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Effect of *Silybum marianum* Seeds Extract Intervention on Biochemical Parameters, Histological Changes, and Apoptosis and Cell Cycle of Liver Tissue in Benzo[*a*]pyrene Injected Rats

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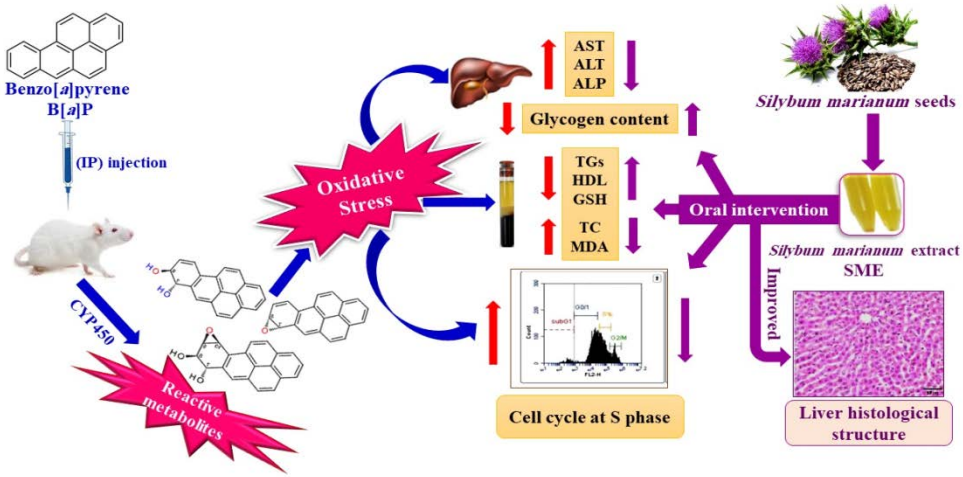
Abstract The present study aims to investigate the effect of *Silybum marianum* seeds extract intervention on biochemical parameters, histological changes, and apoptosis and cell cycle of liver tissue in Benzo[*a*]pyrene injected rats. Rats (n=36), were randomly assigned to six groups of 6 rats per each. Group 1 served as normal control. Groups 2 to 6 were injected intraperitoneally with B[*a*]P for 14 days and group (2) acted as a model control, while groups (3-6) received SME at concentrations of 200, 400, 600, and 800 mg/kg bw/d by oral gavage for 28 days each, respectively. Treatment of rats with B[*a*]P caused a significant increased ($p \leq 0.05$) in liver enzymes functions (AST, 126.07%, ALT, 120.24%, and ALP, 142.32%), compared to normal control rats. Also, B[*a*]P treatment brought a significant ($p \leq 0.05$) decrease in serum triglycerides (TG's), high density lipoprotein (HDL), and liver glycogen, albumin and glutathione (GSH) content by the ratio of -78.48, -39.98, -71.40, -43.03 and - 35.29%, respectively. Furthermore, B[*a*]P treatment brought a significant ($p \leq 0.05$) increase in serum cholesterol and liver malonaldehyde (MDA), the biomarkers of oxidative stress in cells, content by the ratio of 37.47 and 225.34%, respectively. Additionally, adverse molecular (apoptosis and cell cycle) and histopathological changes of liver tissue as the result of B[*a*]P treatment. Supplementation of the rat diets with SME (200 to 800 mg/kg bw/d) prevented the rise of liver function enzymes activities, MDA liver content and serum cholesterol as well as increases in serum TG's and albumin and the liver glycogen and GSH content. Also, improvement in both molecular and histological parameters was recorded. The rate of improving in all of these parameters exhibited a dose-dependent increase with the SME intervention. The results of this study suggest that treatment with SME in the tested concentrations proved beneficial on manipulation of the liver biochemical, molecular and histological injuries induced by B[*a*]P.



Keywords: *liver functions, glycogen, triglycerides, albumin, glutathione, malonaldehyde, flow cytometer*

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1. Introduction

Liver is a vital and principal organ present in all vertebrates. It has a wide range of functions, including detoxification, protein synthesis, and production of biochemical necessary for digestion [1]. Also, liver plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification processes [2]. Furthermore, liver produces bile juice, an alkaline compound which aids in digestion via the emulsification of lipids. The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions [3]. For that, the liver is necessary for survival and its disease create a huge economic, humanistic, and clinical burden all over the world. In Egypt, the burden of liver diseases has been increasing with a doubling of the incidence rate in the past twenty years. This has been attributed to several biological (e.g. virus infection) and environmental/dietary factors (e.g. Aflatoxin, polycyclic aromatic hydrocarbons) [4,5,6].Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides and heavy metals, and endemic infections in the community, as schistosomiasis, may have additional roles in the etiology or progression of the disease [7]. Several studies reported that cooking can produce toxic compounds in foods, if the appropriate precursors are present. Cooking processes have been found to be a major source of toxic compounds in foods such polycyclic aromatic hydrocarbons (PAHs). Although, PAHs can also be formed in curing and processing of raw food prior to [4] [8,9,10,11,12,13]. PAHs from incomplete combustion occur in several foods such as charcoal broiled and smoked goods [5,10] [14,15,16,17,18]. Benzo[*a*]pyrene (B[*a*]P) is a member of the family PAHs that is a by-product of incomplete combustion or burning of organic (carbon-containing) items, e.g., cigarettes, gasoline, and wood. B[*a*]P is commonly found with other PAHs in cigarette smoke, in grilled and broiled foods, and as a by-product of many industrial processes [4,5,13,19]. B[*a*]P is also found in outdoor and indoor air, and in some water sources [20]. Many of PAH compounds including B[*a*]P have been shown to be toxic, mutagenic and/or carcinogenic by extensive *in vivo* and *in vitro* experiments [4,11] [21,22,23,24,25,26,27]. Also, B[*a*]P exposure is associated with the development of liver cancer in all vertebrata [4,11] [24,28,29,30]. It is known that the toxic, tumorigenic and carcinogenic effects of B[*a*]P correlate with the cellular metabolism of this compounds to arene oxides, phenols, quinones, dihydrodiols, and epoxides and with their subsequent formation of reactive intermediates that interact covalently with DNA to form adducts [4] [31,32,33]. While the Fixation of a biochemical changes by cell proliferation is considered the next step. The mutagenicity of B[*a*]P is dependent upon metabolic activation. So, B[*a*]P is considered a promutagen [4] [30,34].

The modern pharmaceutical therapy is costly and associated with multiple side effects resulting in patient non-compliance. For that, there is a need to explore alternative therapies particularly from natural sources as these are cost effective and possess minimal side effects. It was reported that some plant parts are containing different categories of bioactive compounds and exhibited various biological roles including antioxidant, anti-inflammatory, antidiuretic, anticarcinogenic, antimutagenic etc [35,36,37,38,39,40].

Milk thistle (MT, *Silybum marianum* L., Family: *Asteraceae*) is one of the least studied plants yet (Figure 1). It is an annual/biennial plant native of Mediterranean area and now growing and cultivated worldwide including Egypt [41,42]. Plants are gathered each year for their seeds, some for cultivation in the following season and some for consumption. *Silybum marianum* has been used for centuries in medicine, mainly to treat kidney, spleen, liver, and gallbladder diseases [43]. Such as reviewed by Abenavoli *et al.*, [44], physician, pharmacologist and naturalist in the old ages used *Silybum marianum* as juices to remove the bile, as extract to treat liver ailments, as prescriptions against all melancholy diseases and to cure fever, and as prescriptions recommended for varicose veins, menstrual problems, and congestion of the liver, spleen and kidney. *Silybum marianum* is therefore among the best-selling herbal nutritional supplements worldwide [45]. Plant fruits/seeds contain a mixture of flavonolignans collectively known as silymarin. It is a complex mixture of polyphenolic molecules, including seven closely related flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin) and one flavonoid (taxifolin) [46,47]. Traditional *Silybum marianum* extract is made from the seeds, which contain approximately 4–6% silymarin [48]. The extract consists of about 65–80% silymarin and 20–35% fatty acids, including linoleic acid. Regarding the beneficial properties of *S. marianum* and main constituent, silymarin in the treatment of dyslipidemia, diabetes, coronary heart disease and obesity, different animal and human studies have been discussed suggesting that it could be a good candidate in the therapy of these metabolic syndrome disease [49,50,51,52,53]. Also, different pharmacological functions of silymarin were reported in liver diseases: antioxidant, antifibrotic regenerative (stimulate hepatic regeneration, choleretic, hepatoprotective, immunostimulating, and anti-inflammatory [27,40,44]. However, previous literature data suggest that although the preclinical data are encouraging, more well-designed trials are needed to demonstrate the true value of *Silybum marianum* extracts in the treatment of liver disease and associated molecular complications. Issues attributable to liver disease create a huge economic, humanistic, and clinical burden all over the world including Egypt. Reducing liver disease could help dramatically decrease the catastrophic health effect of it which in turn decreases mortality and DALYs lost. Therefore, the current study aims to prepare the ethanolic extract from the seeds of *Silybum marianum* and then investigate the potential protective effects of this extract on biochemical and molecular disorders in liver induced by benzo[*a*]pyrene in experimental rats.

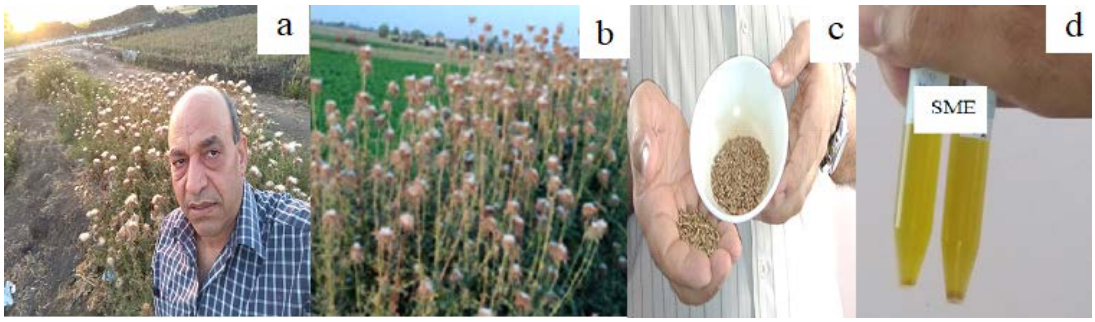

Figure 1. a. Milk-thistle (*Silybum marianum*) plant grows wild on the canals of Met Ghorab Village, Sinbellaween Center, Dakhlia Governorate, Egypt, b. its ripe fruits, c. its seeds, and d. its seeds methanolic extract

2. Material and Methods

2.1. Material

2.1.1. Silybum Marianum Seeds

Mature fruits of wild *Silybum marianum* L. were gathered from the shores of irrigation canals, Met Ghourab Village, Sinbellaween Center, Dakhlia Governorate, Egypt. The fruits were botanically confirmed by plant taxonomy professors, Faculty of Agriculture, Menoufia University, Shebin El-Kom, Egypt. The seeds were removed manually from dry fruits, purified of impurities, dried in lab oven at 50 ^{0}C for 10 h, and kept in sealed polyethylene pages until use.

2.1.2. Reagents and CHEMicals

Benzo[*a*]pyrene (B[*a*]P) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Basal diet constituents (casein, vitamin and minerals mixture etc.) supplied by Morgan Chemical Company, Cairo, Egypt. All other chemicals, reagents and solvents (Except as otherwise stated) in analytical grade were purchased from El-Ghomhorya Company for Trading Drug, Chemicals and Medical Instruments, Cairo, Egypt.

2.1.3. Kits

Kit's assays for Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, glycogen, malondialdehyde (MDA), serum lipids profile (triglycerides, TGs; total cholesterol, TC; high density lipoprotein cholesterol, HDL-c) were purchased from BIODIAGNOSTIC, Dokki, Giza, Egypt. Reduced glutathione (GSH) was assayed by the kits provided by MyBioSource, Inc., San Diego, CA, USA).

2.1.4. Machines

UV-visible-light spectrophotometer (UV-160A; Shimadzu Corporation, Kyoto, Japan) was used for all biochemical analysis.

2.1.5. Animals and Housing

Animals used in this study, adult male albino Sprague-Dawley rats (170±7.42g per each) were obtained from the Laboratory Animal Unit, College of Veterinary Medicine, Cairo University, Egypt. Rats were kept in stainless steel cages under typical laboratory conditions (22±2.5°C temperature, 60±5.9 % relative humidity, and a 12/12 hour light-dark cycle) and kept under normal healthy conditions. All rats were fed on basal diet (BD) for one-week before starting the experiment for acclimatization.

2.1.6. Standard/Basal Diet (BD)

The basic diet prepared according to the following formula as mentioned by Reeves *et al.*, [54] as follow: protein (10%), corn oil (10%), vitamin mixture (1%), mineral mixture (4%), choline chloride (0.2%), methionine (0.3%), cellulose (5%), and the remained is corn starch (69.5%). The used vitamin (Table 1) and minerals (Table 2) mixtures component were formulated according to Reeves *et al.*, [54].

2.2. Methods

2.2.1. *Silybum Marianum* Seeds Ethanolic Extract (SME)

SME was prepared according to the method of Oludemi *et al.*,[55], In brief, *Silybum marianum* seeds were ground in high miller speed (Moulinex Egypt, Al-Araby Co., Egypt) and reduced to powder (20 mesh) and mixed to obtain homogeneous samples. A 5g of seeds powder were extracted in a Soxhlet apparatus (Soxhelt Semiautomatic apparatus Velp company, Italy) for 4-5 h (approximately 20 min per cycle) using 95% ethanol. The solvent was evaporated under reduced pressure (rotary evaporator Büchi R-210, Switzerland) at 40°C and 50 rpm to obtain the dried solvent extract and kept at 4°C until use. The total yield of SME was 6.02% (w/w) in terms of the *Silybum marianum* seeds.

2.2.2. Experimental Design

All biological experiments performed a complied with the rulings of the Institute of Laboratory Animal Resources, Commission on life Sciences, National Research Council [56]. After the acclimatization period (7 days), rats (n=36), were randomly assigned to six groups of 6 rats per each. Group 1: served as normal control, was fed on the BD and given corn oil intraperitoneally at a dosage of (10 ml/kg/b.wt.) on the seventh day only; Groups 2–6: were injected intraperitoneally with a single dose of B[*a*]P (125 mg/kg/b.wt. in corn oil) on the seventh day only following the method described by Shahid *et al.*, [57], and were fed on the BD. After 14 days of treatment with B[*a*]P, group (2) acted as a model control, while groups (3-6) received SME at concentrations of (200, 400,

600, and 800 mg/kg bw/d) by oral gavage for 28 days each respectively.

2.2.3. Blood and Liver Tissues Sampling

At the end of the experiments, the rats were slaughtered under diethyl ether anesthesia after 12 hours of fasting. Blood samples were obtained through the abdominal aorta and placed in centrifuge tubes then serum was carefully separated after centrifugation at 3000 rpm for 10 minutes to assess the biochemical parameters such as described by Stroev and Makarova, [58].Specimens of the liver organ were taken immediately after sacrificing rats and immersed in 10% neutral buffered formalin for the histological examination. Another specimen of the liver samples was promptly snap-frozen in liquid nitrogen before being kept at -70°C for molecular analysis.

2.2.4. Biochemical Analysis

Different tested parameters in serum were determination using the specific methods as follow: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities according to Yound, [59], Tietz, [60], and Yound, [59], respectively. Albumin was determined by a colorimetric method described by Vanessa *et al.*, [61]. Glycogen in liver tissue was determines such as described by Shokri-Afra *et al.*, [62]. Triglycerides (TGs), Total cholesterol (TC) and HDL-Cholesterol were determined in serum according to the methods of Fossati and Prenape [63], Richmod [64], and Lopes-Virella *et al.*, [65] respectively. Reduced glutathione (GSH) was measured colorimetrically in serum samples such as described by Ellman, [66]. Serum malonaldialdehyde (MDA) content was measured by the thiobarbituric acid (TBA) method according to the methods of Buege and Aust, [67].

2.2.5. Molecular Studies

2.2.5.1. Flow cytometer Machine

The flow cytometer used is FACS calibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact air cooked low power 15 mwatt argon ion laser beam (488 nm).

2.2.5.2. Preparation of Single Cells from Tissue

Fresh tissue specimens from different groups were prepared according to Tribukait *et al.*, [68]. Briefly, liver was homogenated and suspended in Phosphate Buffer Saline (PBS) then, the cell suspension was centrifuged at 2000 rpm for 10 mins, where upon the supernatant was aspirated. Flow- cytometry analysis was performed on single cell suspensions washed three times with PBS (pH 7.2). After washing with PBS, the cell viability was determined by flow cytometry.

2.2.5.3. Flow Cytometry Analysis of Apoptosis and Cell Cycle

Single cell suspensions of liver tissue was used for determination of apoptosis and cell cycle. The cell viability was determined by flow cytometry and apoptosis was measured by using the sub G1 peak staining with propidium Iodide [69]. The average number of evaluated nuclei per specimen 20.000 and the number of nuclei scanned was 120 per second. DNA histogram derived from flow cytometry was obtained with a computer program according to Dean and Jett mathematical analysis [70]. Data analysis was conducted using DNA analysis program MODFIT (verity software house, Inc. Po Box 247, Topsham, ME 04086 USA, version: 2.0, power Mac with 131072 KB Registration No.: 42000960827-16193213 Date made: 16-Sep., 1996).

2.2.6. Histopathological Examination

Specimens of liver were taken immediately after sacrificing rats and immersed in 10% neutral buffered formalin. The fixed specimens were then trimmed and dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin, sectioned (4-6 µm thickness), stained with hematoxylin and eosin and examined microscopically [71].

3. Results and Discussion

3.1. Effect of Intervention with *Silybum Marianum* Seeds Ethanolic Extract (SME) OnLiver Function Disorders Induced by B[*A*]P In Rats

Effect of SME intervention on liver functions disorder induced by B[*a*]P in rats were shown in Table 1 and Figure 2. From such data it could be noticed that B[*a*]P-injected rats exhibiting significantly ($p \leq 0.05$) increased levels of AST (126.07%), ALT (120.24%) and ALP (142.32%) compared to the normal group. Intervention with SME (200, 400, 600 and 800 mg/kg bw/day) for 28 days significantly ($p \leq 0.05$) decreased the levels of these enzymes activities by the rate of 111.19, 93.51, 41.88 and 27.98% (for AST); 100.03, 81.15, 50.51 and 18.61% (for ALT), and 123.51%, 105.64, 75.80 and 42.43% (for ALP), compared to the normal controls, respectively. The rate of decreasing in all of these parameters exhibited a dose-dependent increase with the SME intervention. B[*a*]P-induced liver damage is commonly used to experimentally study the hepatoprotective effects of drugs and natural extracts [25,27,37] [72,73,74]. Such liver damage came with the cellular metabolism of this compound to arene oxides, phenols, quinones, dihydrodiols and epoxides, and with their subsequent formation of reactive intermediates/radicals that react covalently with nucleic acids to form adducts [4] [22,31]. While the fixation of a biochemical changes by cell proliferation is considered the next step, some of these reactive intermediates/radicals subsequently cause lipid peroxidation of membrane-bound fatty acids [5,10,19]. Additionally, the structure and function of the cell membrane and intracellular organelles (mitochondria and lysosomes) of the hepatocyte become disrupted [4,75,76]. In the present study, B[*a*]P injection induced severe damage to liver cells, demonstrated by increased the serum AST, ALT and ALP level activities. Such enzymes activity measurement are often considered sensitive markers for determining the course of liver damage due to their presence of the cytoplasm facilitates blood flow after

liver cell damage [77,78,79]. The present data also indicated that SME significantly ($p \leq 0.05$) reduced AST, ALT and ALP levels which demonstrating that it could prevent the live cells damage. Such preventive effects could be attributed to the SME high content of some important bioactive components that have been measured by previous studies [27,80]. The bioactive compounds content of SME indicated that silymarin was the most largest compound followed by phenolics , flavonoids, tannins, chlorophyll, carotenoids and anthocyanin's . Thus, SME samples exhibited several high biological activities which include inhibition of low density lipoprotein (LDL) oxidation and scavenging of free radicals. Also, Mahran and Elhassaneen, [27] reported that extracts contained the same bioactive compounds exhibit protective activities against liver disorders induced by B[*a*]P.

Table 1. Effect of intervention with Silybum marianum seeds ethanolic extract (SME) on liver functiondisorders induced by B[*a*]P in rats

Groups	Aspartate aminotransferase (AST)		Alanine aminotransferase (ALT)		Alkaline phosphatase (ALP)	
	Value (IU/L)	% of change	Value (IU/L)	(% of change)	Value (U/L)	(% of change)
Normal control	63.45 ± 5.32 ^{d}	0.00	41.22 ± 2.40 ^{d}	0.00	131.67 ± 11.18 ^{f}	0.00
Modelcontrol	143.44 ± 3.10^{a}	126.07	90.78 ± 5.17 ^{a}	120.24	319.06 ± 19.65 ^{a}	142.32
SME-1	134.00 ± 3.70 ^{ab}	111.19	82.45 ± 3.63 ^{ab}	100.03	294.30 ± 13.46 ^{ab}	123.51
SME-2	122.78 ± 5.97 ^{b}	93.51	74.67 ± 3.88 ^{b}	81.15	270.77 ± 21.48 ^{b}	105.64
SME-3	90.02 ± 5.34 ^{c}	41.88	62.04 ± 4.34 ^{c}	50.51	231.48 ± 15.92 ^{c}	75.80
SME-4	81.20 ± 3.80 ^{cd}	27.98	48.89 ± 3.30 ^{d}	18.61	187.54 ± 17.33 ^{d}	42.43

Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

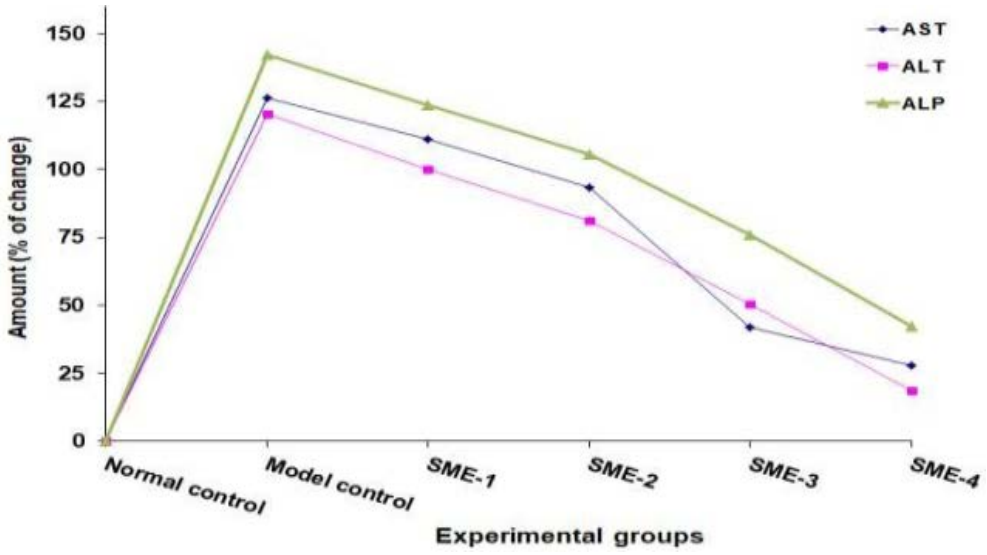

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Figure 2. Effect of intervention with Silybum marianum seeds ethanolic extract (SME) on liverfunction disorders induced by B[a]P in rats

3.2. Effect of Intervention with *Silybum Marianum* Seeds Ethanolic Extract (SME) on Liver Glycogen Changes Induced by B[*A*]P In Rats

Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liver glycogen changes induced by B[*a*]P in rats were shown in Table 2 and Figure 3. Such data indicated that B[*a*]P induced significantly ($p \leq 0.05$) decreasing in liver glycogen content by the ratio of -71.40%. Intervention with SME (200, 400, 600 and 800 mg/kg bw/day) for 28 days significantly ($p \leq 0.05$) leads to decrease the levels of liver glycogen by the rate of -64.68, -56.68, -45.43 and -37.13% compared to the normal controls, respectively. The rate of elevation in glycogen exhibited a dose- dependent increase with SME intervention. Such data are in agreement with that obtained by Fayez, [37] and Elhassaneen *et al.*, [29] as the result of treatment of liver injuries inducing by B[*a*]P with turmeric and curcumin powders. Also, Badawi, [81] recorded the same results with reishi mushroom (*Ganoderma lucidum*) extract. In general, glycogen is represents a complex sugar necessary for production of nutrients and energy in the body. Its storage disorders i.e. glycogen storage disease (GSD) are genetic conditions seen in pediatric population (birth and childhood) which impair the ability of the liver to store and metabolize glycogen [82]. Such disease, GSD, cause varying degrees of liver enzyme abnormalities and also affects the muscles

and other areas of the body. In similar study, Hasegawa *et al.*, [83] found that previous drinking of green tea clearly protected against the changes in liver glycogen content induced by B[*a*]P treatment. Also, Mehram and Sayed Ahmed [26] reported data similar to what were obtained in the current study with mulberry leaves (*Morus alba* L.), and also demonstrated that their protective effects against B[*a*]P could be attributed to their high content of active compounds biologically similar to what is found in SME. Data of the present study with the others suggested that the energy depletion suggested by the marked decrease in glycogen content probably leads to block/hinder the secretion of different biological constituents from the liver to blood such as lipid particles.

Table 2. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liver glycogen changes induced by B[*a*]P in rats

Groups	mg/g tissue	% of control
Normal control	13.25 ± 1.89 [a]	0.00
Model control	3.79 ± 0.97 [d]	-71.40
SME-1	4.68 ± 0.78 [cd]	-64.68
SME-2	5.74 ± 0.92 [c]	-56.68
SME-3	7.23 ± 0.77 [bc]	-45.43
SME-4	8.33 ± 0.90 [b]	-37.13

Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

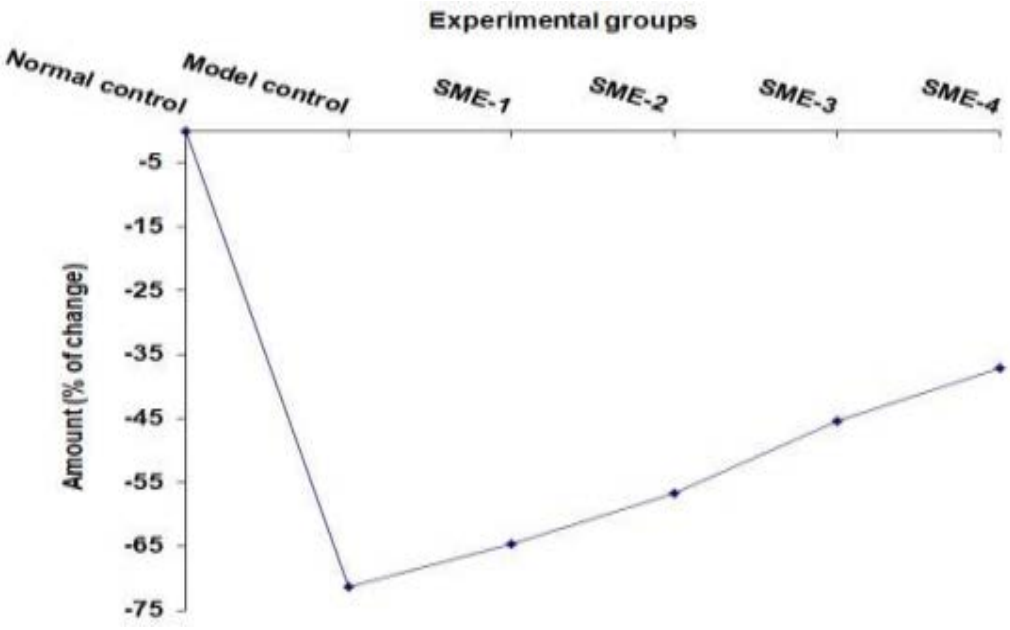

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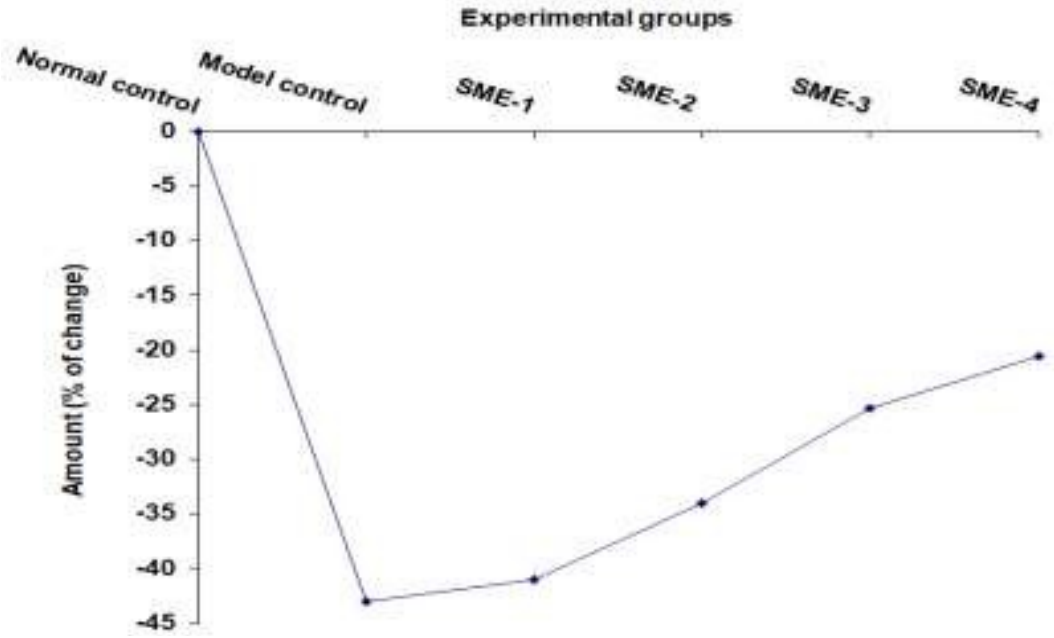
Figure 3. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liverglycogen changes induced by B[*a*]P in rats

Table 3. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on serum albumin changes induced by B[*a*] P in rats

Groups	g/dL	% of control
Normal control	5.02 ± 0.22 [a]	0.00
Model control	2.86 ± 0.14 [c]	-43.03
SME-1	2.96 ± 0.12 [c]	-41.04
SME-2	3.31 ± 0.20 [bc]	-34.06
SME-3	3.75 ± 0.25 [b]	-25.30
SME-4	3.99 ± 0.17 [b]	-20.52

Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.



Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[a]P (Benzo[a]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

Figure 4. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on serum albumin changes induced by B[*a*]P in rats

3.3. Effect of Intervention with *Silybum Marianum* Seeds Ethanolic Extract (SME) on Serum Albumin Changes Induced by B[*A*]P in Rats

Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on serum albumin changes induced by B[*a*]P in rats were shown in Table 3 and Figure 4. Such data indicated that B[*a*]P induced significantly ($p \leq 0.05$) decreasing in serum albumin level by the ratio of -43.03%. Intervention with SME (200, 400, 600 and 800 mg/kg bw/day) for 28 days significantly ($p \leq 0.05$) leads to increase the levels of serum albumin by the rate of -41.04, -34.06, -25.30 and -20.52% compared to the normal controls, respectively. The rate of elevation in serum albumin exhibited a dose- dependent increase with SME intervention. Such data are in agreement with that obtained by Mahran *et al.*, [73]as the result of treatment of liver injuries inducing by B[*a*]P with red onion powder. Also, Wang *et al.*, [84] showed that B[*a*]P induced significant ($p \leq 0.05$) decrease in the serum albumin content. Generally, albumin is an important metal binding protein. It is a sacrificial antioxidant that can bind copper tightly and iron weakly to its surface serving as a target for their related free radical reactions. Thus, it inhibits copper ion dependent lipid peroxidation[85]. It was reported that hypoalbuminaemia is most frequent in the presence of advanced chronic liver diseases. Hence, decline in total protein and albumin can be deemed as a useful index of the severity of cellular dysfunction in chronic liver diseases. In similar study, treatment with onion skin significantly decreased the reduced levels of serum albumin induced by B[*a*]P [29].

3.4. Effect of Intervention with *Silybum Marianum* Seeds Ethanolic Extract (SME) on Serum Lipid Profile Disorders Induced by B[*A*] P in Rats

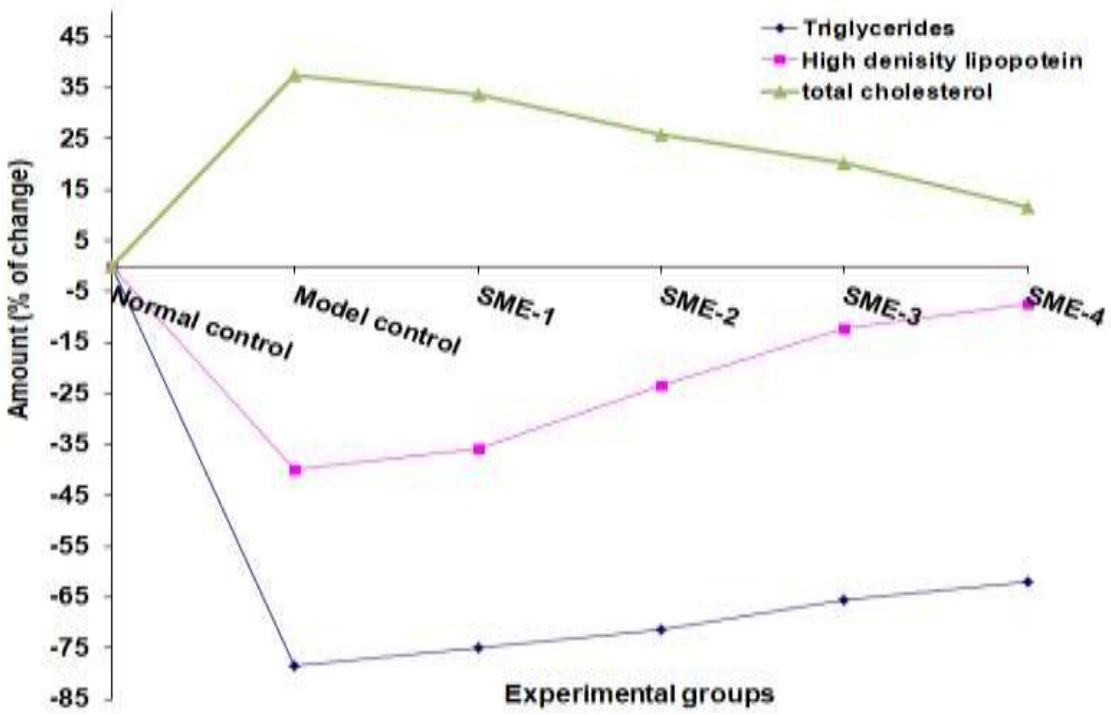
Effect of SME intervention on serum lipid profile disorders induced by B[*a*]P in rats were shown in Table 4 and Figure 5. Such data indicated that B[*a*]P induced significantly ($p \leq 0.05$) decreasing in serum TG and HDL levels by the ratio of -78.48 and -39.98 % whereas, the serum total cholesterol concentration was exhibited the opposite direction. As the result of SME, serum TGs and HDL were significantly ($p \leq 0.05$) increased and total cholesterol was significantly ($p \leq 0.05$) declined. The rate of increasing in TGs and HDL as well as declined in cholesterol exhibited a dose-dependent increase with the SME intervention. Such data are in agreement with the obtained with Fayez [37] as the result of treatment of liver injuries inducing by B[*a*]P with turmeric and curcumin powders. In general, TGs are the most common type of fat in the body. They come from foods and extra calories. When the body needs energy it releases the TGs [2]. Very low density lipoprotein (VLDL) cholesterol particles carry the TGs to the tissues. Having a high level of TGs can raise your risk of heart diseases, such as coronary artery disease. High blood TGs levels can be genetic, or caused by diabetes, thyroid problems, kidney disease, or some medicines [86]. The composition of human diet plays an important role in the management of lipid, cholesterol and lipoprotein concentrations in blood. Reduction in saturated fat and cholesterol intake has traditionally been the first goal hypocholesrerolemic effects

of several dietary components, such as found in SME including phenolics, flavonoids, silymarin, anthocyanins, alkaloids, carotenoids etc., have attracted much interest. [76,80] [87,88,89] Also, such bioactive compounds exerts their beneficial effects on cardiovascular health by antioxidant, radicals scavenging and anti-inflammatory activities. With the same context, Hasegawa et al., [83] found that previous drinking of green tea with high content of the previous bioactive compounds clearly protected against the changes in serum TGs level. Also, Asai and Miyazawa, [90] indicated that dietary curcuminoid lowered liver cholesterol and triacylglycerol, and plasma triacylglycerol. Furthermore, Chuengsamarn et al., [91] found that curcumin lowers the atherogenic risks by reducing the insulin resistance, triglyceride, visceral fat and total body fat. In addition, Mehram and Sayed Ahmed [26] recorded results similar to what were obtained in the current study with mulberry leaves (Morus alba L.), and also demonstrated that their protective effects against B[a]P could be attributed to their high content of active compounds biologically similar to what is found in SME. Data of the present study with the others interpreted the decreasing of the serum TGs and HDL as the result of B[a]P injection by secretion of their components from liver to blood might be blocked because of intracellular structural failure and/or because of the energy depletion suggested by the marked decrease in glycogen content.

Table 4. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on serum lipid profile disorders induced by B[*a*]P in rats

Groups	Triglycerides (TG's)		Total cholesterol (TC, mmol/L)		High density lipoprotein (HDL-c, mmol/L)	
	Value (mmol/L)	% of change	Value (mmol/L)	% of change	Value (mmol/L)	% of Change
Normal control	138.32 ± 10.67 ^{a}	0.00	118.77 ± 7.39 ^{c}	0.00	44.02 ± 5.78 ^{a}	0.00
Model control	29.76 ± 4.22 ^{d}	-78.48	163.27 ± 7.69 ^{a}	37.47	26.42 ±5.99 ^{c}	-39.98
SME-1	34.54 ± 5.01 ^{c}	-75.03	158.74 ± 9.48 ^{a}	33.65	28.25 ± 3.99 ^{bc}	-35.82
SME-2	39.54 ± 4.12 ^{bc}	-71.41	149.56 ± 5.90 ^{ab}	25.92	33.73 ± 5.15 ^{b}	-23.38
SME-3	47.56 ± 5.77 ^{b}	-65.62	142.91 ± 6.04 ^{b}	20.32	38.60 ± 5.30 ^{ab}	-12.31
SME-4	52.54 ± 6.04 ^{b}	-62.02	132.50 ± 8.34 ^{bc}	11.56	40.71 ± 4.69 ^{a}	-7.52

Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight



Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

Figure 5. Effect of intervention with Silybum marianum seeds ethanolic extract (SME) on serum lipid profile disorders induced by B[*a*]P in rats

Table 5. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liver oxidative stress parameters induced by B[*a*]P in rats

Groups	Reduced glutathione concentration (GSH)		Malondialdehyde concentration (MDA)	
	Value (µmol/mg tissue protein)	% of change	Value (nmol/mg tissue protein)	% of change
Normal control	9.18 ± 1.13 [a]	0.00	2.96 ± 0.30 [d]	0.00
Model control	5.94 ± 1.03 [c]	-35.29	9.63 ± 0.64 [a]	225.34
SME-1	6.19 ± 0.89 [c]	-32.57	9.01 ± 0.53 [a]	204.39
SME-2	6.81 ± 1.33 [bc]	-25.82	8.03 ± 0.43 [ab]	171.28
SME-3	7.56 ± 0.56 [b]	-17.65	5.74 ± 0.32 [b]	93.92
SME-4	7.98 ± 0.60 [ab]	-13.07	4.01 ± 0.52 [c]	35.47

Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

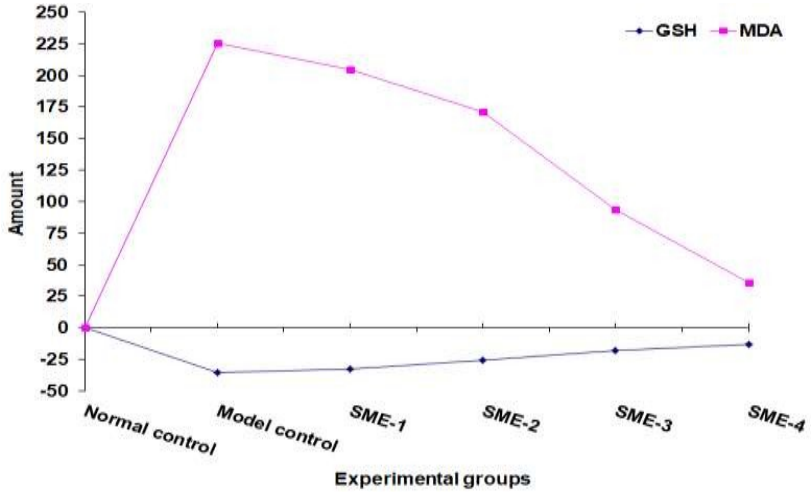
3.5. Effect of Intervention with Silybum Marianum Seeds Ethanolic Extract (SME) on Liver Oxidative Stress Parameters Induced by B[A]P in Rats

Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liver oxidative stress parameters induced by B[*a*]P in rats were shown in Table 5 and Figure 6. Such data indicated that B[*a*]P induced significantly (p≤0.05) decreasing in liver GSH level by the ratio of -35.29% whereas, the liver MDA concentration was exhibited the opposite direction, increased by the rate of 225.34%. As the result of SME, liver GSH content significantly (p≤0.05) increased and MDA was significantly (p≤0.05) declined. The rate of increasing in GSH and declined in MDA exhibited a dose- dependent increase with the SME intervention. Such data are in agreement with the obtained with Fayez [37] as the result of treatment of liver injuries inducing by B[*a*]P with turmeric and curcumin powders. GSH is a tripeptide-thiol (γ-glutamyl cysteinyl-glycine) that has received considerable attention in terms of its biosynthesis, regulation, and various intracellular functions [4] [92,93]. Among of those functions, its role in detoxifications process represent the central role through as a key conjugate of xenobiotics electrophilic intermediates and as an important antioxidant. The antioxidant functions of GSH include its role in the activities of the antioxidant enzymes system such glutathione peroxidase and glutathione reductase. In addition, GSH can apparently serve as a nonenzymatic scavenger of oxyradicals i.e. reactive oxygen species (ROS) [94,95]. The present data revealed that exposing rats to B[*a*]P generated ROS, as demonstrated by decreased GSH levels. These findings are in accordance with that reported by Elhassaneen *et al*., [96], who found that treatment of rats with B[*a*]P resulted in a significant (p≤0.05) reduction in GSH by the rate of -38.35% as compared to normal rats. Also, Elhassaneen [5] and Elhassaneen and El-Badawy [19] found that GSH levels in human erythrocytes were reduced significantly (p≤0.05) after exposure/ingested to B[*a*]P. According to Galkina *et al*., [97] and Mahran and Elhassaneen [27], the GSH level may fall owing to decreased glutathione reductase (GSH-Rd) activity which increases the antioxidant activities of glutathione-*S*-transferase and glutathione peroxidase (GSH-Px), both of which employ GSH as a cofactor in their processes. On the other side, clinical evidences for B[*a*]P-associated oxidative stress have been provided by measurement of either biomarkers or end-products of free radical-mediated oxidative processes [5]. For instance, lipid peroxidation markers such as MDA represent one of the most important compound and major product of the oxidation of polyunsaturated fatty acids. MDA found to be increased in plasma patients exposed to/ingested B[*a*]P [5]. Also, interest in the possible significance of MDA on human health has been documented several decades ago by reports that are mutagenic and carcinogenic compound [98]. On the other side, several studies have reported that the antioxidant activities of bioactive constituents such as measured in SME where by mitigation of lipid peroxidation and oxidative stress in several tissues were demonstrated [29,73]. Therefore, data of the present study prove that SME is a promising successfully tool in the prevention of oxidative stress caused by exposure of cells to B[*a*]P.

3.6. Effect of Intervention with *Silybum Marianum* Extract (SME) on Apoptosis and Cell Cycle of Liver Tissue of B[*A*]P Injected Rats

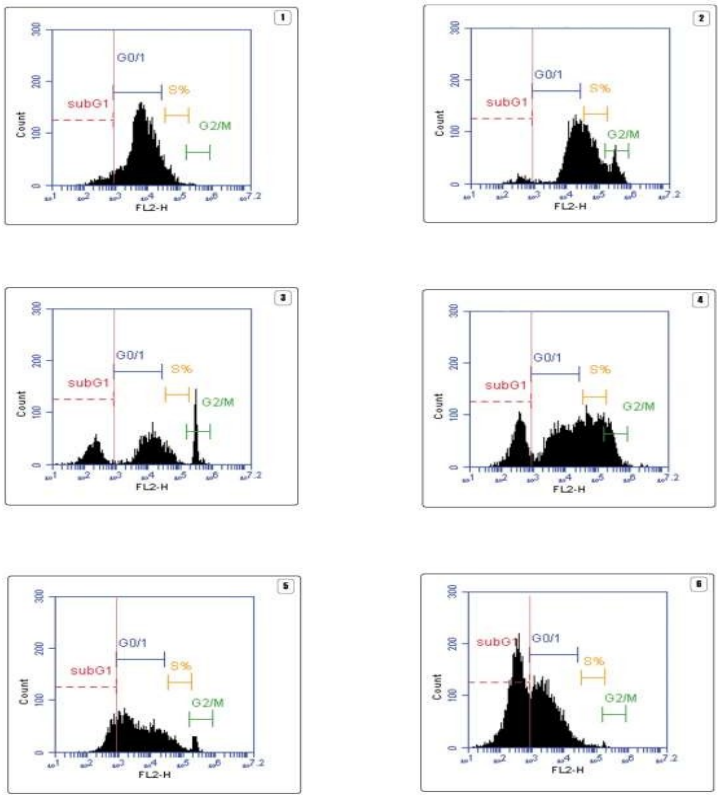
As shown in Table 6 and Figures 7 and 8, by using flow cytometer analysis, result indicated a significant (P≤0.05) decrease in mean percentage of apoptosis in rats injected by B[*a*]P (4.2±0.6) compared with normal control (6.5±0.68). On contrast, rats injected by B[*a*]P and treated with SME (200; 400; 600 and 800 mg/kg) showed significantly increase in mean percentage with values 25.7±1.2, 21±1.1, 17±1.2 and 39.5±1.6, respectively when compared to B[*a*]P rats. Rats injected by B[*a*]P showed a significant decrease in mean percentage of G0/1 with value 30.8±1.6 as compared with normal rats (82.7±1.9). While, SEM treated rats indicated significant increase in mean percentage 0f G0/1 as compared with B[*a*]P group. But, B[*a*]P injected rats showed significant increase in mean percentage of S and G2/M (52.5±2.1 and 12.5±0.4, respectively) when compared to normal group. On the other hand, mean percentage of S phase of SME (200; 400; 600 and 800 mg/kg) treated rats illustrated significant decrease (11±1.4, 27±1.7, 11±1.2 and 9±0.6, respectively) compared to B[*a*]P rats. Also, SME (200 and 400 mg/kg) treated rats indicate significantly increase in mean percentage of G2/M phase as compared with B[*a*]P group. This result agree with Michurina *et al*., [99] who indicated that Intraperitoneal injection of B[*a*]P (in a total dose of 60 mg/kg body weight) reduced activity of nuclear

endonucleases in the liver cells, which attests to inhibition of apoptosis by the nuclear pathway. The previous studies illustrated that BaP up-regulated the expression of Ki67 and PCNA, affecting the differentiation of stromal cells. BaP induced polyploid cells deficiency with enhanced expressions of CyclinA(E)/CDK2, CyclinD/CDK4 and CyclinB/CDK1, which promote the transformation of cells from G_1 to S phase and simultaneously activate the G_2/M phase by using cell cycle progression analysis during decidualization *in vivo* and *in vitro* [100]. On the other hand, Silybin (also known as silibinin), a major bioactive component of milk thistle (*Silybum marianum*), has long been used for the prevention hepatic damage[101]. Lee *et al.*, [102] reported that Silybin induced cell cycle arrest at the G1 phase and inhibited melanoma cell growth *in vitro* and *in vivo*. Silybin suppresses melanoma growth by directly targeting MEK- and RSK-mediated signaling pathways.



Each value represents mean ±SD (n=6). Values under the same column with different superscripts mean significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight; GSH, glutathione; MDA, malonaldehyde.

Figure 6. Effect of intervention with *Silybum marianum* seeds ethanolic extract (SME) on liveroxidative stress parameters induced by B[*a*]P in rats



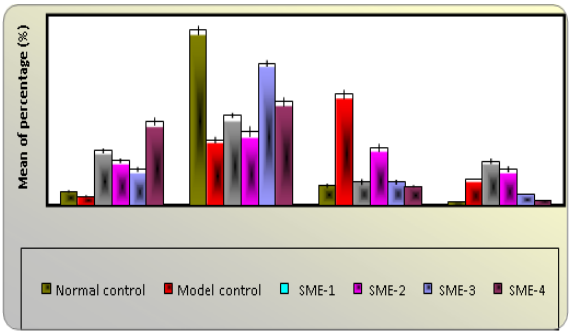
1) Normal control; 2) benzopyrene (B[*a*]P) injected rats; 3) B[*a*]P injected rats and treated with SME (200mg/kg bw/day); 4) B[*a*]P injected rats and treated with SME (400mg/kg bw/day); 5) B[*a*]P injected rats and treated with SME (600mg/kg bw/day);6) B[*a*]P injected rats and treated with SME (800mg/kg bw/day). Sub G1 = percentage of apoptosis

Figure 7. Flow cytometry analysis of apoptosis and cell cycle of liver tissue of B[*a*]P injected rats andtreated with *Silybum marianum* extract (SME)

Table 6. Effect of *Silybum marianum* extract (SME) treatment on apoptosis and cell cycle of livertissue of B[*a*]P injected rats

Groups	Apoptosis	G0/1	S	G2/M
Normal control	6.5±0.68	82.7±1.9	9.3±1	1.5±0.26
Model control	$4.2±0.6	$30.8±1.6	$52.5±2.1	$ 12.5±0.4
SME-1	*25.7±1.2	*42.5±1.2	*11±1.4	*20.8±1.3
SME- 2	*21±1.1	*35±2.5	*27±1.7	*17±1.4
SME- 3	*17±1.2	*67±1.5	*11±1.2	*5±0.3
SME- 4	*39.5±1.6	*49±2	*9±0.6	*2.5±0.13

Each value represents mean ±SD (n=6). *Significant difference compared to Benzopyrene group at ($P \leq 0.05$). $Significant difference compared to normal control. Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.



Each value represents mean ±SD (n=6). Normal control, healthy rats without intervention; Model control, B[*a*]P (Benzo[*a*]pyrene) induced hepatic disorders in rats without intervention; SME-1, SME-2, SME-3 and SME-4, B[a]P induced hepatic disorders in rats with SME intervention by 200, 400, 600 and 800 mg/kg bw/day, respectively; bw, body weight.

Figure 8. Effect of *Silybum marianum* extract (SME) treatment on apoptosis and cell cycle of liver tissue of B[*a*]P injected rats

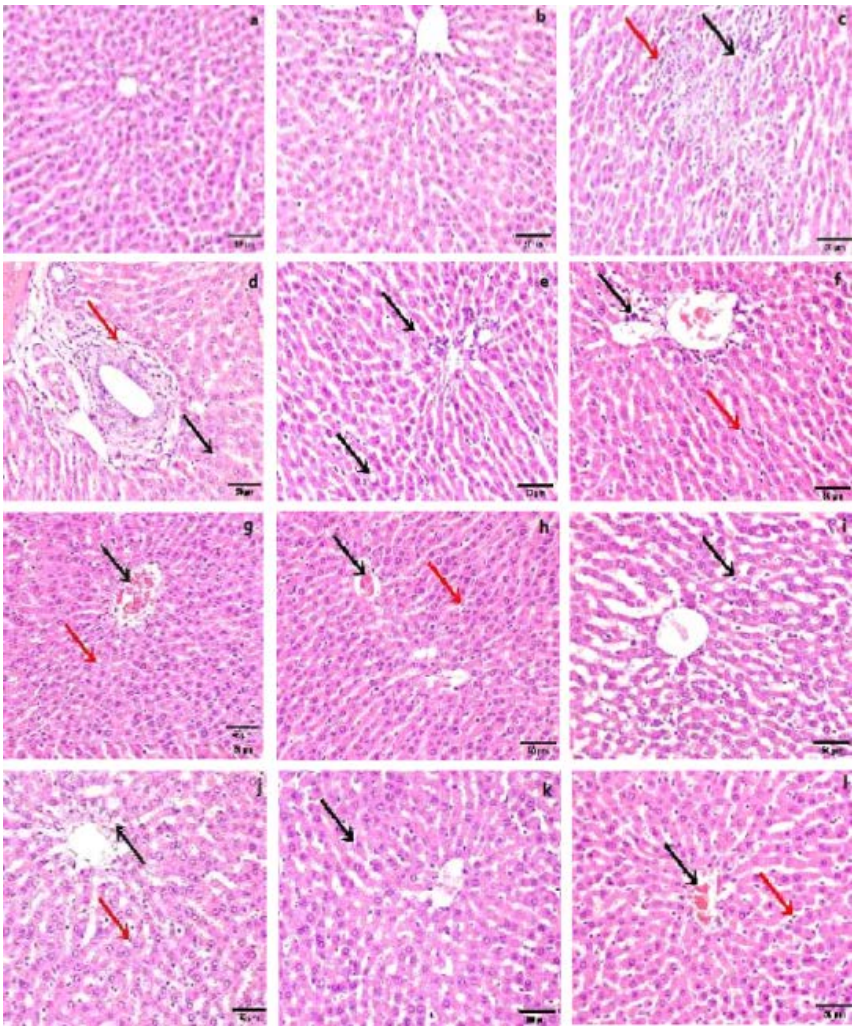


Figure 9. Effect of intervention with *Silybum marianum* extract (SME) on liver histopathological examination of hepatotoxic rats. (H & E X 400, scale bar 50µm). **Photo a)** group 1 showing the normal histoarchitecture of hepatic lobule, **Photo b)** group 1 showing the normal histoarchitecture of hepatic lobule, **Photo c)** group 2 showing focal hepatocellular necrosis and apoptosis (black arrow) associated with inflammatory cells infiltration (red arrow), **Photo d)** group 2 showing hepatocellular vacuolar degeneration (black arrow) and fibroplasia in the portal triad (red arrow), **Photo e)** group 3 showing hepatocellular vacuolar degeneration (black arrow). **Photo f)** group 3 showing small focal hepatocellular necrosis associated with inflammatory cells infiltration (black arrow) and Kupffer cells proliferation (red arrow), **Photo g**) group 4 showing slight congestion of central vein (black arrow) and Kupffer cells proliferation (red arrow), **Photo h)** group 4 showing slight congestion of central vein (black arrow) and Kupffer cells proliferation (red arrow), **Photo i)** group 5 showing slight Kupffer cells proliferation (arrow), **Photo j)** group 5 showing vacuolar degeneration of centrilobular hepatocytes (black arrow) and Kupffer cells proliferation (red arrow), **Photo k)** group 6 showing slight vacuolization of sporadic hepatocytes (black arrow), and **Photo l)** group 6 showing slight congestion of central vein (black arrow) and Kupffer cells proliferation (red arrow).

3.7. Effect of Intervention with *Silybum Marianum* Extract (SME) on Liver Histopathological Changes Induced by B[*A*]P in Rat

The effect of intervention with *Silybum marianum* extract (SME) on liver histopathological changes induced by B[*a*]P in rats was shown in Figure 9. Microscopic examination of liver sections of rats from group 1 revealed the normal histoarchitecture of hepatic lobule (Photos a and b). In adverse, liver of rats from group 2 showed focal hepatocellular necrosis and apoptosis associated with inflammatory cells infiltration (Photo c), hepatocellular vacuolar degeneration and fibroplasia in the portal triad (Photo d). Meanwhile, liver of rats from group 3 manifested hepatocellular vacuolar degeneration (Photo e) and Kupffer cells proliferation (Photos e and f). On the other hand, liver of rats from group 4 showed slight congestion of central vein and Kupffer cells proliferation and necrosis of sporadic hepatocytes (Photos g & h).. Otherwise, improved picture was noticed in liver tissue of rats from groups 5 and 6. Briefly, examined sections from group 5 showed slight Kupffer cells proliferation (Photos i and j), vacuolar degeneration of centrilobular hepatocytes (Photo j). Furthermore, liver of rats from group 6 exhibited slight vacuolization of sporadic hepatocytes (Fig. k), slight congestion of central vein and Kupffer cells proliferation (Photo l). Such data are in accordance with several authors who observed that *Silybum marianum* seeds feeding ameliorate the histopathological changes in liver of rats induced by carbontetrachloride which due to the antioxidant activities exhibited by such plant part [40]. Also, Kolade and Oladiji, [103] reported that treatment of B[a]P induced toxic effects with curcumin helped to restore the normal histological architecture of the liver tissues. Curcumin has been found to manifest its protective benefits against severe histopathological damage via its powerful antioxidant property, whereby it clears off oxygen free radicals.

4. Conclusion

B[*a*]P is considered as a ubiquitous environmental and food contaminants as well as a top risk factor in the development of liver diseases. Intervention with *Silybum marianum* seeds extract are able to prevent or inhibit liver injuries induced by chemical toxin i.e. B[*a*]P. The manipulation process includes improving the liver functions enzymes activities, glycogen and serum synthesis, oxidative stress parameters (GSH and MDA), serum lipid profile, and molecular and histological changes thereby adversely affecting the injuries process to the benefit of the biological system. We recommended that *Silybum marianum* seeds extract by the tested concentrations to be included in our daily dishes, drinks and pharmaceutical preparations/formulae.

Ethical Considerations

The ethical issues of this study was reviewed and approved by the Institutional Animal Care and Use Committee (ICUC, Approval # FGE723), Faculty of Science and Scientific Research Ethics Committee (SREC, Approval # 28-SREC-03-2022), Faculty of Home Economics, Menoufia University, Shebin El-Kom, Egypt.

Conflict of Interest

The authors declare that they have no conflict of interest in publishing this paper.

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Authors' Contribution

Yousif Elhassaneen participated in developing the study protocol, retrieving conceptual information, validating the results, statistical analysis and preparing a draft of the paper, performed a critical revision to structure the content intellectually, and gave approval for the final version to be published. Mona Elhefny conducted the experimental procedures and validations, data acquisition, compilation, analysis, and interpretation and also was involved in retrieving conceptual information and draft paper preparation. Tarek Afifi made significant contributions to the concept and design of the work and draft paper preparation. Asmaa Bayom participated in retrieving conceptual information, validating the molecular biology results, and preparing the draft of the paper.

Abbreviations

ALT, Alanine aminotransferase, ALP, alkaline phosphatase, AST, aspartate aminotransferase, B[*a*]P, benzo[*a*]pyrene, BD, basal diet; GSH, reduced glutathione, HDL-c, high density lipoprotein, MDA: malondialdehyde; ROS: reactive oxygen species; SM: *Silybum marianum*; SME: *Silybum marianum* seeds ethanolic extract, TC, total cholesterol, TG's, triglycerides.

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