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ASSESSING THE PROTECTIVE ROLE OF SILYMARIN FROM MILK THISTLE AGAINST BUSULFAN-INDUCED OXIDATIVE STRESS AND LIVER DYSFUNCTION IN EGYPTIAN RATS

[1]Dr. Amina Mahmoud El-Sayed

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Abstract

Busulfan, a chemotherapeutic agent widely used in conditioning regimens for hematopoietic stem cell transplantation, is known to induce oxidative stress and liver dysfunction. This study aimed to assess the protective role of Silymarin, an herbal extract from Milk Thistle (Silybum marianum), against Busulfan-induced liver damage and oxidative stress in Egyptian rats.

Thirty male Egyptian rats were divided into three groups: a control group, a Busulfan-treated group, and a Silymarin-treated group. The Busulfan-treated group received a single dose of Busulfan, while the Silymarin-treated group received Silymarin orally for 14 days before Busulfan administration. After 14 days, the rats were sacrificed, and liver tissues were collected for analysis.

Our results demonstrated that Busulfan administration led to a significant increase in oxidative stress markers, such as malondialdehyde (MDA) levels, and a decrease in antioxidant enzyme activities, including superoxide dismutase (SOD) and glutathione peroxidase (GPx). Moreover, Busulfan-induced liver damage was evident through elevated liver enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as histopathological changes.

Conversely, the Silymarin-treated group exhibited significantly lower MDA levels and improved antioxidant enzyme activities compared to the Busulfan-treated group. Furthermore, Silymarin pre-treatment ameliorated liver dysfunction, as evidenced by reduced ALT and AST levels and histological improvements in liver tissues.

In conclusion, Silymarin from Milk Thistle demonstrated a protective effect against Busulfan-induced oxidative stress and liver dysfunction in Egyptian rats. These findings suggest the potential utility of Silymarin as an adjunct therapy to mitigate the adverse effects of

[1] Department of Home Economics, Faculty of Specific Education, Zagazig University, Zagazig, Egypt

Busulfan in clinical settings, particularly in the context of hematopoietic stem cell transplantation. Further research is needed to elucidate the underlying mechanisms of Silymarin's protective actions and its clinical applicability.

1. Introduction

Busulfan, a chemotherapy drug, is a cell cycle non-specific alkylating antineoplastic agent, in the class of alkyl sulfonates. It is an alkylating agent that forms DNA-DNA interstrand crosslinks between the DNA bases guanine and adenine and between guanine and guanine [1]**.** DNA crosslinking prevents the replication of DNA across the cell by apoptosis, because DNA crosslinks cannot be repaired by cellular machinery [2]. It was approved by the US Food and Drug Administration (FDA) for treatment of chronic myeloid leukemia (CML) in 1999 and is still in use to a degree as a result of the drug's relative low cost [3]**.** Busulfan is used in pediatrics and adults as a conditioning agent prior to bone marrow transplantation, especially in CML and other leukemias, lymphomas, and myeloproliferative disorders [4, 5]. It can control tumor burden but cannot prevent transformation or correct cytogenic abnormalities. Also, Busulfan used to treat ovarian cancer [6]. Furthermore, it was used in a study to examine the role of platelettransported serotonin in liver regeneration its volume after major tissue loss [7]**.** In spite of this, busulfan has many dangerous side effects on different organs including the liver, skin, bladder, nervous system and gonadal function, and is probably carcinogenic and mutagenic. In this context, Grigg*, et al.* [8] and Brisse*, et al.* [4] reported that busulfan toxicity may include interstitial pulmonary fibrosis, hyperpigmentation, seizures, hepatic or sinusoidal obstruction syndrome emesis, and wasting syndrome. Also, Hayhoe and Kok [9] mentioned earlier that busulfan also induces thrombocytopenia i.e. lowered blood platelet count and activity, and sometimes medullary aplasia. Although the reason busulfan causes such a side effect is still not fully understood, some studies have attributed it, at least in part, to the ability of this drug to trigger oxidative stress in various organs of the body [10, 11].

Oxidative stress (OS) was initially defined by Sies [12] as a serious imbalance between oxidation and antioxidants, “a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage”. So, it reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Reactive species of oxygen, nitrogen and chlorine atoms are represent the most free radicals/oxidants producing by living organisms as a result of normal cellular metabolism [13, 14]. Disturbances in the normal redox state of cells , i.e. OS, can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA [13, 15-18]. OS from oxidative metabolism causes base damage, as well as strand breaks in DNA [19-21]. Base damage is mostly indirect and caused by ROS generated, e.g. O_2^- (superoxide radical), OH (hydroxyl radical) and H_2O_2 (hydrogen peroxide) [20]. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling [22]. Therefore, OS plays a pivotal role in various pathological conditions including hypertension, pulmonary hypertension, obesity, rheumatoid arthritis and neurological disorders, anemia, diabetes, and chronic liver and kidney diseases, with high levels of oxidative stress in target organs such as the heart, pancreas, liver, brain, kidney, and lung [13, 16, 17, 20, 23, 24]. Also, it is contributing to tissue injury following irradiation and hyperoxia as well as in diabetes and is likely to be involved in age-related development of cancer [13]. Infection by Helicobacter pylori which increases the production of reactive oxygen and nitrogen species in human stomach is also thought to be important in the

development of gastric cancer [20, 25]. Furthermore, associations between busulfan treatment and markers of OS and the susceptibility of lipid to oxidative modification have been observed [10, 11, 26].

Many drugs and synthetic chemical compounds have been used to reduce these harmful effects associated with the use of busulfan in therapeutic applications. Many of the side effects and drug interactions associated with these drugs have had a significant impact in the search for safer and more effective alternatives [27-29]. In recent decades, huge studies have been conducted on natural antioxidants extracted from various plant parts which have high antioxidant activity and are even used in many food applications. Among of these substances, phenolic compounds/extracts, which are widely distributed, have the ability to scavenge free radicals and inhibition the lipid peroxidation [30-33].

Milk thistle (MT, *Silybum marianum* L.) is an annual/biennial plant of the *Asteraceae* family, native of Mediterranean area and now growing and cultivated worldwide including Egypt (See Fig.1) [34]. It has been used for centuries in medicine, mainly to treat kidney, spleen, liver, and gallbladder diseases [35]. MT seeds contain a mixture of flavonolignans collectively known as silymarin, being silybin, isosilybin (A and B), silydianin, and silychristin are the main silymarin flavonolignas [36]. The study of Asghar and Masood [37] suggested that silymarin may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement. It is suggested that many of these properties may be related to the antioxidant and free radical scavenging activity of Silymarin. It has been said to be at least ten times more potent in antioxidant capacity than vitamin E [38]. Also, Valenzuela, *et al.* [39] reported that Silymarin increases glutathione in the liver by more than 35% in healthy subjects which is responsible for the detoxification of a wide range of hormones, drugs, and xenobiotics. Furthermore, Tajmohammadi and Bibi [40] reviewed that silymarin also increases the level of the antioxidant defense system including glutathione, enzymes (glutathione peroxidase, superoxide dismutase and catalase) in both subjects and animal models. For the above reasons, the present work was carried out to determine whether busulfan has an oxidative effect after administration to the rats. Also, whether different doses of wild Milk thistle (*Silybum marianum* L.) seed extract intervention can protect different organs from busulfan oxidative-induced damage will be in the scope of this investigation.

2. Materials and Methods

2.1. Materials

2.1.1. Milk thistle (MT) fruits

Dried fruits of MT (*Silybum marianum* L.), wild populations plant growing beside public irrigation canals, were collected with the help of some people living in the area, Mit Ghorab Village, Sinbellaween Center, Dakhlia Governorate, Egypt. The fruits were collected in bags made of plastic threads, and was verified by the professors of plant taxonomy, Faculty of Agriculture, Menoufia University, Shebin El-Kom, Egypt.

2.1.2. Chemicals and Kits

Casein was obtained from Morgan Chemical Co., Cairo, Egypt. Vitamins and salts mixtures in food grade, organic solvents and other chemicals in analytical grade were purchased from El-Ghomhorya Company for Trading Drugs, Chemicals and Medical instruments, Cairo, Egypt. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were assayed by the kits provided by MyBioSource, Inc., San Diego, CA, USA). SOD activity was measured by Ransod kit (Randox laboratories mmited, Germany). Reactive oxygen species (ROS) and protein assayed by the kits provided by Sigma-Aldrich, St. Louis, Missouri, USA.

2.1.3. Machines

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

2.2. Methods

2.2.1. Preparation of *Silybum marianum* Seed Ethanol Extracts (SMSEE)

Dried fruits of *Silybum marianum* samples were transferred to the laboratory and the seeds were extracted manually. Seed were sorted to remove foreign bodies and damaged seeds then ground in high miller speed (Moulinex Egypt, Al-Araby Co., Egypt) to a reduced powder (20 mesh) as well as mixed to obtain homogeneous samples. SMSEE was prepared such as mentioned in Tajmohammadi and Bibi [40]. In brief, five grams of *Silybum marianum* powder were extracted in a Soxhlet apparatus (Soxhelt Semiautomatic apparatus Velp company, Italy) for 5-6 h (25 ± 5 min per cycle) using 80% ethanol. Finally, the solvent was evaporated under reduced pressure (rotary evaporator Büchi R-210, Switzerland) to obtain the dried solvent extract and stored at 4 ^{0}C before use. The total yield of SMSEE was 6.31% (w/w) in terms of the *Silybum marianum* seeds.

2.2.2. Biological Experimental

2.2.2.1. Ethical Approval

All experiments for this study especially the biological ones were ethically approved by the Scientific Research Ethics Committee (Animal Care and Use), Faculty of Home Economics, Menoufia University, Shebin El-Kom, Egypt (Approval no. 07- SREC- 02-2021).

2.2.2.2. Animals

Adult male albino rats (150.67±7.34 g per each) were used in this study and purchased from Helwan Station, Ministry of Health and Population, Helwan, Cairo, Egypt.

2.2.2.3. Basal Diet

The basic diet (BD) for rats feeding was prepared according to the following formula (per kg) as modified of AIN. American Institute of Nutrition [41] as follow: corn starch (465.692g), casein-85% protein (140g), dextrinized corn starch (155g), sucrose (100g), soybean oil (40g), fiber (50g), mineral mixture (35g), vitamin mixture (10g), Lcystine (1.8g), choline bitartrate (2.5g) and tert-butylhydroquinone (0.008g). Also, vitamins and minerals mixtures component were formulated according to the same reference.

2.2.2.4. 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 [42]. Rats (n=42 rats), were housed individually in wire cages in a room maintained at 25 ± 3 ^{0}C, relative humidity (56±4%), a 12-hr lighting cycle and kept under normal healthy conditions. All rats were fed on BD for one-week before starting the experiment for acclimatization. After one week period, the rats were divided into sex groups as follow: group 1 (Normal control), healthy rats without intervention; group 2 (control SMSEE), given by gavage 400 mg/kg/day SMSEE for 14 days; group 3 (positive control group), received 20 mg/kg/day i.p. busulfan; group 4 (experimental group 1), received 20 mg/kg/day busulfan i.p. for 14 days and after 2 weeks of 200 mg/kg/day SMSEE was gavage for 14 days; group 5 (experimental group 2), received 20 mg/kg/day busulfan i.p. for 14 days and after 2 weeks of 400 mg/kg/day

SMSEE was gavage for 14 days, group 6 (experimental group 3), received 20 mg/kg/day busulfan i.p. for 14 days and after 2 weeks of 600 mg/kg/day SMSEE was gavage for 14 days; and group 7 (experimental group 4), received 20 mg/kg/day busulfan i.p. for 14 days and after 2 weeks of 800 mg/kg/day SMSEE was gavage for 14 days. SMSEE extract concentrations were selected for experiments based on many of the results of previous studies [43]. At the end of the treatment period, rats were anesthetized by using ether then killed; after sacrifice the peritoneal cavity was opened through a lower transverse abdominal incision and their organs (liver, kidneys, pancreas, heart, spleen and testis) were promptly collected.

2.2.2.5. Organs Homogenate Preparation

Organs homogenate were prepared such as mentioned by Nencini, *et al.* [44]. In brief, small pieces of each organ (liver, kidneys, pancreas, heart, spleen or testis) were then transferred to a sterile vessel containing cell lysis buffer (phosphate buffer 0.025M, pH 7.4) solution. Then the organs were immediately ground to make a tissue homogenate (1g/4ml). The homogenates were centrifuged at 750g for 15 minutes at 4 ^{0}C and the supernatant was collected to a new microcentrifuge tube and the the total protein concentration was measure according to the method of Lowry, *et al.* [45]. The samples were diluted to 10 mg protein/mL with 1X phosphate buffer and used for biochemical assays.

2.2.2.6. Oxidative Stress Determination

Glutathione fractions (GSH and GSSG) were measured colorimetrically in serum samples such as described by Ellman [46]. Glutathione peroxidase (GSH-Px) and catalase (CAT) activities were measured as mentioned by Splittgerber and Tappel [47] and Aebi [48], respectively. Superoxide dismutase (SOD) activity was measured according to the method of Mett and Müller [49]. GSH-Rd activity was determined according to the method recommended by the International Committee for Standardization in Haematology [50]. Malondialdehyde (MDA) was measured using the colorimetric method described by Buege and Aust [51] based on the reaction of thiobarbituric acid (TBA) with MDA, one of the aldehyde products of lipid peroxidation. The absorbance of the MDA-TBA adduct thus produced was measured spectrophotometrically at 535 nm. The results were expressed in terms of the mmol of malondialdehyde (MDA) / mg of tissue homogenates total protein**.** Reactive oxygen species (ROS) was determined by a colorimetric method described by Erel [52].

2.2.3. Statistical Analysis

Results were expressed as means ± SD with Microsoft Excel Software (version 15.0, 2013). The data were analyzed statistically using Student *t*-test and MINITAB 12 computer program statistical software (Minitab Inc., State College, PA). A value of $P \leq 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1. Effect of Busulfan Administration and SMSEE Intervention on Changes in MDA Level Profile

The effect of busulfan administration and SMSEE intervention on changes in ROS level profile was shown in Table 1 and Fig 2. The results showed that the level of ROS recorded the highest values in the normal group (group 1) in liver tissues, followed by testis, kidney, pancreas, spleen and heart tissues, respectively. The intervention with SMSEE extract in normal group (Group 2) resulted in significant ($p \leq 0.05$) decrease in the degree of ROS level in different degrees except heart. The injecting with busulfan (group 3) led to a significant ($p \leq 0.05$) increase in the level of ROS in all tissues of the organs ranged 45.01 to 53.66%. Also, the intervention with SMSEE extract in busulfan-treated rats (groups 4-6) led to a significant ($p \leq 0.05$) decrease in the degree of ROS level in the tissues of all organs, which reached its maximum in the liver, testis and spleen tissues. The rate of decreasing in ROS levels in all tissues were exhibited a dose- dependent increase with SMSEE intervention. Also, similar behaviors were reported for the MDA levels (Table 2 and Fig 3).

The present study shows that the ROS and MDA levels of different organs (liver, spleen, pancreas, testis, heart and kidney) in the busulfan control group was significantly increased as compared to other groups. The elevated tissue of ROS and MDA levels as markers of oxidative stress and decreased antioxidant enzymes activity simultaneously in rats treated only with busulfan indicated the pro-oxidant role of busulfan in producing ROS and lipid peroxidation. Such data is in agreement with the findings of Ray [10]; Ray [26]; Hosseini Ahar, *et al.* [53]. Who find that reduce body and testicular weight, and increase serum MDA and there could be side effects in reproduction process in rats administrated busulfan. Considering that MDA is produced from ROS, the higher level of MDA may promote polyunsaturated fatty acid peroxidation, lipid hydro peroxides and conjugated dienes

[54]. Thus, the controlling of ROS and MDA levels induced by SMEE intervention would be helpful in maintaining a suitable level of oxidative stress. In line with this, Sajedianfard, *et al.* [55]**.** Found that treatment of rats with *Silybum marianum* bioactive compound, silymarin, leads to decrease the levels of MDA and increase the levels SOD and GSH-Px. Also, Asghar and Masood [37]. Suggested that silymarin (Main bioactive compound in SMSEE) may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement. It is suggested that many of these properties may be related to the antioxidant and free radical scavenging activity of Silymarin. It also increases the activity of the important antioxidant enzymes such as superoxide dismutase, catalase, and inhibits lipid peroxidation [56, 57]. Furthermore, MDA, a harmful degredative product, can be formed in cell membranes as a result of lipid peroxidation [5]. Cross linking of MDA with the membrane components leads to changes in its properties including disturbance in membrane fluidity, inactivation of enzymes and receptors in membranes, cell injury and may cause the formation of atherosclerotic plaques [31, 58, 59]. Some *in vitro* studies have shown that the bioactive compound of SMSEE, silymarin's, have the protective effect through the preserving of the integrity of the plasma membrane which can affect Ca^{2+} modulation, as an essential role in hepatoprotection [60]. In our opinion, if there were no change in the antioxidant defense system of the busulfan administration rats intervented with SMSEE, it would be difficult to observe low levels of ROS and MDA.

3.2. Effect of Busulfan Administration and SMSEE Intervention on Changes in GSH Level Profile in the Different Organs of Rats

The effect of busulfan administration and SMSEE intervention on changes in GSH level profile was shown in Table 3 and Fig 4. The results showed that the level of GSH concentration recorded the highest values in the normal group (group 1) in liver tissues, followed by spleen, kidney, pancreas, heart and testis tissues, respectively. The intervention with SMSEE extract in normal group (Group 2) resulted in an increase in the degree of GSH level in different degrees in all organs, which represented a significant ($p \leq 0.05$) increase in almost tissues. The injecting with busulfan (group 3) led to a significant ($p \leq 0.05$) decrease in the level of GSH in all tissues of the organs ranged 25.83 to -49.56 %. Also, the intervention with SMSEE extract in busulfan-treated rats (groups 4-6) led to a significant ($p \leq 0.05$) increase in the degree of GSH level in the tissues of all organs, which reached its maximum in the heart and testis tissues. With the except of pancreas and spleen, the rate of increasing in GSH levels were exhibited a dose- dependent increase with SMSEE intervention. Also, similar behaviors were reported for the GSSG levels (Table 4 and Fig 5).

Among GSH functions are two constructing roles in detoxifications including: as a key conjugate of electrophilic intermediates, principally via glutathione-*s*-transferase activities in phase II metabolism, and as an important antioxidant. The antioxidant functions of GSH include its role in the activities of GSH enzymes family including glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd), and can apparently serve as a nonenzymatic scavenger of oxyradicals [17, 61, 62]. Therefore, determination of GSH is very important for presentday nutrition, medicine and pharmacy. Data of the present study with the others suggested that levels of GSH fractions might be decreased as the busulfan administration. For example, DeLeve, and Wang (2000) found that busulfan given *in vivo* or *in vitro* decreased hepatocyte GSH by 60 and 50%, respectively. Various components of SMSEE, in particular silymarin, exerts hepatoprotective effect through its antioxidant and increasing intracellular and liver GSH level and scavenging free radicals [63]**.** Also, data of the present study are accordance with that reported by Valenzuela, *et al.* [39], silymarin increases GSH in the liver by more than 35% in healthy subjects and by more than 50% in rats. Silymarin also increases the activity of the important GSH antioxidant enzymes family including GSH-PX and GSH-Rd [56]. Therefore, data of the present suggested that busulfan toxicity was prevented by SMSEE through its antioxidant activity which led to increased production of oxidized

GSH in different organs tissues. Therefore, data of the present suggested that busulfan toxicity was prevented by SMSEE through its antioxidant activity which led to increased production of oxidized GSH and thiobarbituric acid reactive substances.

3.3. Effect of Busulfan Administration and SMSEE Intervention on Changes in Antioxidant Enzymes Activity Profile in the Different Organs of Rats

3.3.1. Glutathione Peroxidase (GSH-PX) and Glutathione Reductase (GSH-Rd)

The effect of busulfan administration and SMSEE intervention on changes in GSH-Px activity profile was shown in Table 5 and Fig 6. The results showed that the level of GSH-Px activity recorded the highest values in the normal group (group 1) in spleen tissues, followed by kidney, liver, pancreas, heart and testis tissues, respectively. The intervention with SMSEE extract in normal group (Group 2) resulted in an increase in the degree of enzyme activity in different degrees in all organs, which represented a significant ($p \leq 0.05$) increase in the tissues of the heart, kidneys and pancreas. Injecting with busulfan (group 3) led to a significant ($p \leq 0.05$) decrease in the degree of GSHPx activity in all tissues of the organs studied (except testes). Also, the intervention with SMSEE extract in busulfantreated rats (groups 4-6) led to a significant ($p \leq 0.05$) increase in the degree of enzyme activity in the tissues of all organs, which reached its maximum in the testis and heart tissues. The rate of increasing in GSH-Px activities were exhibited a dose- dependent increase with SMSEE intervention. Also, similar behaviors were reported for the GSHRd enzyme (Table 6 and Fig 7).

3.3.2. Catalase (CAT)

The effect of busulfan administration and SMSEE intervention on changes in CAT activity profile was shown in Table 7 and Fig 8. From such data it be noticed that the level of CAT activity recorded the highest values in the normal group (group 1) in heart tissues, followed by liver, spleen, pancreas, kidney and testis tissues, respectively. The intervention with SMSEE extract in normal group (Group 2) resulted in an increase in the degree of enzyme activity in different degrees in all organs, which represented a significant ($p \leq 0.05$) increase in the tissues of the liver, heart and spleen. Injecting with busulfan (group 3) led to a significant ($p \leq 0.05$) decrease in the degree of CAT activity in all tissues of the organs studied (except testes). On the other hand, the intervention with SMSEE extract in busulfan-treated rats (groups 4-6) led to a partially significant ($p \leq 0.05$) increase in the degree of enzyme activity in the tissues of all organs, which reached its maximum in the heart, pancreas and spleen tissues. All the tissue organs except kidney, exhibited a dose- dependent increase in CAT activities with SMSEE intervention.

3.3.3. Superoxide Dismutase (SOD)

The effect of busulfan administration and SMSEE intervention on changes in SOD activity profile was shown in Table 8 and Fig 9. Such results showed that the level of SOD activity recorded the highest values in the normal group (group 1) in heart tissues, followed by spleen, pancreas, kidneys and liver tissues, respectively. The intervention with SMSEE extract in normal group (Group 2) resulted in an increase in the degree of enzyme activity in different degrees in all organs, which represented a significant ($p \leq 0.05$) increase in the tissues of the liver, kidney and heart. Injecting with busulfan (group 3) led to a significant ($p \leq 0.05$) decrease in the degree of SOD activity in all tissues of the organs with the rate ranged -40.35 to -64.63%. Also, the intervention with SMSEE extract in busulfantreated rats (groups 4-6) led to a significant ($p \leq 0.05$) increase in the degree of enzyme activity in the tissues of all organs, which reached its maximum in the testis and heart tissues. The rate of increasing in GSH-Px activities were exhibited a dose- dependent increase with SMSEE intervention.

It is customary that the organism has developed antioxidant defense systems largely based on antioxidant enzymes (GSH-Px, GSH-Rd, CAT and SOD) able to scavenge ROS to prevent the free radical damages i.e. oxidative stress activities [17, 64, 65]. The antioxidant defense systems is conducted as follows: SODs are responsible for the reduction of $O_2^{\cdot-}$ to H_2O_2 and multiple enzymes will remove H_2O_2 including GSH-Px and CAT. The GSH reduces

the Se and the reduced form of the enzyme then react with H_2O_2. The ratio of nine *GSH/GSSG* in normal cells are kept high. So there must be a mechanism of reducing GSSG back to GSH. This is achieved by GSH-Rd enzyme which catalyze the reaction: $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$. Mammalian RBC's operate the pentose phosphate pathway in order to provide NADPH for GSH reduction. GSH-Rd can also catalyze reduction of certain mixed disulphides such as that between GSH and Co-enzyme A [62]. Many studies such reported that antioxidant enzymes systems are active in different organs tissue cells [15, 66, 67]. Decreasing the activity of the antioxidant enzymes in both *in vitro* and *in vivo* systems results in increased ROS production and mitochondrial dysfunction [15, 55, 68, 69]. Tissue antioxidant enzymes results indicate that GSH-Px, GSH-Rd, CAT and SOD activities of different organs in liver, kidney, heart, testis, pancreas and spleen is significantly decreased due to Busulfan administration and this event shows that Busulfan could induce oxidative stress and reduction of antioxidant enzymes as the main antioxidant defense system for neutralization of ROS produced by Busulfan. There was partially dose-dependent manner in some organs tissues but others were not. The selected SMSEE in the present study intervention are rich in bioactive compounds such silymarins which exhibited antioxidant activities in different biological systems [70]. Such antioxidant activities are important in manipulation of the diseases development/complications through ROS scavenging processes in RBC's [13, 71]. In the present study, in all organs administration of different doses of SMSEE in combination with Busulfan could increase the activities of GSH-Px, GSH-Rd, CAT and SOD by different rates. This case may be due to different content of such enzymes in organs. Another reason is probably that tissues responses to SMSEE treatment are different from each other. According to our knowledge, the studies that have been conducted so far on the effect of busulfan on the activity of enzymes (GSH-PX and SOD) are very limited, and as for the activity of other enzymes (CAT and GSHRd), there are no studies, so it is difficult to compare the results obtained. But anyway, data of the present study are going well with other experiments. For example, Das and Mukherjee [38] reported that chronic ethanol consumption increased MDA and glutathione-*S*-transferase (GST) and decreased the activity of catalase and SOD and GSH-Px and Silymarin treatment reduced MDA and GST and elevated SOD, GSH-Px and CAT. Also, Kris-Etherton, *et al.* [59] found that Silymarin could decrease the activity of plasma liver functions (AST and ALT) and increase the activity of liver SOD and GSH-Px in comparison to mice that received CCl4 intraperitoneally. Finally, several studies reported that silymarin (Main bioactive component in SMSEE) is a vital antioxidant activity and plays a role by increasing antioxidant enzymes level in different organs [55, 70].

3.4. Correlation Studies

Correlation analysis between oxidative stress (ROS and MDA) and antioxidant defense systems (glutathione fractions and antioxidant enzymes) parameters in busulfan rat's administration and intervention with SMEE was shown in Table (9) and Figs (10 and 11). When all treatments were included in the statistical analysis important differences were found between oxidative stress and antioxidant defense systems parameters. There was a negative significant ($p \leq 0.05$) relationship between glutathione fractions concentration in plasma [GSH ($r^2 = -0.4505$) and GSSG ($r^2 = -0.4461$)], and antioxidant enzymes in organs tissue [GSH-Px ($r^2 = -0.3479$), GSH-Rd ($r^2 = -0.3555$), CAT ($r^2 = -0.2601$) and SOD ($r^2 = -0.7890$)], and ROS levels in different rats organs. The same behavior i.e. correlations/relationship were observed between all of those parameters and MDA concentrations in the same organs. These correlations confirm that if there were no change in the antioxidant defense systems parameters of busulfan administration rats, it would be difficult to observe high concentrations of ROS and MDA. In similar study, Sajedianfard, *et al.* [55] found that treatment of rats with *Silybum marianum* bioactive compound, silymarin, leads to decrease the levels of MDA and increase the levels SOD and GSH-Px. Also, Heidarian and Rafieian-Kopaei [56] and Tzeng, *et al.* [57] found that silymarin increases the activity of the important antioxidant enzymes such as superoxide dismutase, catalase, and inhibits lipid peroxidation.

4. Conclusion

Busulfan is a chemotherapy drug used to manage and treat several diseases including cancer. Like other drugs, it has side effects along with its therapeutic effects and some of these effects could be due to its pro-oxidative effect in inducing oxidative stress in non-target tissues including liver, kidney, heart, testis, pansies, and spleen. Such side effects lead to normal tissue injury. On the other hand, *Silybum marianum* ethanolic extract (SMSEE) could be a useful agent to protect against oxidative damages induced by busulfan. Such protection effects mainly due to its powerful antioxidant which increasing the antioxidant defense system (glutathione fractions and antioxidant enzymes), scavenging free radicals (ROS) resulted from oxidative stress, and inhibited the formation of lipid oxidation products (MDA). These results provide a basis for the use of *Silybum marianum* extracts as promising tools in the future for many important nutritional and therapeutic applications.

Abbreviations

BD, basic diet, CAT, catalase, CML, chronic myeloid leukemia, FDA, food and drug administration, DNA, deoxyribonucleic acids, GSH, reduced glutathione, GSSG, oxidized glutathione, GSH-Px, glutathione peroxidase, GSH-Rd, MDA, malonaldehyde, glutathione reductase, RNA, ribonucleic acids, ROS, reactive oxygen species, OS, oxidative stress, SMSEE, *Silybum marianum* seed ethanol extract, SOD, superoxide dismutase, TBA, thiobarbituric acid.

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Conflict of Interests

Authors declared no competing of interest whatsoever

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Fig-1. Wild milk thistle (*Silybum marianum* L.): the different stages of plant growth, taking study samples from the plant by the author (Prof. Yousif Elhassaneen) and the alcoholic extract that was prepared from the plant's seeds

Table-1. Effect of busulfan administration and SMSEE intervention on changes in ROS level (U/mg protein) profile

Groups	Liver	Kidney	Heart	Testis	Pancreas	Spleen

1	61.54±	3.50 c	59.67±	6.63 c	50.82±	5.91 b	60.25±	4.51 c	58.45±	3.64 c	52.89±	5.95 d
2	55.56±	5.34 d	54.32±	4.52 d	48.32±	5.99 b	53.65±	1.68 d	50.05±	1.97 d	47.11±	4.50 e
3	94.56±	4.21 a	86.53±	3.43 a	78.04±	2.17 a	91.65±	2.90 a	85.19±	3.70 a	78.54±	5.16 a
4	79.90±	5.23 b	67.67±	5.36 b	75.03±	3.09 a	75.54±	4.44 b	69.63±	2.42 b	73.27±	7.36 a
5	68.54±	4.89 c	60.32±	2.69 c	69.43±	2.88 ab	69.32±	5.49 b	60.51±	1.19 c	65.05±	2.94 bc
6	64.87±	5.96 c	60.23±	5.74 c	54.2±	4.77 b	60.53±	2.98 c	60.16±	6.48 c	58.87±	3.36 c
7	62.98±	6.1c	58.04±	7.49 c	47.54±	3.43 b	62.43±	3.52 c	56.98±	3.65 c	58.08±	1.89 c

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference ($P \leq 0.05$). group 1, Normal control: healthy rats without intervention; group 2.control SMSEE, 200 mg/kg/day SMSEE; group 3, positive control group received 20 mg/kg/day busulfan, group 4, treated group received 20 mg/kg/day busulfan + 200 mg/kg/day SMSEE; group 5, treated group received 20 mg/kg/day busulfan+ 400 mg/kg/day SMSEE; group 6, treated group received 20 mg/kg/day busulfan + 600 mg/kg/day SMSEE; group 7, treated group received 20 mg/kg/day busulfan + 800 mg/kg/day SMSEE.

Fig-2. Effect of busulfan administration and SMSEE intervention on changes in ROS (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

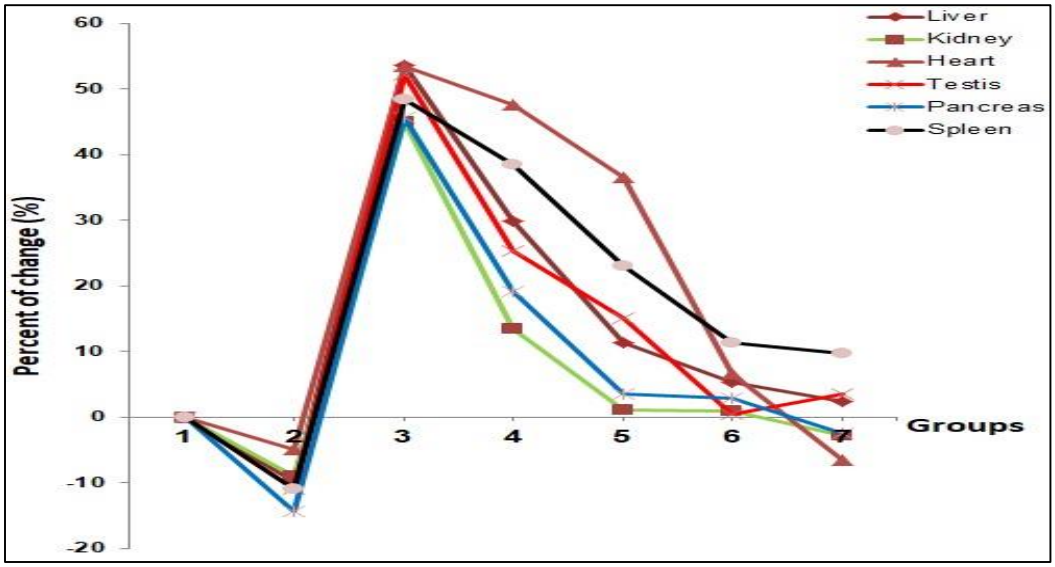


Table-2. Effect of busulfan administration and SMSEE intervention on changes in MDA level (µmol/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	6.01±	1.21 b	6.13±	0.89 b	6.32±	0.76 b	6.21±	0.68 b	6.64±	0.34 b	5.94±	0.98 b

2	5.81±	0.91 [b]	5.82±	0.32 [b]	5.82±	0.36 [b]	5.78±	0.71 [b]	5.71±	0.41 [b]	5.79±	0.76 [b]
3	9.53±	0.69 [a]	9.24±	1.03 [a]	9.13±	1.04 [a]	9.43±	1.14 [a]	9.58±	0.98 [a]	9.32±	1.02 [a]
4	5.92±	1.00 [b]	6.72±	1.23 [b]	5.98±	0.66 [b]	5.71±	0.98 [b]	6.05±	0.62 [b]	6.01±	0.33 [b]
5	5.82±	0.85 [b]	6.15±	0.67 [b]	5.86±	0.48 [b]	5.67±	0.28 [b]	5.89±	0.29 [b]	5.89±	0.29 [b]
6	5.60±	0.76 [b]	5.83±	0.32 [b]	5.71±	0.59 [b]	5.79±	0.55 [b]	5.72±	0.22 [b]	5.70±	0.32 [b]
7	5.62±	0.38 [b]	5.72±	0.15 [b]	5.67±	0.38 [b]	5.80±	0.60 [b]	5.80±	0.54 [b]	5.70±	0.54 [b]

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference (P≤0.05). Detailing the numbers for the groups as shown under Table 1.

Fig-3. Effect of busulfan administration and SMSEE intervention on changes in MDA (% of change from nrmal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

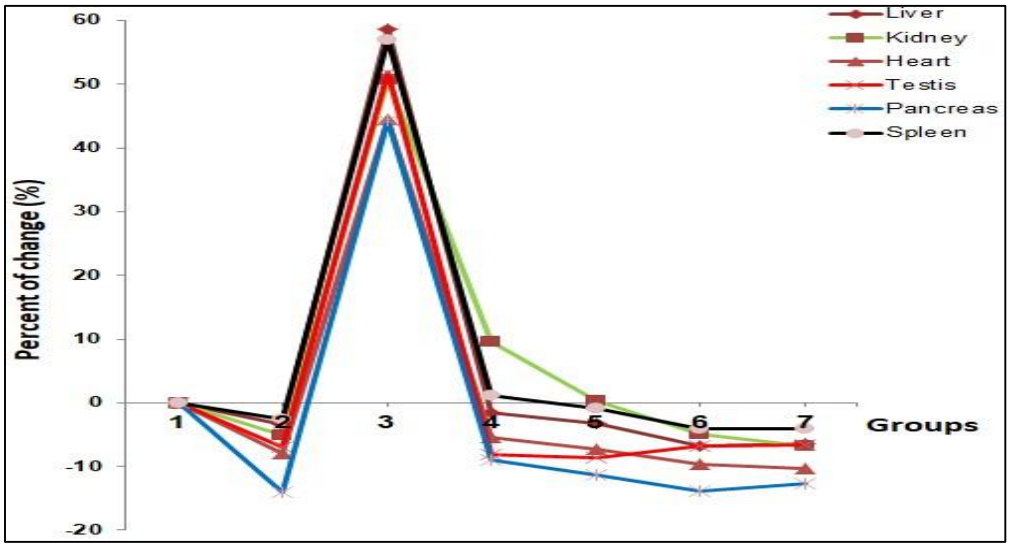


Table-3. Effect of busulfan administration and SMSEE intervention on changes in GSH level (mmol/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	10.98±	0.11 [a]	10.06±	0.06 [a]	7.83±	0.05 [a]	5.94±	0.05 [a]	7.91±	0.27 [b]	10.70±	0.34 [a]
2	11.70±	0.09 [a]	11.12±	0.12 [a]	9.15±	0.14 [a]	6.73±	0.09 [a]	9.47±	0.21 [a]	11.67±	0.21 [a]
3	6.02±	0.04 [c]	5.07±	0.08 [c]	4.22±	0.05 [d]	4.40±	0.10 [b]	4.54±	0.08 [e]	6.04±	0.23 [bc]
4	6.85±	0.10 [c]	7.42±	0.10 [b]	5.68±	0.08 [c]	4.96±	0.07 [b]	4.08±	0.11 [e]	6.96±	0.12 [b]

5	6.62±	0.06 c	7.53±	0.08 b	6.44±	0.12 b	5.77±	12.0 a	5.86±	0.05 d	7.83±±	0.10 b
6	7.96±	0.08 b	8.75±	0.13 b	7.80±	0.07 a	6.11±	0.08 a	5.83±	0.10 d	7.50±	0.14 b
7	8.03±	0.13 b	7.91±	0.06 b	8.51±	0.21 a	6.15±	0.03 a	6.03±	0.06 c	7.65±	0.09 b

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference ($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-4. Effect of busulfan administration and SMSEE intervention on changes in GSH level (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

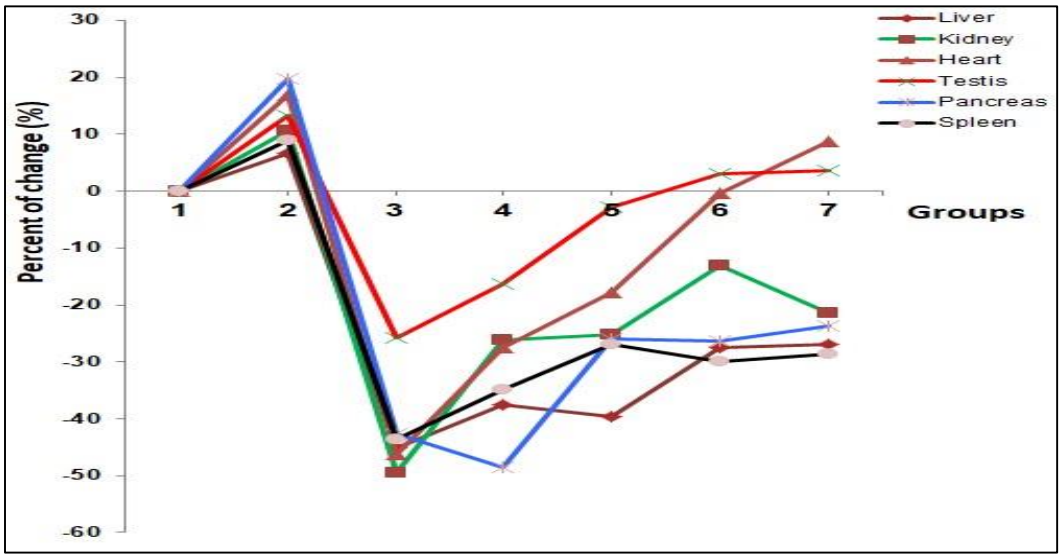


Table-4. Effect of busulfan administration and SMSEE intervention on changes in GSSG level (mmol/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	0.907±	0.11 a	0.909±	0.06 a	0.662±	0.05 b	0.486±	0.05 a	0.670±	0.27 b	0.922±	0.34 a
2	0.938±	0.09 a	0.979±	0.12 a	0.754±	0.14 a	0.545±	0.09 a	0.808±	0.21 a	0.984±	0.21 a
3	0.466±	0.04 d	0.410±	0.08 c	0.298±	0.05 d	0.374±	0.10 c	0.391±	0.08 d	0.528±	0.23 c
4	0.586±	0.10 c	0.594±	0.10 bc	0.431±	0.08 d	0.412±	0.07 b	0.336±	0.11 d	0.582±	0.12 bc
5	0.597±	0.06 c	0.611±	0.08 b	0.503±	0.12 c	0.498±	0.12 a	0.475±	0.05 c	0.636±	0.10 b
6	0.677±	0.08 b	0.696±	0.13 b	0.633±	0.07 b	0.526±	0.08 a	0.489±	0.10 c	0.621±	0.14 b
7	0.682±	0.13 b	0.653±	0.06 b	0.713±	0.21 a	0.514±	0.03 a	0.510±	0.06 c	0.629±	0.09 b

Results are expressed as means ± SD (n=5). Means with ±different superscript letters on the same column indicate ±significant difference
($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-5. Effect of busulfan administration and SMSEE intervention on changes in GSSG level (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

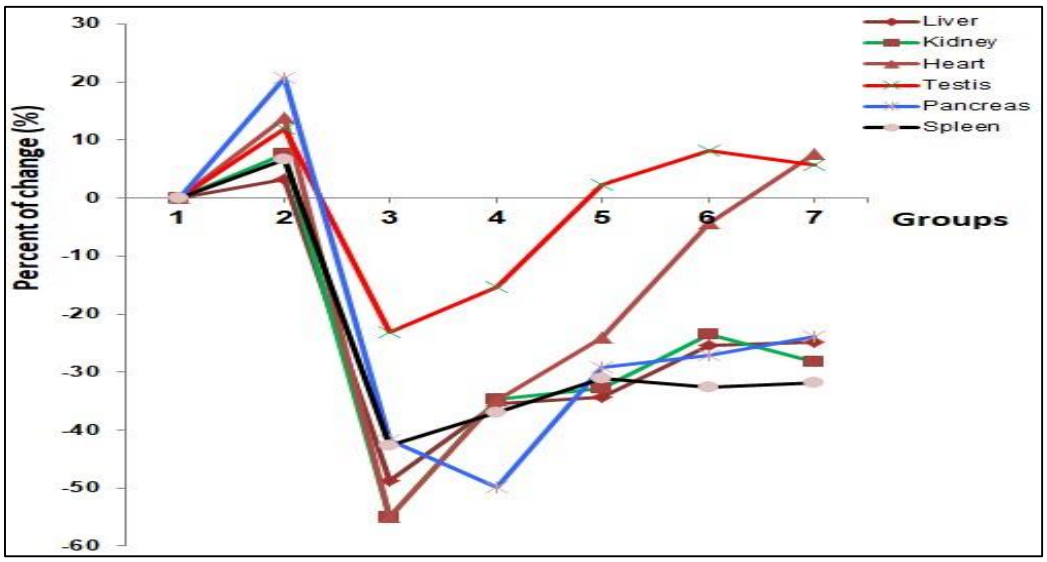


Table-5. Effect of busulfan administration and SMSEE intervention on changes in GSH-Px activity (U/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	16.98±	3.01 a	17.04±	2.11 a	12.05±	0.95 c	4.76±	0.65 b	11.76±	0.95 b	26.67±	3.11 a
2	17.76±	2.11 a	18.54±	1.23 a	19.53±	2.76 a	6.12±	1.14 a	14.65±	0.27 a	27.65±	3.10 a
3	8.03±	2.02 b	7.52±	0.67 c	3.98±	0.84 e	3.93±	0.78 bc	4.71±	0.75 d	15.05±	3.76 b
4	8.01±	1.09 b	10.98±	0.54 b	7.85±	0.65 d	5.43±	0.65 b	5.74±	0.77 cd	12.56±	1.09 c
5	7.88±	0.99 b	12.02±	1.05 b	9.01±	1.65 d	6.17±	1.42 a	6.98±	1.02 c	13.67±	0.85 bc
6	8.92±	0.56 b	12.34±	2.10 b	12.98±	0.78 c	7.24±	0.64 a	7.54±	1.21 c	16.90±	2.05 b
7	9.11±	1.06 b	12.07±	0.98 b	15.01±	1.73 b	6.98±	0.56 a	8.84±	2.24 c	16.78±	1.43 b

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference ($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-6. Effect of busulfan administration and SMSEE intervention on changes in GSH-Px activity (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

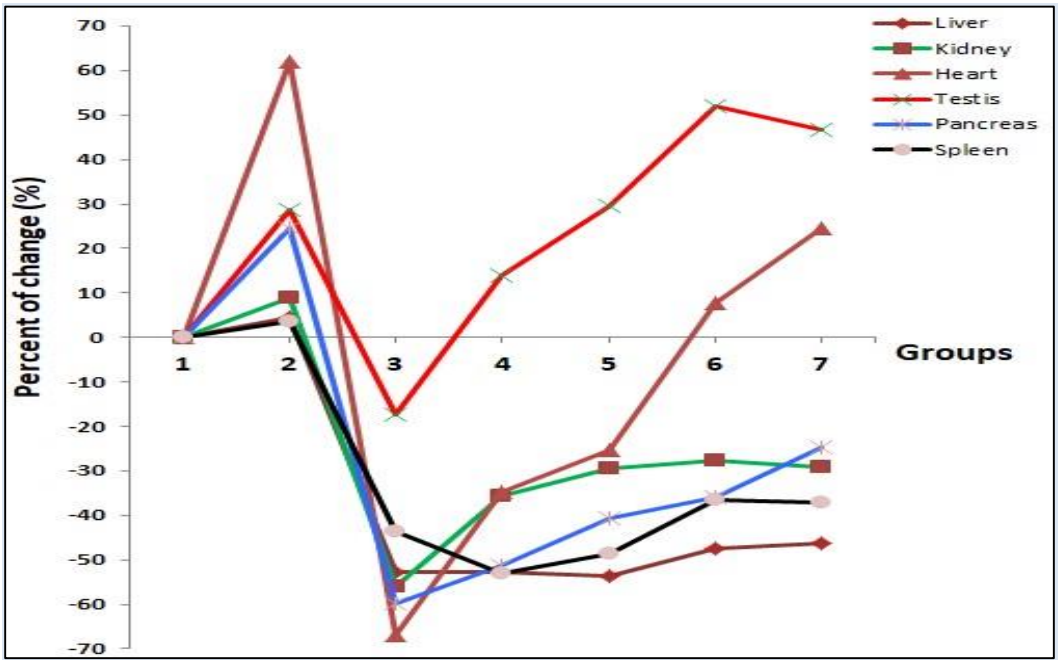


Table-6. Effect of busulfan administration and SMSEE intervention on changes in GSH-Rd activity (U/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	8.81±	1.51 a	8.40±	1.06 a	6.16±	0.48 c	2.61±	0.33 b	5.60±	0.48 b	12.14±	1.56 b
2	8.91±	1.06 a	8.89±	0.62 a	9.73±	1.38 a	3.26±	0.57 a	7.61±	0.14 a	14.83±	1.55 a
3	3.99±	0.63 d	3.76±	0.34 e	2.90±	0.42 e	1.85±	0.39	2.58±	0.38 d	7.87±	1.88 d
4	4.14±	0.55 c	5.49±	0.27 c	3.98±	0.33 d	2.81±	0.33 b	2.97±	0.39 d	6.01±	0.55 e
5	3.81±	0.50 d	5.99±	0.53 bc	4.73±	0.83 d	3.09±	0.71 a	3.55±	0.51 c	7.02±	0.43 d
6	5.72±	0.28 b	6.38±	1.05 b	6.49±	0.39 c	3.73±	0.32 a	3.87±	0.61 c	8.95±	1.03 c
7	5.94±	0.53 b	6.04±	0.49 b	7.44±	0.87 b	3.32±	0.28 a	4.98±	1.12 b	8.87±	0.72 c

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate ±significant difference ($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-7. Effect of busulfan administration and SMSEE intervention on changes in GSH-Rd activity (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

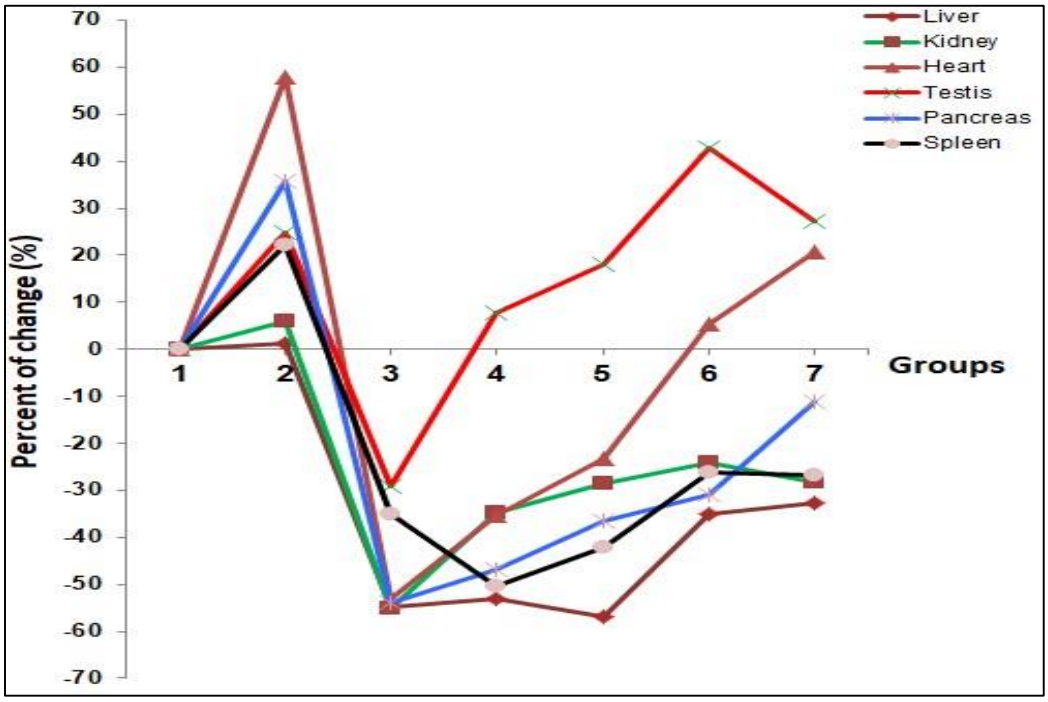


Table-7. Effect of busulfan administration and SMSEE intervention on changes in CAT activity (U/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	58.23±	2.23 ab	34.21±	5.12 [a]	58.67±	4.23 [b]	11.04±	6.21 [a]	50.04±	3.11 [a]	54.90±	7.34 ab
2	65.76±	4.77 [a]	35.16±	4.11 [a]	67.53±	5.16 [a]	13.98±	3,87 [a]	53.11±	2.54 [a]	60.42±	5.33 [a]
3	31.65±	3.76 [d]	29.73±	3.12 [b]	20.75±	1.87 [d]	6.16±	4.05 [b]	20.45±	3.16 [d]	32.75±	4.23 cd
4	41.83±	4.67 [c]	29.21±	4.87 [b]	46.89±	2.66 [c]	8.17±	3.67 ab	32.56±	2.92 [c]	38.43±	6.03 [c]
5	49.90±	6.15 bc	32.07±	6.08 [b]	55.17±	5.93 [b]	10.56±	6.19 [a]	40.17±	1.87 ab	49.15±	4.05 [b]
6	56.74±	5.32 [b]	32.58±	5.22 [b]	59.34±	4.11 ab	12.08±	4.94 [a]	44.72±	5.54 ab	54.89±	2.75 ab
7	52.59±	7.31 [b]	31.67±	4.08 [b]	61.67±	5.54 [a]	12.13±	2.91 [a]	48.42±	8.25 [a]	56.40±	1.96 [a]

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference ($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-8. Effect of busulfan administration and SMSEE intervention on changes in CAT activity (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

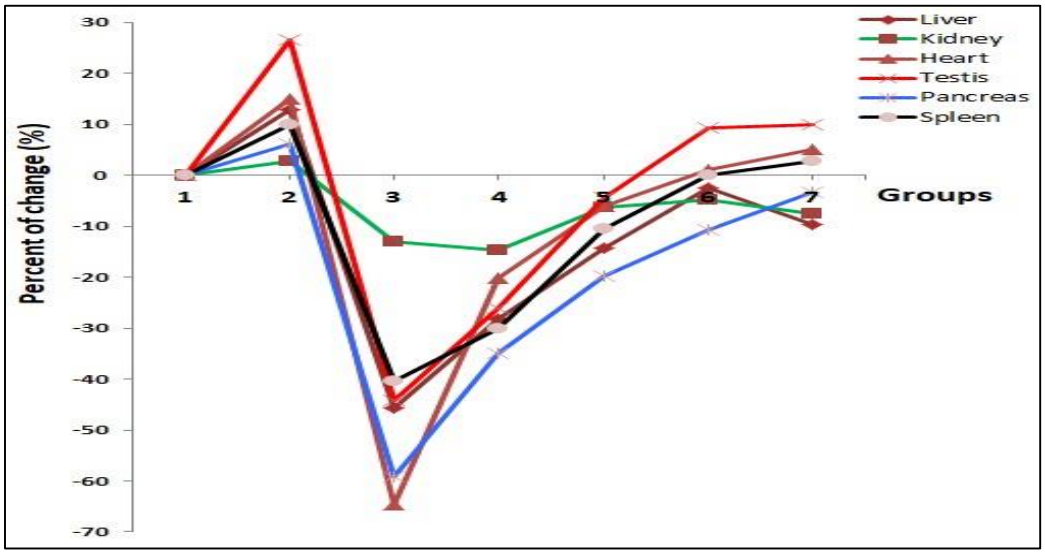


Table-8. Effect of busulfan administration and *Silybum marianum* ethanolic extract (SMSEE) intervention on changes in SOD activity (U/mg protein) profile

Groups	Liver		Kidney		Heart		Testis		Pancreas		Spleen	
1	42.67±	2.23 ^{b}	46.89±	5.12 ^{b}	58.67±	4.23 ^{b}	44.85±	6.21 ^{a}	50.04±	3.11 ^{a}	54.90±	7.34 ^{a}
2	52.56±	4.77 ^{a}	59.30±	4.11 ^{a}	67.53±	5.16 ^{a}	49.56±	3,87 ^{a}	53.11±	2.54 ^{a}	60.42±	5.33 ^{a}
3	23.12±	3.76 ^{c}	16.98±	3.12 ^{d}	20.75±	1.87 ^{d}	22.89±	4.05 ^{c}	20.45±	3.16 ^{d}	32.75±	4.23 ^{b}
4	40.67±	4.67 ^{b}	38.04±	4.87 ^{c}	46.89±	2.66 ^{c}	37.89±	3.67 ^{b}	32.56±	2.92 ^{c}	38.43±	6.03 ^{b}
5	42.01±	6.15 ^{b}	44.76±	6.08 ^{b}	55.17±	5.93 ^{b}	42.01±	6.19 ^{a}	40.17±	1.87 ^{b}	49.15±	4.05 ab
6	44.01±	5.32 ^{b}	48.05±	5.22 ^{b}	59.34±	4.11 ab	47.01±	4.94 ^{a}	44.72±	5.54 ab	54.89±	2.75 ^{a}
7	45.65±	7.31 ^{b}	51.67±	4.08 ^{b}	61.67±	5.54 ^{a}	48.05±	2.91 ^{a}	48.42±	8.25 ^{a}	56.40±	1.96 ^{a}

Results are expressed as means ± SD (n=5). Means with different superscript letters on the same column indicate significant difference ($P \leq 0.05$). Detailing the numbers for the groups as shown under Table 1.

Fig-9. Effect of busulfan administration and *Silybum marianum* ethanolic extract (SMSEE) intervention on changes in SOD activity (% of change from normal group) profile. Results are expressed as means (n=5). Detailing the numbers for the groups as shown under Table 1

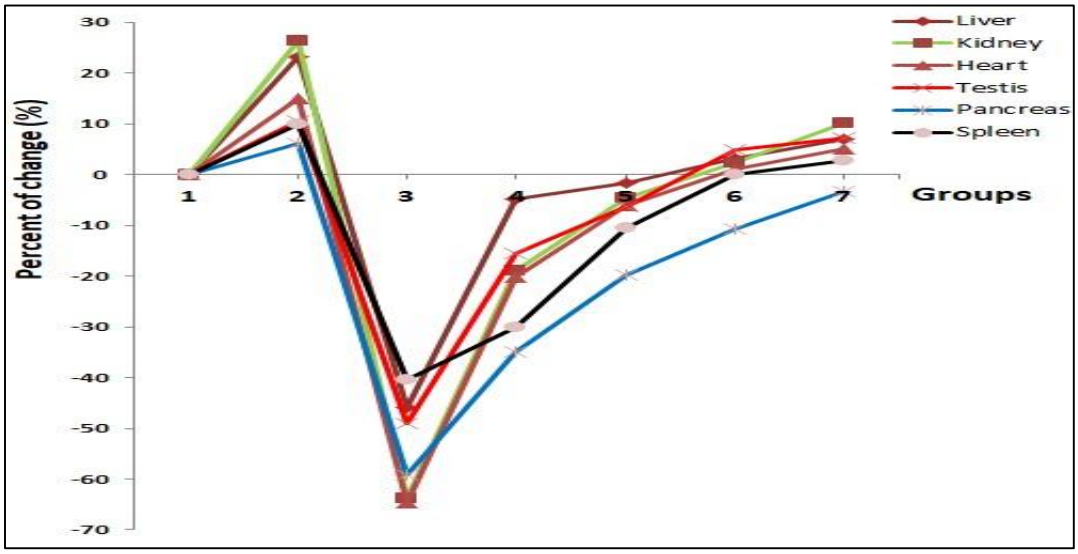


Table-9. Correlation between OS and antioxidant defense systems parameters in busulfan administration rats intervention with *Silybum marianum* ethanolic extract (SMSEE)

Parameters	Equation	*r2**
ROS/GSH	GSH = -0.1147 (ROS) + 14.718	- 0.4505
ROS/GSSG	GSSG = -0.01 (ROS) + 1.2528	- 0.4461
ROS/GSH-Px	GSH-Px = -0.2817 (ROS) + 29.528	- 0.3479
ROS/GSH-Rd	GSH-Rd = -0.1406 (ROS) + 14.896	- 0.3555
ROS/ CAT	CAT = -0.7636 (ROS) + 88.771	- 0.2601
ROS/SOD	SOD = -0.8926 (ROS) + 102.63	- 0.789
MDA/GSH	GSH = - 0.7388 (MDA) + 12.052	- 0.2108
MDA/GSSG	GSSG = - 0.0646 (MDA) + 1.0235	- 0.2112
MDA/GSH-Px	GSH-Px = -1.3718 (MDA) + 20.146	- 0.093
MDA/GSH-Rd	GSH-Rd = - 0.6859(MDA) + 10.223	- 0.0954
MDA/ CAT	CAT = - 5.4212 (MDA) + 74.231	- 0.1478
MDA/SOD	SOD = - 7.4549 (MDA) + 92.786	- 0.6204
ROS/MDA	MDA = 0.0797 (ROS) + 1.2574	+ 0.5637

* $P \leq 0.05$.

Fig-10. Correlation between ROS and MDA in busulfan administration rats intervention with SMEE.

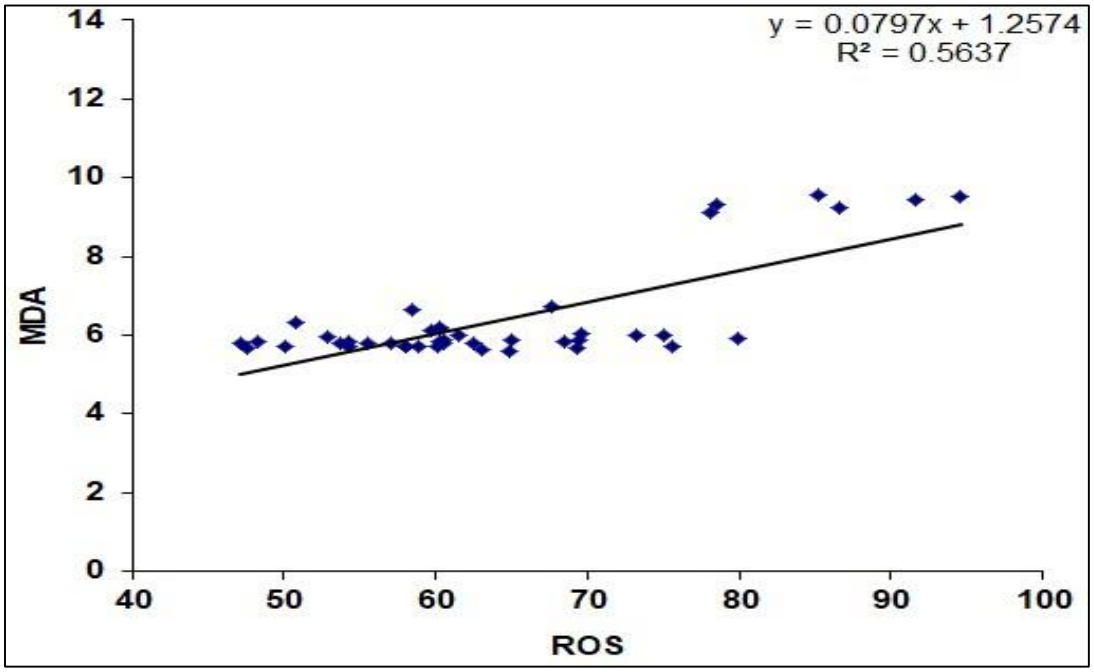
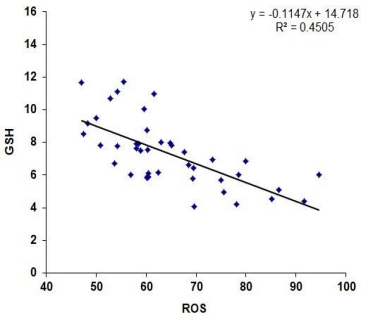
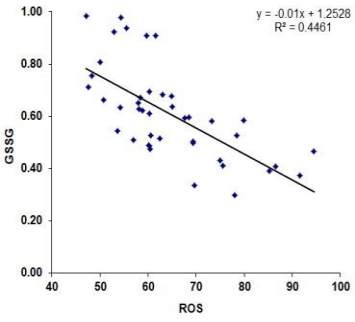


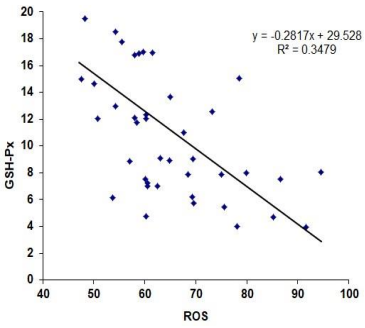
Fig-11. Correlation between oxidative stress and antioxidant defense systems parameters in busulfan administration rats' intervention with SMSEE



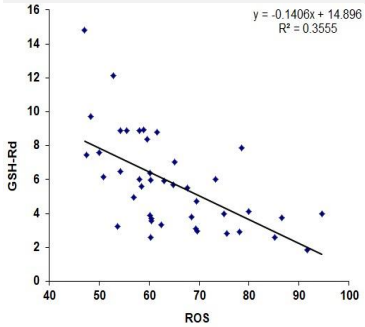
ROS vs GSH



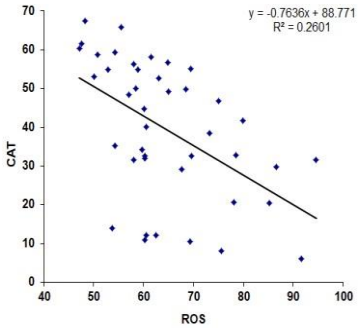
ROS vs GSSG



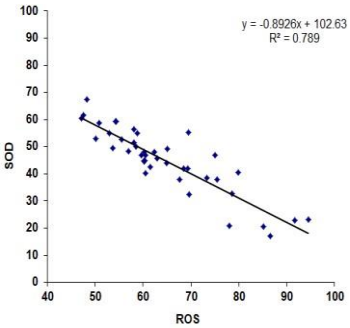
ROS vs GSH-Px



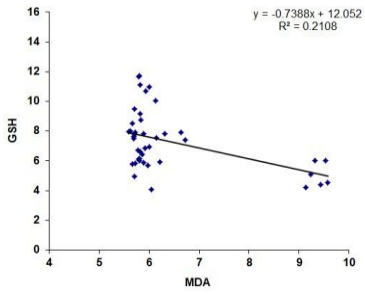
ROS vs GSH-Rd



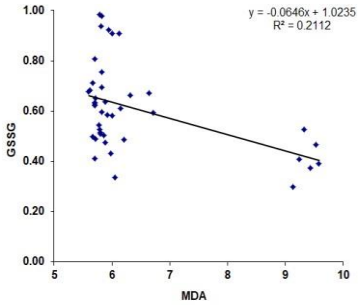
ROS vs CAT



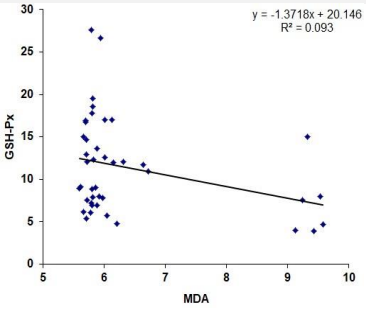
ROS vs SOD



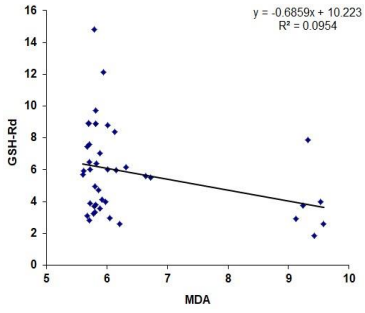
MDA vs GSH



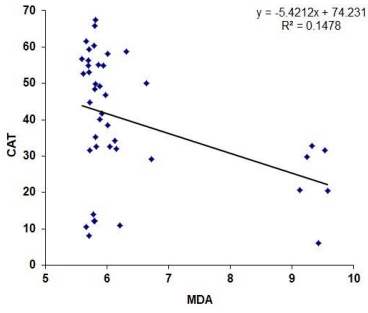
MDA vs GSSG



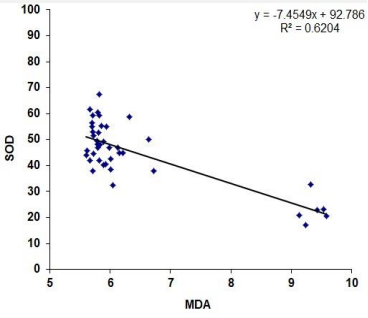
MDA vs GSH-Px



MDA vs GSH-Rd



MDA vs CAT



MDA vs SOD