates apoptosis by controlling integrity of mitochondria preventing cytochrome c spillage ***(Kluck et al., 1997; Yang et al., 1997***) or by impeding caspase stimulation (***Marsden et al., 2002***). It was known that, caspase-3 is involved in the apoptosis cell by intrinsic (mitochondrial), and extrinsic (death ligand) pathways ***(Salvesen, 2002; Ghavami et al., 2009***). During mitochondrial pathway of apoptosis, cytochrome C released from mitochondria that associated with activation of apoptosis related factor 1 and caspase-9, then activate procaspase-3 and subsequently caspase-3 activation (***El-Sayed et al., 2011; Guo et al., 2013***). Regarding death ligand pathway, TNF-α is intervened through two distinct cell receptors TNFR1 and TNFR2. TNFR1 that activate caspase-3 which plays a role in condensation of chromatin and DNA breakdown (***Porter and Jänicke, 1999; Guo et al., 2015***). Finally, DNA breakage is dependent on mitochondrial Endonuclease G triggering apoptosis **(*Li et al., 2010*)**. Hepatic histopathological changes indicating hepatotoxicity after BPA administration which reveals hepatic apoptosis with pyknotic nuclei specially after 8 weeks. Moreover, liver damage stimulates TGF-β production from macrophage that enhance hepatic stellate cell and fibroblast activation resulting in production of myo-fibroblast and extracellular matrix deposition and fibrosis (***Dooley and Dijke, 2012***; ***Yuslianti et al., 2016***). These results confirmed by histopathology which illustrate Severe intrahepatic damage, apoptosis and fibroblasts proliferation and in portal area were observed all the experimental period specially after 8 weeks.

Herbal drugs highlights importance in hepatocyte regeneration, acceleration of healing process and hence management of many liver disorders. Silymarin is very important hepatoprotective drug of plant origin and recorded their ameliorative efficacy versus CCL4- induced hepatic damage (***Farghali et al., 2002; Lieber et al., 2003).*** In the current work**,** Silymarin has hepatoprotective impacts and normalized serum liver enzymes all over the experimental period that could be refereed to its antioxidant and free radical scavenging properties and stabilizing action on plasma membrane **(*Pradhan and Girish, 2006*).** As well as, Silymarin can interact with components of cellular membrane to prevent any abnormalities in lipid content maintaining normal membrane fluidity (***Muriel and Mourelle, 1990***). Moreover, Silymarin has antioxidant effects through increased GSH in liver and prevent entrance of xenobiotics from entering hepatocyte by protecting cell membrane (***Mayer et al., 2005***). Basis on such data, Silymarin exert antioxidant activity as referred by elevated TAC activity although it doesn't significantly affect GSH-PX& GST gene expression. These may clear by an attempt to modulation pathways rather than mediated by those enzymes. This explanation was agreeing with findings of ***Flora et al., 1996***; ***Hanafy et al., 2016*** whom recorded free radicals scavenging properties of flavonoids in silymarin. Furthermore, hepatic 4HNE level was declined in group received silymarin with BPA all over the experiment that agree with (***Kim et al., 2016***). Hepatic TNF-α & TGF-β level were declined after Silymarin administration that could be related to anti-inflammatory efficacy through stabilized mast cell, impedes migration of neutrophil, kupffer cell, leukotrienes and cytokines production (***Jeong et al., 2005; Malih et al., 2009; Alkaladi and Abdelazim, 2013***)**.** Additionally, Silybinin supressed IL-2, IL-4, TNF-α, and IFN-γ production and inhibited hepatic apoptosis that contributing to NF-κB down regulation (***Saller***

et al., 2007; Morishima et al., 2010; Baeeri et al., 2011). Furthermore, Silibinin can inhibit on the transformation of stellate cells into myo-fibroblasts which are responsible for the deposition of collagen fibers resulting in fibrosis **(*Fuchs et al., 1997)***. From the previous published data, we can confirm the antioxidant, anti-inflammatory and antifibrotic effect of silymarin. Further confirmation was done by histopathology where mild hydropic degeneration, apoptosis and fibrosis were observed. These results are clearly evidenced that, silymarin play very important role in liver protection and repairing of damaged hepatocytes.

In the current work, administration of *J. montana* ethanolic extract with bisphenol A exerted hepatoprotective efficacy that revealed by amelioration of oxidative damage- induced by BPA. These observations could be related to that flavonoid and phenolic compounds exert their antioxidant efficacy through scavenging peroxyl, alkoxyl radicals and donation of hydrogen to free radicals, thus preventing hydroxyl radical production and inhibiting lipid peroxidation (***Bors et al., 1990; Jovanovic et al., 1998; Tawfeq et al., 2005; Hussein, 2008***). Interestingly, quercetin as one of flavonoids present in *J. montana* could be upregulated Nrf2 gene expression that control antioxidant enzymes expression such as CAT& SOD (***Arredondo et al., 2010; Barcelos et al., 2011***). As well as, its ability to modulate cytochrome P450 that disrupted by BPA (***Ferguson, 2001***; ***Wang et al., 2011; Padgett et al., 2013***). Subsequently, a reduction in hepatic 4HNE production was recorded. Since ROS stimulates NF-κB expression that mediates cytokines production such as TNF-α, IL-6 and TGF-β. In contrary, the antiinflammatory activity of flavonoids is mediated by inhibition of ROS and nitric oxide, cyclooxygenase and down regulate cytokines expression through modulate NF-κB (***Tunon et al., 2009; González-Gallego et al., 2010; Kumar and Pandey, 2013; Ribeiro et al., 2015***). Basis on such data, ethanolic extract of *J. montana* exert antioxidant activity as referred by elevated TAC activity although it doesn't significantly affect GSH-PX& GST gene expression. These may clear by an attempt to modulation pathways rather than mediated by those enzymes. As well as ethanolic extract of *J. montana* possess anti-inflammatory effect as indicated by reduced TNF-α, antiapoptotic effect as referred by reduced hepatic caspace-3 along with increased BCL-2 expression and antifibrotic effect as expressed by decreased TGF-β expression. Collectively, an amelioration in hepatic oxidative stress, apoptosis, fibrosis cascade was established in BPA+*J. Montana*, then BPA+ silymarin, then BPA+ silymarin+*J. Montana* - treated groups respectively all the experiment all over the experimental period specially after 6& 8 weeks.

Conclusions

In conclusion, exposure to bisphenol at a dose rate of (50 mg/kg b.w/ day) represent harmful impact on liver health affecting antioxidant system depending on the time of exposure. In this way, we are in need to minimize usage of plastic bottles that release bisphenol A into food and drink. Our results evoke that *J.Montana* ethanolic extract supplementation showed hepatoprotctive effects, while silymarin can be served as a novel hepatoprotective medication. As well as, combination of both silymarin and *J. Montana* ethanolic extract was better than each alone due to their synergistic antioxidant properties.

Conflict of interests

All authors declare that there is no conflict of interests.

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